Investigation of the Effect of Melatonin in *Arabidopsis thaliana* and Soil Microbes

Submitted by

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List of abbreviations

AA	Antimycin A
ABA	Abscisic acid
ASDAC	*N*-acetylserotonin deacetylase
ANOVA	Analysis of variance
AOX	Alternative oxidase
ASMT	*N*-acetylserotonin *O*-methyltransferase
CK	Cytokinin
cv.	Cultivar
DEGs	Differentially expressed genes
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
DR5	Direct repeat 5
DW	Dry weight
ELISA	Enzyme-linked immunosorbent assay
ET	Ethylene
EtOH	Ethanol
FDR	False discovery rate
FW	Fresh weight
GBAs	Gibberellins
GC-MS	Gas chromatography-mass spectrometry
HDAC	Histone deacetylase
IAA	Indole-3-acetic acid
JA	Jasmonic acid
L.	Linnaeus
LI	Light intensity
MS	Murashige and Skoog
MT	Melatonin
NAA	1-naphthalene acetic acid
NO	Nitric oxide

N/A	Not available
p-value	Probability value
pv.	Pathovar
qRT-PCR	Quantitative reverse transcriptase-polymerase chain reaction
RH	Relative humidity
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RNase	Ribonuclease
RNA-Seq	Ribonucleic acid-sequencing
ROS	Reactive oxygen species
SA	Salicylic acid
SNAT	Serotonin *N*-acetyltransferase
SPSS	Statistical Package for the Social Sciences
T5H	Tryptamine 5-hydroxylase
TDC	Tryptophan decarboxylase
TF	Transcription factor
UHPLC	Ultra-high-performance liquid chromatography
UHPLC-MS/MS	Ultra-high-performance liquid chromatography-Tandem mass spectrometry
v/v	volume by volume
WT	Wild-type
ng	nanogram
µM	micromolar
µg	microgram
$\mu mol \cdot m^{-2} \cdot s^{-1}$	micro moles per square meter per second
g	gram
mL	millilitre
mM	millimolar
°C	degree celsius
%	percentage

Abstract

Melatonin is a tryptophan-derived indoleamine conserved across all life kingdoms that acts as an antioxidant and a signalling molecule. Phyto-melatonin, discovered in 1995, is linked to the regulation of growth and development, and tolerance to biotic and abiotic stresses. Owing to this, melatonin has potential for use in agriculture. The phytohormone auxin is structurally and functionally similar to melatonin, but the cross-talk in the molecular responses by both indoleamines in plants is unclear. Also, the importance of the genotype effect into the phenotypic and transcriptomic response to melatonin has not been investigated. Moreover, limited information exists about the biological function of melatonin in microbes and in trans-kingdom signalling, such as between plants and microbes.

In this context this project aimed to: analyse whether melatonin regulates gene expression in a similar way to auxin by direct comparison using *Arabidopsis thaliana* treated with melatonin and auxin; determine the phenotypic and transcriptomic variation among several geographically diverse *Arabidopsis thaliana* ecotypes and a melatonin biosynthetic gene *serotonin-N-acetyltransferase1* mutant (*atsnat1*); assess in vitro growth and motility responses of selected plant-growth promoting rhizobacteria (PGPRs) and a pathogenic bacterium, *Pseudomonas aeruginosa* PAO1.

The results of these investigations revealed distinct gene expression responses by melatonin versus auxin treatments, with predominant effects of melatonin related to biotic stress defence. Melatonin treatment revealed differences in transcriptional responses in terms of differentially expressed genes between *atsnat1* mutant and wild-type indicating the potential role of AtSNAT1 in diverse processes regulated by melatonin related to growth and development and stress tolerance. A set of core-conserved genes were revealed in response to melatonin between the *Arabidopsis* ecotypes indicating the potential role of melatonin as a conserved signalling molecule. Moreover, extensive variation in patterns of gene expression were observed among the ecotypes, suggesting the link between melatonin with local habitat adaptation of the ecotypes. It was also found that melatonin restored the motility growth of *P. aeruginosa* PAO1 under ethanol treatment, while the growth of PGPRs was unaffected. In conclusion, this study strengthens the potential use of melatonin for future agricultural practices.

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Chapter 1: General Introduction

1.1 Introduction

1.1.1 A three-billion-year old molecule

Melatonin (*N*-acetyl-5-methoxytryptamine) is a ubiquitous low molecular weight (MW 232.3) indole molecule present in all cellular life forms studied to date including humans, plants, bacteria and fungi (Lerner et al., 1958, Dubbels et al., 1995, Hattori et al., 1995a, Hattori et al., 1999, Sprenger J, 1999). Melatonin is an ancient molecule (3.2-3.5 billion years) that has been retained throughout evolution in all organisms (Tan et al., 2010a).

Melatonin was first discovered in 1958 by the dermatologist, Aaron Lerner and colleagues who isolated it from the beef pineal gland (a small endocrine gland in the center of the brain) and reported the skin-lightening effect of melatonin extract on the amphibian, Northern leopard frog (*Rana pipiens*) (Lerner et al., 1958, Arendt, 2007). A year later, the presence of melatonin was demonstrated in extracts of human peripheral nerves (Lerner et al., 1959). The chemical structure was subsequently deduced in 1960 (Lerner et al., 1960) (Figure 1.1). Alongside, the pioneering work on melatonin synthesis pathway was ongoing. *N*-acetylserotonin was established as a metabolic by-product of serotonin (and precursor of melatonin) (Mcisaac and Page, 1958). Soon after, melatonin synthesis in animals was deduced where *N*-acetylserotonin was shown to be *O*-methylated to form melatonin (Axelrod and Weissbach, 1960).

Since its discovery in animals as a neurohormone in 1958, it has been extensively studied for its biological roles in mammalian systems, such as regulation of circadian rhythms, immune system, improvement in sleep, physiology of retina, and mood and oncostasis (i.e. anti-cancer effect) (Lerner et al., 1958, Lerner et al., 1960, Reiter et al., 1980, Jan et al., 2009, Carrillo-Vico et al., 2013, Hevia et al., 2015, Fathizadeh et al., 2019, Hao et al., 2019). The effects of exogenous melatonin studied in humans were principally on sleep disorders due to underlying conditions, or those arising from rotating shift-work or jet lag. Melatonin became available in the market from mid-1990s as a sleep-aid in the form of a prescribed-only medicine (as in Australia in accordance to guidelines by Therapeutic Goods Authority or over-the-counter pills/tablets (as available in the United States of America) (Grigg-Damberger and Ianakieva, 2017).

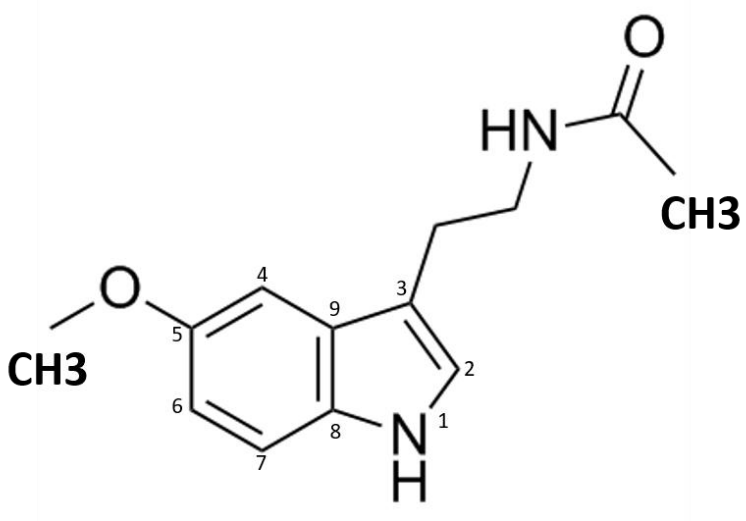


Figure 1.1: Chemical structure of melatonin (*N*-acetyl-5-methoxytryptamine).
The structure of melatonin was first deduced by (Lerner et al., 1960).

1.1.2 Functional evolution of melatonin in model systems (mammals, plants and yeasts)

It is speculated that evolution of melatonin resulted at the time organisms began transitioning from an anerobic to aerobic metabolism (Tan et al., 2010a). Melatonin is believed to have evolved in bacteria and its production has been reported in both alpha-proteobacteria and in photosynthetic cyanobacteria (Manchester et al., 1995, Hattori et al., 1999).

Universal antioxidant

Mitochondria and chloroplasts are the major sites where reactive oxygen species (ROS), including free radicals, are generated as by-products of aerobic metabolism arising from respiration and photosynthesis, respectively (Inupakutika et al., 2016). The primary biological function of melatonin was therefore proposed to be that of an antioxidant to protect cells from damage caused by toxic free radicals (Poeggeler et al., 1993). The antioxidant property of melatonin was first shown in vitro where melatonin scavenged hydroxyl radicals that were generated by hydrogen peroxide (H_2O_2) upon light exposure. This was measured by 5,5-dimethyl-pyrroline N-oxide (DMPO) spin-trapping method combined with HPLC electro-chemical detection and electron spin resonance (EPR) spectroscopy. The ROS scavenging (hydroxyl radical) potency of melatonin was significantly higher (approximately 6-fold) than other ROS scavengers

such as glutathione (Tan et al., 1993). Since then, many studies have shown that melatonin is a potent antioxidant (Tan et al., 2015, Reiter et al., 2016, Reiter et al., 2017).

Structure-activity relationship studies have shown that the indole moiety, methoxy and amide side chains in the melatonin structure contribute to the antioxidative capacity of melatonin (Figure 1). The carbonyl group in the N-C=O structure of the C3 amide side chain has been shown to be crucial for melatonin to scavenge the ROS. It directly scavenges free radicals by mechanisms such as transfer of single electron and hydrogen and radical adduct formation at the C3 side chain of the melatonin structure (Poeggeler et al., 1994, Tan et al., 2002, Zhang and Zhang, 2014). Melatonin also regenerates other classical antioxidants such as vitamin C, vitamin E, nicotinamide adenine dinucleotide (NAD) + hydrogen (H) (NADH) and glutathione by reducing their oxidised states to their original form (Tan et al., 2005, Tuzcu and Baydas, 2006). High membrane permeability has been shown for melatonin owing to its amphiphilic nature (Yu et al., 2016). Unlike other antioxidants, the subcellular distribution of melatonin is not limited as it can easily distribute both in lipid-rich membranes and aqueous cytosol (Shida et al., 1994, Catala, 2007, Venegas et al., 2012). This property enhances the antioxidant function of melatonin and gives it an edge over other classical antioxidants.

One molecule of melatonin can scavenge up to 10 ROS molecules whereas other classic antioxidants can scavenge up to one ROS (Tan et al., 2003). This shows the superiority of melatonin as an antioxidant over other compounds as tested in in vivo studies conducted on animal models (Yilmaz et al., 2002). The strong detoxification ability of melatonin lies partially in the fact that it can form a cascade reaction with its own metabolites including 2-hydroxymelatonin, cyclic-3-hydroxymelatonin and N^1-acetyl-N^2-formyl-5-methoxyknuramine upon interaction with ROS resulting in neutralization of toxic reactive radicals (Tan et al., 2015). In fact, studies have shown that some of the melatonin metabolites such as cyclic 3-hydroxymelatonin (C-3HOM) and *N*-acetyl-5-methoxy-knuramine (AMK) are more potent in scavenging ROS than melatonin (López-Burillo et al., 2003, Ressmeyer et al., 2003, Byeon et al., 2015). Understanding the importance of melatonin metabolism and melatonin derivatives is an emerging topic area for investigation.

The ROS scavenging function of melatonin (and its derivatives) represents a chemical property and is therefore not organism dependent. This property is hence not lost and

is conserved across organisms (Tan et al., 2010a). The antioxidant action of melatonin has been shown in organisms such as humans, plants and yeasts under oxidative stresses (Guo et al., 2017, Szewczyk-Golec et al., 2017, Bisquert et al., 2018, Vazquez et al., 2018, Zampol and Barros, 2018, Huang et al., 2019, Wang et al., 2020a). The mechanism of melatonin actions has remained the same across organisms which included ROS scavenging such as H_2O_2 and O_2^- (detected by histochemical techniques) and enhanced activities of antioxidants including superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and ascorbate peroxidase (APX) (detected spectrophotometrically) (Szewczyk-Golec et al., 2017, Vazquez et al., 2018, Huang et al., 2019).

Circadian rhythms

Circadian clocks are endogenous, time-keeping systems in biological life forms and adapt functions such as sleep/wake cycle, hormone release and body temperature to specific times within the day or night (Patke et al., 2020). In mammals, the information on photoperiodic status is received by suprachiasmatic nucleus (SCN) cells in the brain through retina and sent to the pineal gland which then releases melatonin (Alberts et al., 2008). The blood levels of melatonin in mammals exhibit a rhythm with low levels during the day and high at night (Reiter, 1986, Paul et al., 2015). For this reason, melatonin has been referred to as the 'hormone or chemical expression of darkness' (Reiter, 1991). Light exposure at night is the major suppressive factor of melatonin levels in mammals (Lewy et al., 1980). The disturbance in the circadian rhythm of melatonin levels has shown to be one of the causes of illnesses such as sleep disorders and have been linked to increasing risk of cancers in shift-workers who are repeatedly exposed to light during night-time (Chang et al., 2015).

Reports on the role of melatonin in regulating circadian rhythms and photoperiodic responses in plants are few and unclear. High, night-time melatonin levels were found in the short-day flowering weed, *Chenopodium rubrum* (Kolář et al., 1999). In contrast, melatonin levels in water hyacinth (*Eichornia crassipes* (Mart) Solms) exhibit circadian rhythms, but the levels peak during the end of the light period, rather than at night-time (Tan et al., 2007). Moreover, the melatonin levels in *Arabidopsis thaliana* (hereafter referred to as *Arabidopsis*) leaves (wild-type, (WT); ecotype Columbia (Col-0)) were found to be not diurnally regulated over a light/dark cycle of long day (14 hours light/10

hours dark) as analysed by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) (Hernández et al., 2015). Similar results were obtained by radioimmunoassay (RIA) detection of melatonin in the cotyledons and shoots of tomato (*Lycopersicon esculentum* Mill, cultivar cv. T5 and Castlemart) and morning glory (*Pharbitis nil* Choisy, cv. Violet) in light/dark cycle of 12/12 hours (Van Tassel et al., 2001). In contrast, melatonin levels assessed by enzyme-linked immunosorbent assay (ELISA) were shown to be rhythmic in a light/dark cycle of long day (16 hours/8 hours) and short day (8 hours/16 hours) in seedlings of *Arabidopsis* (WT; Col-0) grown on Murashige and Skoog (MS) medium (Shi et al., 2016a). Notably, the discrepancies between studies could be attributed to differences in age of the plants and/or analytical method in melatonin detection. An important factor is also likely to be the differences in the time-of-day for harvesting tissues and extracting melatonin for measurements. As reported earlier, Hernandez et al., 2015 show that the melatonin levels do not exhibit circadian rhythmicity in *Arabidopsis* leaves (Col-0) grown over a 14 hours light/10 hours dark period when measured every three hours from 1:30 am to 22:30 pm. No significant variation in melatonin levels (80 – 120 ng/g DW) were found in the leaves during the tested period (Hernández et al., 2015). In contrast, a recent study has tested by HPLC that melatonin levels exhibit circadian rhythmicity in 10-day old *Arabidopsis* seedlings (Col-0) grown over a 12 hours light/12 hours dark period of the day where the main peak (approximately 0.3 ng/g FW) is observed four hours into the morning and a second peak at night (20 hours into the day) (Li et al., 2020). While Hernandez et al., were careful in extracting melatonin at dim light levels (2 µmol m^2 s^{-1}) to avoid potential analyte degradation, the same has not been reported by Li and colleagues (Hernández et al., 2015, Li et al., 2020).

Recently, human gut bacterium (*Enterobacter aerogenes*) has shown to respond to melatonin (100 pM and 1 nM) with circadian rhythmicity in vitro indicating that human systems can modulate their microbiome by utilising bacterial clocks (Paulose et al., 2016).

Senescence

Melatonin levels in mammals including human subjects decline with age due to the reduced function of the SCN cells in the brain (Reiter et al., 1980, Mahlberg et al., 2006, Reiter et al., 2008, Tan et al., 2018). Melatonin administration has been shown to

increase life-span in animals (Karasek, 2004, Magnanou et al., 2009). For example, an earlier report showed that melatonin (provided in drinking water at 20 µg/mL or as injections at 100 µg/mL) enhanced longevity in female mice (*Mus musculus*; strain New Zealand Black (NZB)) (Pierpaoli and Regelson, 1994). Since then, many studies have confirmed the link between melatonin and aging (Karasek, 2004, Magnanou et al., 2009, Hardeland, 2013, Tamura et al., 2020). Clinical research also indicates therapeutic potential of melatonin in age-related neurological disorders such as Alzheimer's and Parkinson's or decline in reproductive (i.e. oocyte) development with age (Rosales-Corral et al., 2012, Alghamdi, 2018). This is mostly due to the efficient antioxidant properties of melatonin as aging involves excessive free radical accumulation and damage (Tan et al., 2015, Tamura et al., 2020).

It is not surprising that, similar to mammals, melatonin application in plants also delays senescence caused as part of aging or due to the negative impact of an environmental stressor in a variety of species such as in *Arabidopsis*, apple leaves (*Malus domestica* Borkh.), barley (*Hordeum vulgare* Linnaeus L.) and rice (*Oryza sativa* L.) (Arnao and Hernández-Ruiz, 2009b, Wang et al., 2012, Liang et al., 2015, Shi et al., 2015c). Delayed senescence in plants by melatonin is also achieved by a similar mechanism as that reported to occur in mammals, by improving antioxidant capacity (Wang et al., 2012, Liang et al., 2015, Liang et al., 2018). For example, melatonin application (10 mM) as a soaking solution delayed dark-induced senescence in detached apple leaves (*Malus domestica* cv. Golden Delicious) by lowering H_2O_2 content and enhancing the state of antioxidants such as ascorbate peroxidase (APX) and glutathione (GSH) (Wang et al., 2012). The effect of melatonin in delaying senescence is of importance as the longevity of plants can greatly result in enhanced grain yields which are otherwise diminished by the negative environmental impacts (Liang et al., 2018, Joshi et al., 2019).

The anti-senescence function of melatonin in microbes has not been studied. Reviews on functional evolution of melatonin hypothesise that melatonin co-existed with other important molecules such as sirtuins and this resulted in a functional co-operation between the two molecules (Mayo et al., 2017, Reiter et al., 2017, Zhao et al., 2019a). The link between melatonin and sirtuins has been shown in mammals where melatonin treatment modulates the expression or activity of sirtuins to confer cytoprotective effects including in aging, immunomodulation and cancer (Chang et al., 2009, Zhou et

al., 2015, Bonomini et al., 2018). For example, melatonin (100 µM) reverses oxidative stress (H_2O_2)-induced senescence in human bone-marrow stem cells in vitro by increasing the mRNA and protein levels of sirtuin-1 (SIRT1). The protective effects of melatonin are counteracted by inhibition of SIRT1 indicating dependency of melatonin on SIRT1 (Zhou et al., 2015). Sirtuins, are known to cause longevity in yeast (*Saccharomyces cerevisiae*) (Wierman and Smith, 2014). It is probable that, like in mammals, melatonin also works in co-operation with sirtuins to modulate aging effects in yeast. However, this is speculative at this stage and needs to be investigated in detail to identify and understand the anti-aging effects of melatonin to establish link of functional evolution of melatonin across kingdoms.

Immunomodulation

During evolution, organisms have developed mechanisms to survive under extreme environmental conditions. The seasonal variations such as changes in day length and temperatures requires organisms to accordingly mount an immune response (Nelson and Demas, 1996). It was shown that melatonin rhythms serve a purpose to mediate adaptation to seasonal variations in the immune system (Nelson and Drazen, 1999). Immunological research has uncovered the role of melatonin in immunomodulation and anti-inflammatory activities in mammals also highlight the potential clinical relevance of melatonin to enhance immune-system (Carrillo-Vico et al., 2013, Yu et al., 2017). These have been reviewed in detail by (Carrillo-Vico et al., 2005, Calvo et al., 2013, Carrillo-Vico et al., 2013). Moreover, direct anti-bacterial effects of melatonin application in vitro on nosocomial clinical pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* have been reported (Tekbas et al., 2008). The protective effect of melatonin against viral infections such as Semliki Forest virus, encephalitis and respiratory syncytial viruses have been experimentally shown in rodents (Ben-Nathan et al., 1995, Boga et al., 2012). The current threat the world is facing is from the 2019 novel coronavirus (2019-nCov, also known as COVID-19 and severe acute respiratory syndrome SARS-CoV-2) pandemic (Viglione, 2020). In light of this, a recent computational study has shortlisted 16 potential drug candidates including the combination of melatonin and meracaptopurine for COVID-19 (Zhou et al., 2020). However, it only remains a hypothesis at this stage and experimental evidence is needed to test the potential efficacy and/or any side-effects of melatonin-mercaptopurine combination against COVID-19.

The diurnal change of endogenous melatonin levels and resulting plant immunity have only been shown in *Arabidopsis* against bacterial pathogen *Pseudomonas syringae* pathovar (pv.) tomato DC3000 (Shi et al., 2016a). Nevertheless, the effect of melatonin in enhancing the basal plant immune system under a variety of abiotic and biotic (pathogens) stresses is well-documented (Bajwa et al., 2014, Shi et al., 2015d, Ke et al., 2018, Li et al., 2018). This section is covered in further detail in this review chapter.

1.2 Discovery and content of melatonin in plants

Melatonin was discovered in plants in 1995 using radioimmunoassay (RIA) and confirmed by high-performance liquid chromatography (HPLC) or gas chromatography/mass spectrometry (GC/MS) by two simultaneous studies in a range of twenty-four edible plants including: oat (*Avena sativa* L.); rice (*Oryza sativa* L. japonica); barley (*Hordeum vulgare* L.); tomato (*Lysopersicon esculentum* L.); cucumber (*Cucumis sativus* L.); and carrot (*Paucus carota* L.), among others (Dubbels et al., 1995, Hattori et al., 1995b). Ingestion of the identified melatonin-rich foodstuff also increased blood plasma melatonin levels (>0.03 ng/mL) in mammals as compared to the low levels (<0.01 ng/mL) upon ingestion of low-melatonin containing foods (Hattori et al., 1995b). The levels of melatonin in mammals is dependent on many factors such as age, time of the day, organs and tissues along with precision of analytical technique for measurement (Tan et al., 2010b). The topic of endogenous levels of melatonin in mammals is covered in depth in the reviews by (Tan et al., 2010b, Chen et al., 2011, Reiter et al., 2014). Melatonin has been detected in a variety of non-edible and edible plant species in tissues such as; fruits, vegetables, cereals, and nuts (Table 1.1).

The range of concentration of melatonin in plants has been detected to be widely distributed with substantial differences within and across species and organs (Murch et al., 1997, Reiter et al., 2005, Oladi et al., 2014). The fact that melatonin concentrations vary in plants has been the topic of much discussion. This has been discussed by researchers as potential differences in analytical techniques for extraction and quantification of melatonin, different growth conditions, and/or natural variation in different varieties of plants representing different habitats such as differences in expression of melatonin biosynthetic genes between different varieties (Arnao and

Hernández-Ruiz, 2013, Hardeland, 2016). The differences could also be due to differences in time-of-day in harvesting tissues and extracting melatonin (Hernández et al., 2015, Li et al., 2020). The wide distribution of melatonin in organs of the plant can be attributed to the organ-specific functions that melatonin exerts. For example, in mustard seeds, melatonin has been observed to be at high endogenous concentrations (129-189 ng/g dry weight, (DW)) which can be explained by the potential need for strong protective action of melatonin due to reduced metabolism during seed stage (Table 1.1).

Table 1.1 Content of endogenous melatonin (MT) in selected plant species and related foodstuffs.
DW=Dry Weight; FW=Fresh Weight; WT=Wild-Type; N/A=information 'not available' as not specified in the study; LI=light intensity; RH=relative humidity; Time of tissue harvesting and melatonin extraction for measurements are N/A except where stated

Plants and Plant-based foods	**Tissue**	**Age**	**Growth conditions**	**Concentration**	**Detection Method**	**References**
Plants						
Thale cress (*Arabidopsis thaliana*) (Col-0)	leaves	5 weeks	14h l/10h d,800 LI, 23°C (Time of harvesting every 3 hours: 1:30 – 22:30)	80-120 ng/g DW	UHPLC-MS/MS	(Hernández et al., 2015)
	seedlings	8 days	16h l/8h d, 120 LI, 23°C, 65% RH	0.6 ng/g FW	ELISA	(Shi et al., 2015d)
	seedlings	5 days	16h l/8h d, 150 LI, 23°C	2.6 ng/g FW	ELISA	(Chen et al., 2017)
	seedlings	10 days	12H l/12H d, 100 LI, Time of harvesting every 4 hours: zeitgeber time (ZT) 0-24 hours	ZT4 = 0.3; ZT8 and ZT12= 0.1; ZT 16=0.125; ZT20=0.15 and ZT24 <0.01 (all measured in ng/g FW)	HPLC	(Li et al., 2020)
Tomato (*Solanum lycopersicum* L. cv. Micro-tom)	leaves	34 days	N/A	11-16.5 ng/g FW	HPLC	(Wang et al., 2014)
Rice (*Oryza sativa* cv. Dongjin)	shoots; roots	10 days	150 LI, 28°C	0.5 ng/g FW; 0.2 ng/g FW	HPLC	(Park and Back, 2012)
Rice (*Oryza sativa* cv. Japonica)	seeds	N/A	N/A	1 ng/g FW	HPLC	(Hattori et al., 1995a)
Mustard (*Brassica nigra; Brassica hirta*)	seeds	N/A	N/A	129 ng/g DW; 189 ng/g DW	HPLC	(Manchester et al., 2000)
Wheat (*Triticum aestivum* L.)	coleoptiles	4 days	24°C, darkness	124.7 ng/g FW	HPLC-ECD	(Hernández-Ruiz et al., 2005)
Cotton (*Gossypium hirsutum* L.) cv. GXM9	seeds	2 days	N/A	80 ng/g DW	ELISA	(Chen et al., 2020)
Mungbean (*Vignaradiata* L.)	seedlings	N/A	N/A	0.17 ng/g DW	ELISA	(Aguilera et al., 2015)
Brocolli (*Brassica oleraceae* L.)	seedlings	N/A	N/A	0.44 ng/g DW	ELISA	(Aguilera et al., 2015)
Cucumber (*Cucumis sativus* L.) cv. Jingyu-1	seeds	2 days	N/A	17.3 ng/g FW	UHPLC-ESI-MS/MS	(Zhang et al., 2014a)

Table 1.1 contd.

Plants and Plant foods	Tissue	Age	Growth conditions	Concentration	Detection Method	References
Cereals						
Corn (*Zea mays*) (YM001-) 58 cultivars	kernel	N/A	N/A	10-2034 ng/g DW	HPLC	(Jinying et al., 2009)
Rice (*Oryza sativa*) (SD001-) 25 cultivars	grain	N/A	N/A	0-264 ng/g DW	HPLC	(Jinying et al., 2009)
Plant-based foods (Fruits and vegetables)						
Kiwi (*Actinidia chinensis*)	whole	N/A	N/A	0.02 ng/g FW	RIA and HPLC	(Hattori et al., 1995a)
Apple (*Malus domestica Borkh* cv. Red Fuji)	whole	1-2 weeks	N/A	5 ng/g FW	HPLC	(Lei et al., 2013)
Grape (*Vitis vinifera* L. cv. Merlot)	fresh	N/A	N/A	3.9 ng/g FW	UPLC-MS/MS	(Vitalini et al., 2011)
Tomato (*Lycopersicon esculentum* var. Bond)	fruit	N/A	N/A	23.87 ng/g FW	GC-MS	(Sturtz et al., 2011)
Black olive (*Olea europaea*)	whole	Purchased from local market		0.01 ng/g DW	LC-MS/MS	(Kocadagli et al., 2014)
Nuts						
Pistachio (*Pistacia vera* L.) 4 varieties	whole	collected from natural habitat		226,900-233,000 ng/g DW	GC-MS	(Oladi et al., 2014)
Walnuts (*Juglans regia* L.) 6 varieties	whole	Purchased from local market		0.14-1.77 ng/g FW	HPLC-MS, LC-MS/MS, HPLC-ECD	(Tapia et al., 2013)
Coffee beans						
Coffee (*Coffea canephora*)	beans	Purchased from local market		5800 ng/g DW	HPLC/ (LC/ESI-MS	(Ramakrishna et al., 2012)
Beverages						
Beer	N/A	Purchased from local market		0.09 ng/mL	LC-MS/MS	(Kocadagli et al., 2014)
Wine (*Vitis*) (Cabernet Sauvignon)	N/A	Winemaking (Spain) according to international standard		0.23-14.2 ng/mL	ELISA, HPLC-MS/MS	(Rodriguez-Naranjo et al., 2011)
Gropello wines (*Vitis vinifera* L.)	N/A	Winemaking (Italy) according to international standard		5.2 ng/mL	UHPLCMS/MS	(Vitalini et al., 2011)
Food-related micro-ogranisms						
Yeast (*Saccharomyces cerevisiae*)	dried brewer	Obtained from Nestlé Purina (USA).		2.2 ng/g	HPLC	(Tan et al., 2014)
Mushroom (*Agaricus bisporus*)	whole	Collected from natural habitats		4300-6400 ng/g DW	RP-HPLC	(Muszynska et al., 2016)

1.3 Melatonin biosynthesis in plants

The amino acid tryptophan is the precursor for melatonin synthesis in all taxa. While plants and microorganisms have the ability to synthesise tryptophan via the shikimic acid pathway, animals have to acquire tryptophan as an essential amino acid through diet as this pathway is not present in them. This is the most likely reason why melatonin is present at relatively higher levels in plants compared to mammals (Zhao et al., 2019a). From tryptophan, melatonin biosynthesis consists of four enzymatic steps in all the organisms although divergencies of biosynthesis exists in diferent kingdoms (Zhao et al., 2019a). In animals, tryptophan is first hydroxylated to 5-hydroxytryptophan by enzyme tryptophan hydroxylase (TPH) followed by acetylation to serotonin by aromatic amino acid decarboxylase (AADC) (Tan et al., 2016). In contrast, the pathway has begun to be deduced just recently in plants and appears to be more complicated than in animals (Byeon et al., 2014b, Byeon et al., 2016a). The major difference between plant and mammalian biosynthetic pathways of melatonin is the reverse order of first two steps. In plants, decarboxylation of tryptophan is followed by hydroxylation of tryptamine to form the key intermediate, serotonin. *N*-acetlyation of serotonin to *N*-acetlysetrotonin by Serotonin *N*-acetyltransferase (SNAT) occurs which is followed by the formation of melatonin by enzyme Acetyl-*O*-methyltransferase (ASMT).

Sites of melatonin synthesis

There are six enzymes present in the melatonin biosynthetic pathways. These are tryptophan-5-hydroxylase (TDC); tryptophan-5-hydroxylase (T5H), SNAT, ASMT, caffeic acid-*O*-methyl transferase (COMT) and *N*-acetylserotonin deacetylase (ASDAC) (Figure 1.2) (Back et al., 2016). A reversible reaction also exists in the melatonin biosynthetic pathway in *Arabidopsis* and rice (*Oryza sativa*) plants in which the enzyme *N*-acetylserotonin deacetylase (ASDAC) catalyzes the conversion of *N*-acetylserotonin (NAS) to serotonin thereby restricting the synthesis of melatonin. This reverse reaction ensures that an optimum balance of melatonin production is maintained that is crucial for efficient growth and development in plants (Lee et al., 2018). The subcellular localisation of melatonin biosynthetic enzyme TDC was reported in the cytoplasm in the leaves of the dicotyledonous Madagascar periwinkle (*Catharanthus roseus* L. G. Don) and eudicotyledonous Coffee rose (*Tabernaemontana divaricata* L. R. Br. Ex roem. Et Schult) (Stevens et al., 1993). The localisation of T5H enzyme was investigated in the leaves of the monocotyledonous rice plant (*Oryza sativa* Japonica cv. Kinmaze) and shown

to be localised in the endoplasmic reticulum (Fujiwara et al., 2010). The SNAT has been shown to be a chloroplastic enzyme tested in the leaves of a variety of plant species such as rice (*O. sativa*), dicotyledonous *Arabidopsis* and grapevine (*Vitis vinefera* L.) and non-flowering plant loblolly pine (*Pinus taeda*) (Byeon et al., 2014b, Lee et al., 2014, Park et al., 2014). The ASMT and COMT enzymes have shown to localise in the cytoplasm in both rice and *Arabidopsis* (Byeon et al., 2014a, Byeon et al., 2014b). Moreover, the recently identified enzymes ASDAC involved in the reversible reaction of melatonin biosynthesis are shown to localise in chloroplasts in both rice and *Arabidopsis* (Lee et al., 2018). Based on the enzyme kinetics, two types of pathways for melatonin synthesis have been proposed which are same until the point of serotonin synthesis, but diverge after that (Back et al., 2016). Pathway I may occur under normal growth conditions where serotonin is *N*-acetylated by SNAT to form *N*-acetylserotonin which is then *O*-methylated by ASMT to form melatonin. On the other hand, pathway II may dominate under stressful conditions such as senescence or cadmium stress when serotonin levels are increased. This involves *O*-methylation of serotonin by ASMT to form 5-methoxytryptamine which is *N*-acetylated by SNAT to produce melatonin (Back et al., 2016, Ye et al., 2019). The final sub-cellular sites of melatonin synthesis are determined by the subcellular localisations of the enzymes involved in the final step and the type of pathway route i.e. pathway I (final enzyme ASMT) leads to synthesis of melatonin in cytoplasm and pathway II (final enzyme SNAT) in chloroplasts (Back et al., 2016).

1.3.1 Melatonin biosynthetic pathway genes in plants

The genes encoding the melatonin biosynthetic enzymes have been identified in plants and are nuclear-encoded (Lee et al., 2014, Back et al., 2016, Byeon et al., 2016a). *SNAT, ASMT* and/or *COMT* have been extensively studied for their role in synthesising melatonin in plants. The effects of the genetic modification of the melatonin biosynthetic genes in plants are summarised in Table 1.2

Serotonin N-acetyltransferase (SNAT)

Serotonin *N*-acetyltransferase (SNAT) belongs to GCN5-related N-acetyltransferase (GNAT) family in plants. The nuclear-encoded *SNAT* gene has been identified and cloned in a variety of plant species including rice (*O. sativa*), *Arabidopsis* and grapevine (*V. vinifera*) (Kang et al., 2013, Lee et al., 2014, Yu et al., 2019). The *SNAT* gene has been

found to be highly conserved across the plant kingdoms and lineages based on sequence and phylogenetics analyses (Kang et al., 2013). For example, the progenitor of chloroplasts, cyanobacterium (*Synechocystis* sp. PCC 6803) *SNAT* gene (*csSNAT*) shows 56% amino acid identity with that of rice (*O. sativa*) (Byeon et al., 2013). Much of the information about the *SNAT* gene comes from its characterization in rice (*O. sativa*) and *Arabidopsis* at the enzymatic and transcript level (Kang et al., 2013). Plants contain two *SNAT* isogenes where *SNAT1* genes have been well-studied (Back et al., 2016). The presence of *SNAT2* genes has been recently demonstrated in rice (*O. sativa), Arabidopsis* and grapevine (*V. vinifera L.).* The amino acid sequences of these *SNAT2* genes are 48-55% identical between rice, *Arabidopsis* and grapevine plant species (Byeon et al., 2016b, Lee et al., 2019, Yu et al., 2019).

Expression patterns and functional role

The *SNAT* isogenes have distinct expression patterns where *AtSNAT1* (*AT1G32070*) is highly expressed in the mature leaves and *AtSNAT2* (*AT1G26220*) has a high expression in the flowers (Lee et al., 2019). Recently, AtSNAT1 encoded by *AtSNAT1* has been shown to play an integral role in light state transitions in *Arabidopsis* by post-translationally modification (acetylation of lysine residues) of chloroplast and photosynthesis-related proteins. This activity is lost in the T-DNA insertional *atsnat1* mutant (SALK_020577) plants of *AtSNAT1 (AT1G32070)* (Koskela et al., 2018). In another study, the *atsnat1* (SALK_020577) mutant is also significantly more suceptible to damage caused by high light intensity, than wild-type (Lee and Back, 2018). Moreover, ectopic overexpression of the *AtSNAT1* gene in *Arabidopsis* has shown to enhance growth and tolerance to stresses such as pathogenic infection from biotrophic bacterium *Pseudomonas syringae,* cadmium, salt and senescence (Lee and Back, 2017, Zhao et al., 2019b).

1.3.2 Metabolism of melatonin

Multiple reports show that melatonin is an intermediate rather than a final product in the sysnthesis of indole compounds in plants (Figure 1.2). After being biosynthesised in plants, melatonin is converted into other metabolites which include 2-hydroxymelatonin, cyclic-3-hydroxymelatonin and N^1-acetyl-N^2-formyl-5-methoxyknuramine (AFMK) (Hardeland, 2017). The formation of these metabolites is either through enzymatic processes or direct interactions between melatonin and ROS. It is believed that during oxidative stresses such

as caused by ultra-violet (UV) light stress, the metabolism of melatonin occurs through direct interactions between melatonin and ROS (Tan et al., 2015). (Yu et al., 2018). The enzymes, melatonin-2-hydroxylase (M2H) and melatonin-3-hydroxylase (M3H) which are the members of 2-ODD (2-oxoglutarate-dependent dioxygenase) superfamily have been shown to hydroxylate melatonin to form metabolites, 2-hydroxymelatonin and cyclic-3-hydroxymelatonin in rice (*O. sativa*) (Byeon and Back, 2015, Lee et al., 2016). The metabolite AFMK is thought to be produced via the catalysis of indoleamine 2,3-dioxygenase (IDO) enzymes. A study conducted in 24 different plant species including rice (*O. sativa*) cv. Dongjin has reported that 2-hydroxymelatonin is the dominant metabolite of melatonin and accumulates at much higher levels (13-fold) than melatonin (Byeon et al., 2015). The physiological signficance of melatonin metabolites in plants is an emerging topic of much interest. The positive role of 2-hydroxymelatonin treatment in enhancing resistance to multiple stresses (cold, drought, cadmium) has been recently shown in rice (*O. sativa*) cv. Dongjin and cucumber (*Cucumis sativus*) seedlings. An important mechanism for stress tolerance by 2-hydroxymelatonin was shown to be the enhancement of antioxidant enzymatic activities (Lee and Back, 2016a, Shah et al., 2020a, Shah et al., 2020b). The metabolite, 2-hydroxymelatonin has also been shown to activate mitogen-activate protein kinase (MAPK) signalling in *Arabidopsis*, albeit with lesser degree than melatonin, and is likely to play a role in melatonin-mediated defence to biotrophic bacterial pathogen, *Pseudomonas syringae* (Lee and Back, 2016b). The contribution of other melatonin metabolites, cyclic-3-hydroxymelatonin and AFMK in enhancement of stress resistance remains unexplored. However, it has been recently shown that stable transgenic rice plants overexpressing the *M3H* gene encoding enzyme for cyclic-3-hydroymelatonin production resulted in growth phenotypes in the form of increased panicles and secondary tiller numbers. (Choi and Back, 2019). Melatonin metabolites thus have the potential for enhancing agronomic traits as well as imparting stress resistance in plants.

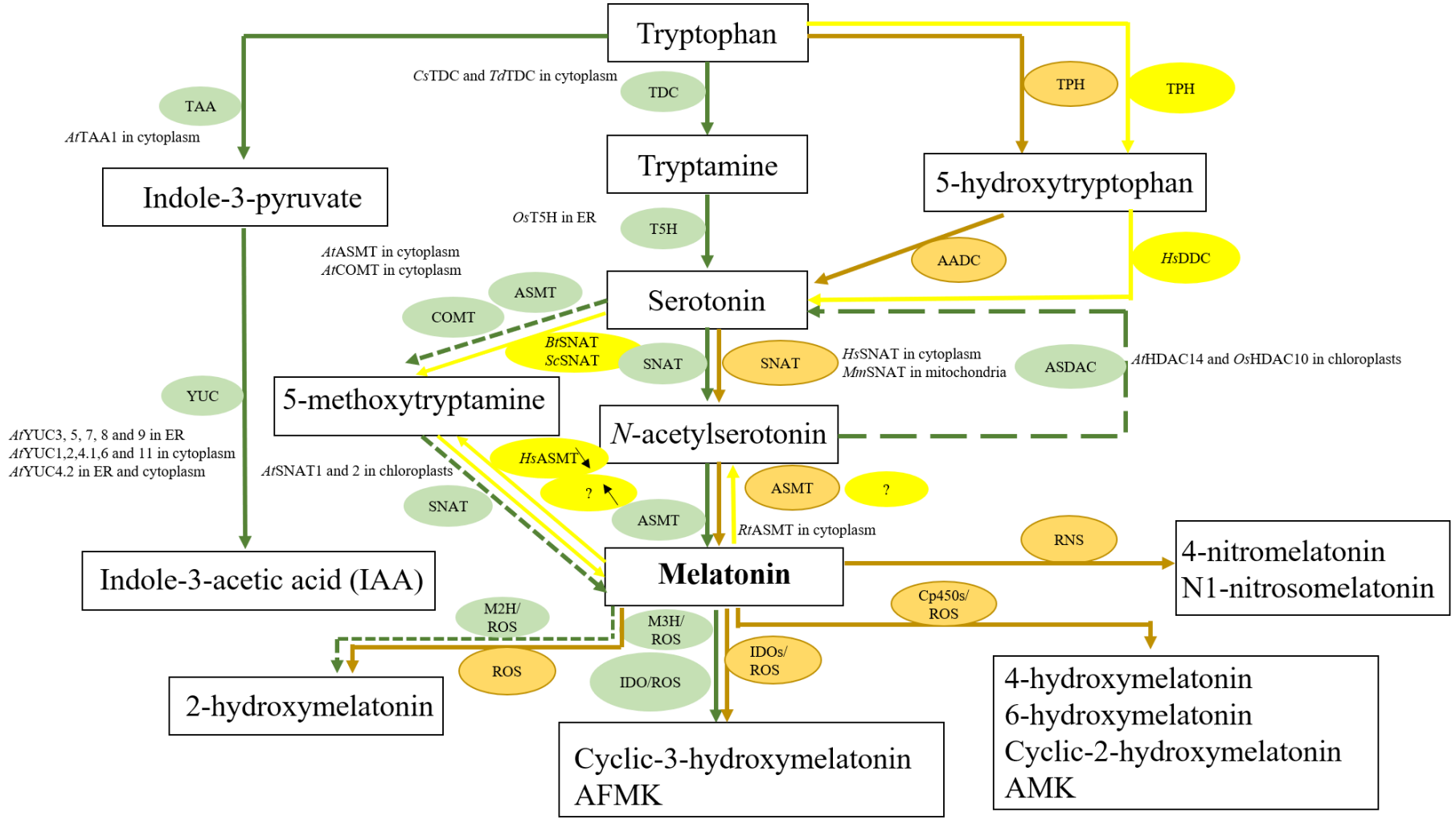


Figure 1.2: The biosynthetic and metabolic pathway of melatonin in plants (green) in comparison with mammals (brown) and heterologous expression in yeast (yellow)

All the biosynthetic enzymes are represented in oval shape. The enzymes that are not yet known are indicated by a question mark. The subcellular localisation of enzymes is stated in text beside each enzyme, where known. Solid arrows (green) represent pathway I. Square dotted lines (green) represent alternate pathway (Pathway II). Long dash lines (green) represent reverse reaction for melatonin biosynthesis.

Figure 1.2 (contd): The enzymes involed in synthesis and metabolism are: TDC, tryptophan decarboxylase; T5H, tryptophan-5-hydroxylase; SNAT, serotonin *N*-acetyltransferase; ASMT, *N*-acetylserotonin methyltransferase; COMT, caffeic acid *O*-methyltransferase; ASDAC, *N*-acetylserotonin deacetylase. TAA= tryptophan aminotransferase; YUC=; YUCCA flavin monnooxygenase; Cp450s=cytochrome P450s; DDC=dope decarboxylase; TPH=tryptophan hydroxylase; IDOs=indoelamine 2,3-dioxygenase; AADC= aromatic amino acid decarboxylase; HDAC=histone deacetylase; M2H=melatonin-2-hydroxylase and M3H=melatonin-3-hydroxylase.

Species abbreviations are *At=Arabidopsis thaliana*; Cs=*Catharanthus roseus; Os=Oryza sativa*; *Td= Tabernaemontana divaricata; Hs=Homo sapiens*; Bt= *Bos taurus*; *Rt=Rattus norvegicus domestica* and Mm=*Mus musculus*. Other abbreviations include ER=Endoplasmic reticulum; ROS=reactive oxygen species, RNS=reactive nitrogen species; AFMK=N^1-acetyl-N^2-formyl-5-methoxykynuramine and AMK=N^1-acetyl-5-methoxykynuramine (Stevens et al., 1993, Ganguly et al., 2001, Stepanova et al., 2005, Kang et al., 2007, Fujiwara et al., 2010, Kang et al., 2013, Byeon et al., 2014b, Lee et al., 2014, Back et al., 2016, Byeon et al., 2016a, Kriechbaumer et al., 2016, Rath et al., 2016, Choi et al., 2017, Lee et al., 2018, Yu et al., 2018, Muniz-Calvo et al., 2019).

Table 1.2: Effect of genetic perturbation of melatonin (MT) biosynthetic genes.

Plant	**MT biosynthetic genes (mutation and/or gene silencing)**	**Type**	**Effects and mechanisms (Relative to WT)**	**References**
Rice (*Oryza sativa*)	*tdc-1, tdc-3*	T-DNA insertion	Stunted growth, dark brown leaves, high serotonin levels	(Kanjanaphachoat et al., 2012)
	tdc; t5h; comt	RNAi	Decreased MT levels; semi-dwarf phenotypes, lower expression levels of BR-biosynthetic *DWARF4* gene; IAA contents unaffected	(Lee and Back, 2019)
	snat1+snat2	RNAi	Retarded seedlings growth, decreased MT levels; accelerated seed aging	(Hwang and Back, 2020)
Thale cress (*Arabidopsis thaliana*)	*snat1* (*snat* (SALK_020577))	T-DNA insertion	Smaller leaf sizes and lower root biomass; MT levels unaffected; decreased SA levels; susceptible to *(Pst-avrRpt2)* infection; downregulation of expression of biotic defence-related genes *PR1, ICS1, PDF1.2*	(Lee et al., 2015)
	snat1 (*snat* (SALK_020577))	T-DNA insertion	No distinct phenotype; Inability to undergo 'state transitions' in response to illumination; inability to post-translationally modify (i.e. acetylate photosynthesis-related genes	(Koskela et al., 2018)
	snat1 (*snat* (SALK_020577))	T-DNA insertion	High sensitivity to iron (Fe) deficiency	(Wan et al., 2018)
	snat2 (SALK_062388)	T-DNA insertion	Reduced MT levels (50%) in flowers only; delayed flowering; reduced leaf area; decreased expression of GA-biosynthetic gene, *ent-kaurene synthase* (KS); enhanced expression of GA-responsive negative TF *MYB33*	(Lee and Back, 2019)

Table 1.2: contd.

Plant	MT biosynthetic genes (over-expressors)	Effects and mechanisms (Relative to WT)	References
Rice *(Oryza sativa)*	*OsSNAT1*	Increased MT levels; increased grain yield; resistance to cadmium (0.2 mM $CdCl_2$) and senescence	(Lee and Back, 2017)
Thale cress (*Arabidopsis thaliana*)	*MzSNAT1*	Enhanced salt tolerance; enhanced expression of genes ion homeostasis-related genes, *sodium hydrogen exchanger 1* (*NHX1*) and *salt overly sensitive1* (*SOS1*).	(Zhao et al., 2019b)
Thale cress (*Arabidopsis thaliana*)	*MsSNAT*	Inhibition of the JA-induced anthocyanin biosynthesis	(Ai and Zhu, 2018)
Tomato (*Solanum lycopersicum*)	CaMV35S::*SlSNAT*	Higher MT levels; enhanced heat (42°C for 3-5 hrs) tolerance; decreased ROS levels; upregulated expression of heat shock-responsive transcription factors	(Wang et al., 2020a)
Thale cress (*Arabidopsis thaliana*)	CaMV35S::*TaCOMT*	Higher MT levels; enhanced drought (100 mM D-Mannitol) tolerance; higher proline content; reduced MDA levels	(Yang et al., 2019)
Thale cress (*Arabidopsis thaliana*)	UBQ::*MzASMT1*	Higher MT levels; lower ROS levels; enhanced drought (water withheld) tolerance	(Zuo et al., 2014)
Thale cress (*Arabidopsis thaliana*)	*MzASMT9*	Higher MT levels , enhanced salt tolerance and photosynthetic rate; lowered MDA levels	(Zheng et al., 2017)
Cassava (*Manihot esculenta*)	CaMV35S:*MeTDC1*, *MeTDC2, MeSNAT*, *MeT5H*, *MeASMT1*, *MeASMT2* and *MeASMT3*	Regulation of expression levels of plant-immunity-related genes *PR1*, *PR2*, *PR5*	(Wei et al., 2018b)

The mutants and overexpressing lines in *Arabidopsis thaliana* are in the background ecotype (Col-0). MT=melatonin; RNAi=RNA interference; TF=Transcription factor; *Ms*=alfalfa (*Medicago sativa* L. cv. Biaogan); *Ta*=wheat (*Triticum aestivum* L.); *Mz*=apple (*Malus zumi* Mats); MDA=Malondialdehyde; T-DNA=Transfer-DNA; UBQ=Ubiquitin promoter; CaMV35S=Cauliflower mosaic

1.4 Biological roles

1.4.1 Melatonin regulates growth and developmental aspects

There are several factors (stimuli) such as external (environmental cues) and/or internal (growth regulators, hormones) that can affect the growth and development throughout the lifecycle of plants. The use of plant growth regulators (PGRs) both naturally-occurring or their synthetic analogues started in 1930's (Rademacher, 2015). These have been employed in agriculture for regulating aspects such as increased growth, seed dormancy, germination, flowering, fruiting and acceleration or delaying of senescence, among others (Rademacher, 2015). Commonly used PGRs are the classical plant hormones such as auxin, cytokinins, gibberellins, ethylene and abscisic acid that are used as active ingredients. Some examples for the commercial product names for these are Rhizopon AA and Obsthormon 24a (auxins), ProGibb (gibberellins), Maxcel and Fulmet (cytokinins), Ethrel and Cyrone (ethylene) and ProTone (abscisic acid) (Rademacher, 2015).

Melatonin was first demonstrated to exhibit plant growth regulating properties in *Lupinus albus* L. (Hernández-Ruiz et al., 2004). Since then the effect of melatonin in regulating morphogenesis and developmental processes has been shown in a variety of plants (Figure 1.3) (Table 1.3). Most of the studies are effect-based where melatonin was applied exogenously, although the regulation of growth and developmental processes has also been shown by changes in the melatonin internal levels over the course of time, upon a particular stress or by molecular transgenic approaches.

1.4.1.1 Growth-related aspects

Many studies have shown the effects of exogenous melatonin in improving growth in both aerial and below-ground parts of plants of a variety of species such as *Brassica juncea*, *O. sativa*, *Arabidopsis*, *Glycine max,* and *Cucumis sativus* (Chen et al., 2009, Park and Back, 2012, Zhang et al., 2013, Bajwa et al., 2014, Wei et al., 2015). The increased growth response in biomass (wet or dry weight), height (e.g. whole plant), length (e.g. root), area (e.g. rosette, leaf), and seed and lateral roots numbers, have been documented. Growth regulation caused by melatonin is dramatically enhanced, or is sometimes only observed, under stress conditions, e.g. high salt (NaCl), drought and cold (Shi et al., 2015b, Ke et al., 2018). An important point is the concentration-dependent effects of melatonin that

show marked variation between and within plant species. These are not just limited to the type of plant, but also the tissue studied, method of melatonin application and growth conditions among other factors (Wang et al., 2012, Bajwa et al., 2014, Hardeland, 2016, Wang et al., 2019, Chen et al., 2020). For example, the variation has been profoundly seen for in vitro studies conducted with *Arabidopsis* where the overall growth promoting effect of melatonin application in terms of increase in primary root length, fresh weight and lateral root numbers in seven to fourteen day-old seedlings is in the concentration range of 0.1 µM – 50 µM (Bajwa et al., 2014, Hernández et al., 2015, Shi et al., 2015d). In contrast, similar effects on lateral roots have been reported at a much higher concentration range of melatonin (100 – 600 µM) (Pelagio-Flores et al., 2012). The cause of the variation between the studies is unknown. It is also crucial to consider and report the important experimental fine details, such as the choice of solvent and its inclusion as a control, growth conditions and the genotype of the plant, all of which could affect the results (Janeczko, 2011, Hernández et al., 2015, Hardeland, 2016).

Above-ground tissues

Exogenous melatonin, like auxin, stimulates coleoptile length in wheat (*Triticum aestivum* L.), oat (*Avena sativa* L.), barley (*Hordeum vulgare* L.) and canary grass (*Phalaris canariensis* L.) in a concentration-dependent manner (0.01-100 µM) (Hernández-Ruiz et al., 2005). This observation generated the hypothesis in the phytomelatonin research community that melatonin acts like an auxin (a plant hormone also derived from tryptophan). This hypothesis has since then garnered much debate and confusion in the literature. This will be discussed in detail in the research Chapter 2 of this thesis. Seed coating of soil-grown soybean plants (*Glycine max*, SuiNong 28, SN28) with melatonin (50 and 100 µM) resulted in increased leaf sizes (leaf area (cm^2), area of unifoliate leaves (cm^2)) and plant height (Wei et al., 2015). In *Arabidopsis*, exogenous melatonin applied through in vitro solid growth medium specifically targets the rosette leaves at higher concentrations (500 – 1000 µM), with resulting reduced growth as commonly measured in the form of leaf area (Bajwa et al., 2014, Hernández et al., 2015, Wang et al., 2017). The leaf development at higher concentration is a result of reduced cell proliferation, in terms of cell size and number, inhibited rate of cell division, and delay in the onset of endoreduplication (the process of DNA replication in the absence of mitosis which can lead to organ size enlargement). However, the negative regulation of leaf development in *Arabidopsis* by melatonin is only to put the plant on a potential 'stand-by mode' as is also the case with exogenous treatment of plant hormone, jasmonate (Noir et al., 2013).

Melatonin-treated plants maintain their potential to recover once the external treatment is removed, as melatonin upregulates the transcripts of several of histone and ribosomal genes such as genes encoding for histone H2A (*HTA10)*, erbb-3-binding protein 1 (*EBP1)* and ribosomal protein S6 (*RPS6A).* These proteins play roles in DNA replication and repair (Wang et al., 2017).

Below-ground tissues

Lateral roots form an integral part of the entire root system for the plants to grow and survive. They serve as the main contact between the plant and the soil to provide anchorage and acquire water and nutrients (Tian et al., 2014). Melatonin (50 µM) when applied through in vitro solid growth medium increases lateral root numbers by enhancing the expression of the key lateral root marker and cell-wall remodelling genes such as LOB-domain protein gene, *LBD16* and *xyloglucan endotransglucosylase*, *XTR6* in *Arabidopsis* (Wan et al., 2018). Foliar spray application of melatonin (100 µM) resulted in enhanced primary root elongation of maize (*Zea mays* L. cv. Zhengdan 958) seedlings under limited watering-induced drought stress by upto 44% compared to no-melatonin control (Ahmad et al., 2019). Moreover, melatonin treatment (1 and 10 µM) through in vitro growth medium mitigated the negative effects of aluminium (0-200 µM $AlCl_3$) on root growth of *Arabidopsis* by reducing the nitric oxide production which was the contributory factor to aluminium toxicity (Zhang et al., 2019b). It is commonly accepted that lateral root formation is dependent on the plant hormone, auxin as its application (in the form of IAA, indole-3-acetic acid; NAA, 1-naphthalene acetic acid and 2,4-D, 2,4-dichlorophenoxyacetic acid) stimulates the number of lateral roots in a variety of plant species including *Arabidopsis* (Blakely et al., 1988). The role of auxin in melatonin-mediated lateral root formation has been the subject of investigation but with contrasting reports. Two reports show that the induction of lateral roots in *Arabidopsis* by melatonin is dependent on auxin signalling by the negative regulation of auxin biosynthesis and polar auxin transport (PIN-formed (PIN1/3/7) (Wang et al., 2016, Ren et al., 2019b). On the contrary, Pelagio-Flores et al., demonstrate that the effect of melatonin on *Arabidopsis* root architecture is appreciably independent of auxin signalling (Pelagio-Flores et al., 2012). While auxin promotes lateral root formation by inhibiting the primary root (Rahman et al., 2007), the same effect by melatonin is relatively poorly-established (Pelagio-Flores et al., 2012, Bajwa et al., 2014, Hernández et al., 2015, Wan et al., 2018). In fact, the role of ROS signalling, particularly H_2O_2 in melatonin-mediated lateral root formation is demonstrated in alfalfa (*Medicago sativa*), tomato (*Solanum lycopersicum*)

and *Arabidopsis* seedlings (Chen et al., 2018b, Chen et al., 2019a). Recently, melatonin was also shown to enhance nitrogen fixation in soybean (*G. max*) by increasing nodulation (Ren et al., 2019a).

1.4.1.2 Developmental-related aspects

Melatonin is also known to affect many natural and stress-affected vital developmental processes in plants such as germination, alteration of flowering time, fruit ripening and senescence (Arnao and Hernández-Ruiz, 2015).

Seed germination

Positive effects of melatonin in enhancing seed germination have been extensively reported in a variety of plant species (Park and Back, 2012, Zhang et al., 2013, Hernández et al., 2015, Cao et al., 2019). Melatonin application (1000 µM) in vitro resulted in improved seed viability and germination after heat stress by up to a 60% as compared to the untreated stressed seeds in *Arabidopsis* (Hernández et al., 2015). In cucumber (*Cucumis sativus* L.), melatonin-treated seeds (100 µM) exhibited improved germination rate upon polyethylene glycol-induced drought (Zhang et al., 2013). Seeds of edible plants such as mustard (*Brassica nigra; Brassica hirta*) contain the highest melatonin content ranging from 2 to 200 ng/g dry weight (Manchester et al., 2000) (Table 1.1). Since seeds are particularly vulnerable to stresses, presence of melatonin at high levels in seeds may play a vital role in the antioxidant defence system by protecting the germ tissue of seeds from oxidative damage due to its high ROS scavenging ability. This has been recently shown where exogenous melatonin (20 µM) improved seed germination in cotton (*Gossypium hirsutum* L.) by enhancing the antioxidant enzymatic activities of superoxide dismutase and peroxidase. Moreover, melatonin also regulated the content of gibberellins (GAs) and abscisic acid (ABA) which are the plant hormones essential in seed germination process (Xiao et al., 2019).

Flowering

The first involvement of melatonin in plant reproductive growth was demonstrated in the short-day plant, *Chenopodium rubrum* where melatonin application delayed flowering (Kolář et al., 1999). Since then the alteration of flowering time by melatonin has been reported in a range of higher plants such as rice (*O. sativa*), apple (*Malus pumila* Mill.) and *Arabidopsis* (Byeon and Back, 2014, Shi et al., 2016b, Zhang et al., 2019a). Melatonin treatments (500 and 1000 µM) have shown to repress the floral transition in *Arabidopsis*

(Landsberg ecotype) plants by stabilizing the DELLA proteins and inducing the transcription of *FLOWERING LOCUS C* (*FLC)* both of which are strong repressors of plant flowering (Shi et al., 2016b).

Fruit ripening

The role of melatonin in delaying flowering and senescence also indicates a role in increasing the shelf life of fruits and vegetable crops (Sun et al., 2015, Hu et al., 2017, Xu et al., 2018, Xia et al., 2020). Exogenous melatonin treatments as an immersion or soaking solution of fruits improved the post-harvest preservation (Hu et al., 2017, Gao et al., 2018, Liu et al., 2019b). This topic is reviewed in detail by (Xu et al., 2019). Briefly, melatonin application (200 and 500 μM) delay the ripening of banana (*Musa acuminata*) varieties postharvest by elevating endogenous melatonin content and reducing the production of ethylene (ET) which otherwise stimulates the ripening process (Hu et al., 2017). Similarly, melatonin-treated (100 μM) peach fruit had lower chilling injury and reduced flesh browning compared to untreated fruit. The effect was shown to be due to increased phenolic anabolism and induced enzymatic activities of polyphenol oxidase (PPO) and peroxidase (POD) upon melatonin treatment (Gao et al., 2018).

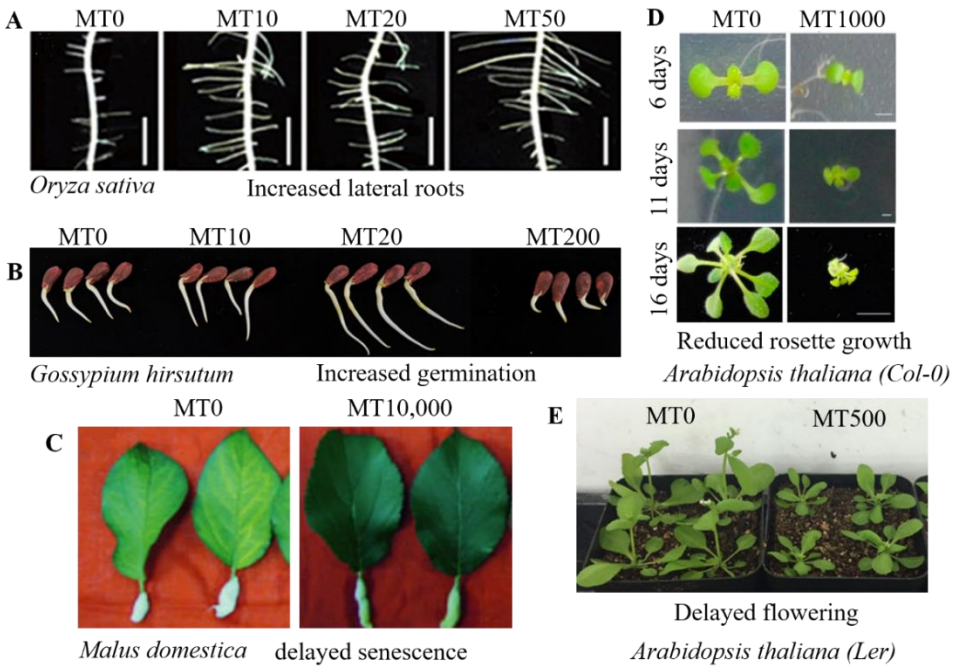


Figure 1.3: Effect of exogenous melatonin (MT) on the growth and development in different plant species. All concentrations are in micro molar (μM). (A) rice (*Oryza sativa* ssp. *Japonica*) (Liang et al., 2017); (B) cotton (*Gossypoum hirsutum* L.) (Xiao et al., 2019); (C) apple (*Malus domestica* Borkh. cv. Golden Delicious) (Wang et al., 2012); (D-E) Thale cress (*Arabidopsis thaliana*) (Shi et al., 2016b, Wang et al., 2017).

Table 1.3: Growth and developmental effects of exogenous melatonin (MT) application in selected plant species

Plant	Age	Concentration (MT)	Application method (MT)	Effect	References
Growth					
Monocotyledons					
Oat (*Avena sativa L.*)	4	0.01 µM	Incubation of seedlings in MT-containing liquid (24 hrs)	▲Coleoptile elongation	(Hernández-Ruiz et al., 2005)
Rice (*Oryza sativa*)	NA	0.5 and 1 µM	Direct germination on MT-containing agar medium (4 days)	▲ Seminal root growth	(Park and Back, 2012)
Dicotyledons					
Lupin (*Lupinus albus L.*)	6	10 µM	Incubation in MT-containing buffer solution (24 hrs)	▲Hypocotyl elongation	(Hernández-Ruiz et al., 2004)
Lupin (*Lupinus albus L.*)	2	0.1 &1 µM	Etiolated cotyledons placed in MT-containing liquid medium	▲Cotyledon expansion	(Hernández-Ruiz and Arnao, 2008)
Brown mustard (*Brassica juncea*)	2	0.1 µM	Etiolated seedlings incubated (24 hrs) in MT solution	▲Root growth	(Chen et al., 2009)
Thale cress (*Arabidopsis thaliana*)	NA	10-40 µM	Direct germination on MT-containing agar medium (12 days)	▲Fresh weight and primary root length	(Bajwa et al., 2014)
Thale cress (*Arabidopsis thaliana*)	NA	150 – 600 µM	Direct germination on MT-containing agar medium (10 days)	▲Lateral root number	(Pelagio-Flores et al., 2012)
Thale cress (*Arabidopsis thaliana*)	1	600 & 1000 µM	Germination on MT-containing agar medium (6 days)	▼Leaf area	(Wang et al., 2017)
Thale cress (*Arabidopsis thaliana*)	NA	200 µM	Direct germination on MT-containing agar medium	▼Fresh weight and Primary root length	(Bajwa et al., 2014)
Soybean (*Glycine max*)	NA	50 and 100 µM	Seeds coated with MT (37 and 90 days)	▲Pods and seeds number, leaf area and plant height	(Wei et al., 2015)

Table 1.3: contd.

Plant	Age	Concentration (MT)	Application method (MT)	Effect	References
Development					
Monocotyledons					
Barley (*Hordeum vulgare L.*)	12	1 mM	Incubation in MT-containing buffer solution (48 hr)	▼Senescence	(Arnao and Hernández-Ruiz, 2009b)
Dicotyledons					
Cucumber (*Cucumis sativus L.*)	N/A	100 µM	Sterilised seeds primed for 12 hrs	▲Germination	(Zhang et al., 2013)
Soybean (*Glyxine max*)	N/A	50 and 100 µM	Seeds coated with MT	▲Germination	(Wei et al., 2015)
Apple (*Malus domestica*)	730	10 mM	Detached leaves treated with MT solution (4, 8, 12 and 16 days)	▼Senescence	(Wang et al., 2012)
Peach fruit (*Prunus persica*)	135	100 µM	Immersed in MT-containing solutions (2 hrs)	▼Post-harvest internal browning	(Cao et al., 2018)
Alfalfa (*Medicago sativa)*	3	1, 10 and 50 µM	Treatment on MT-containing medium (60 hrs)	▲Lateral root primordia	(Chen et al., 2018a, Chen et al., 2018b)
Thale cress (*Arabidopsis thaliana*)	N/A	100 and 200 µM	Direct germination on MT-containing agar medium (7 days)	▼Lateral root primordia	(Pelagio-Flores et al., 2012)
Sour cherry (*Prunus cerasus L.*)	N/A	1 µM; 10 µM	Shoot explants grown in MT-containing solution (11 weeks)	▼Adventitious root regeneration	(Sarropoulou et al., 2012)
Eudicotyledons					
Red goosefoot (*Chenopodium rubrum L.*)	5	100 and 500 µM	MT solutions were applied to the cotyledons and plumules (12 hr)	▼Flowering	(Kolář et al., 1999)

1.4.2 Stress tolerance

The growth and yield of plants are adversely affected by the negative impacts of environmental stresses. Plants have developed sophisticated mechanisms to activate various kinds of growth regulating molecules and subsequent signalling processes to counteract the effects of stresses (Mosa et al., 2017). Exogenously applied melatonin has resulted in tolerance to a wide range of stresses such as salinity, drought, extreme temperatures, heavy metals, UV and pathogenic infection. The effects of melatonin and the underlying mechanisms are summarized in Table 1.4.

Climatic extremes such as heat, cold and chilling negatively alter membrane proteins and enzymatic activities leading to abnormalities in growth and development of plants (Sung et al., 2003). Drought and salinity-induced osmotic and ionic toxicity stresses cause growth retardations particularly impairment of seed germination and a significant decline in the photosynthetic rate (Golldack et al., 2014).

Toxic heavy metals such as arsenic, lead and cadmium in the environment can lead to deleterious effects such as plant cell damage (Nagajyoti et al., 2010). The resulting damage leads to excessive overproduction of ROS molecules in the form of oxidative stress that is damaging to the cell components (Demidchik, 2015).

Up to 20 – 40% of crop losses occur as a result of pathogenic diseases, as is evident from some of the historically significant plant disease epidemics in the world, such as the late blight epidemic that led to the Irish potato famine (Savary et al., 2012, Goss et al., 2014). Efforts are continuously being made to develop cultivars resistant to disease and/or develop applications such as bio-stimulators to combat plant disease (Yakhin et al., 2016). Apart from conferring resistance to abiotic stresses, melatonin also leads to enhanced immunity to biotic stresses caused by fungal, bacterial, and viral plant pathogens spanning biotrophic, necrotrophic and hemibiotrophic pathogenic lifestyles (Moustafa-Farag et al., 2019). The applicability of melatonin in negating the deleterious effects of stresses in agricultural crops is promising.

Table 1.4: Effect of exogenous melatonin (MT) in alleviating abiotic and biotic stresses in selected plants species.

Stress	Plant	(MT)	Application method (MT)	MT effect and mechanisms	References
Abiotic					
Heat (37°C ~ 2 hours)	Thale cress (*Arabidopsis thaliana*)	5 and 20 µM	Supplementation in growth medium	▲Heat tolerance; transcript levels of heat-shock transcription factors (*AtHSFA1s*); heat-stress responsive genes (*HSP90, HSA32*)	Shi et al., 2015
Cold (2°C ~ 3 days)	Wheat (*Triticum aestivum*)	1 mM	Foliar spray	▲Cold tolerance; membrane stability; antioxidant enzyme activity	Sun et al., 2018
Drought (PEG-induced)	Grapevine (*Vitis vinifera*)	50, 100 and 200 nM	Immersed in MT-containing nutrient solutions	▲Drought tolerance;▼O_2^-, H_2O_2 contents; ▲proline content ; *Fv/Fm* ; improved ultrastructure of chloploasts; stomatal length and width	Meng et al., 2014
Drought (withholding watering)	Maize (*Zea mays* L.)	20 and 200 µM	Root irrigation	▲Drought tolerance and chlorophyll content proline content ;▼MDA content and electrolyte leakage	Huang et al., 2019
Salt (150 mM NaCl)	Naked oat (*Avena nuda* L.)	50 and 100 µM	Pre-treatment with MT-containing nutrient solutions	▲Salt tolerance and seedlings growth;▼MDA content;▲transcript levels of transcription factor-genes (*NAC, WRKY1, WRKY3*)	Gao et al., 2019
Light*	Thale cress (*Arabidopsis thaliana*)	10 – 1 mM	Incubated with MT-solutions	▲High light tolerance and chlorophyll content; ▼anthocyanin content, proline content and SOD enzymatic activity	Bychkov et al., 2019
Al (0 – 200 µM)	Thale cress (*Arabidopsis thaliana*)	0-10 µM	MT-supplemented agar media	▲ Aluminum tolerance, cell division and root growth	Zhang et al., 2019
Cd ($CdCl_2$100 µM)	Tobacco (*Nicotiana tabacum* L.)	100 µM	Foliar spray	▲Cd tolerance ; Cd sequestration; transcript levels of Cd-transport genes (*HMA2, HMA4, and HMA3*)	Wang et al., 2018
Paraquat (75 µM)	Pea (*Pisum sativum L.*)	50 and 200 µM	Seeds priming	▲*Fv/Fm* ; preservation of photosynthetic pigments	Szafrańska et al., 2016

Table 1.4: contd.

Stress	Plant	Melatonin	Melatonin application	MT effect and mechanisms	References
Pathogen					
Fungi					
Diplocarpon mali	Apple (*Malus prunifolia*)	0.05-0.5 mM	Root irrigation	▲Enhanced disease resistance; transcript levels of plant defence-related genes	Yin et al., 2013
Fusarium oxysporum	Banana (*Musa acuminata* L.)	100 µM	Root pre-treatment (2 days)	▲Enhanced disease resistance by regulating the epxression of *MaHSP90s*	Wei et al., 2017
Bacteria					
Pseudomonas syringae (pv.) tomato DC3000	Thale cress (*Arabidopsis thaliana*)	1-50 µM	MT-supplemented agar growth medium (5 days)	▲Disease resistance; cell wall invertase (CWI); hexose, cellulose, xylose and galactose expression of defence-related genes *PR1*, *PR5* and plant defensin *PDF1.2*	Zhao et al., 2015; Lee et al., 2014
Xanthomonas oryzae (pv.) *oryzicola*	Rice (*Oryza sativa*)	200 µg/mL	MT-supplemented growth medium	▲Disease resistance ;▼bacterial biofilm formation and swimming motility	Chen et al., 2019
Viruses					
Tobacco Mosaic Virus	Tomato *(S. lycopersicum)* and tobacco (*Nicotiana glutinosa*)	100 µM	Root irrigation (3 days)	▼virus concentrations ;▲SA concentrations ; expression levels of *PR1* and *PR5*	Zhao et al., 2019
Rice stripe virus	Rice (*Oryza sativa* L. *cv*.)	10 µM	Root irrigation (12 hours)	▲ disease resistance; MT content; NO-dependent pathways; expression levels of *OsPR1b* and *WRKY 45*	Lu et al., 2019
Oomycete					
Phytophthora infestans	Potato (*Solanum tuberosum* L.)	1-5 mM	MT-supplemented agar growth medium	▼mycelial growth ; pathogenecity stress tolerance of *P. infestans* to abiotic stresses ; metabolic processes	Zhang et al., 2017

MT = exogenous melatonin concentration; PEG = Polyethylene glycol. *Light energy flux = LEF (250 µE m−2 s−1). Al=Aluminium; Pb=lead. *Fv/Fm*= Chlorophyll fluorescence; ▲ indicate 'enhanced' and ▼indicate 'decreased'

1.5 Melatonin-mediated signalling

Basic understanding of molecular mechanisms of melatonin-mediated signalling has benefited from studies using 'omics' approaches where melatonin signalling has been shown to have a direct modulation of the transcriptome, proteome and metabolome of a range of plant species such as *Arabidopsis*, cucumber *(C. sativus*), bermudagrass (*Cynodon dactylon* L. Pers.) and rice (*O. sativa*) (Weeda et al., 2014, Shi et al., 2015b, Liang et al., 2017, Zhang et al., 2017b). However, the lack of knowledge about the melatonin receptor in plants was impeding the full understanding of melatonin signalling. A recent study was the first to report the discovery of a melatonin receptor (PMTR1/CAND2) in plants through a melatonin binding assay (Wei et al., 2018a). In this study, it was shown that stomatal closure regulated by melatonin was mediated by the candidate G-protein coupled receptor (PMTR1/CAND2) through H_2O_2 and calcium (Ca^2)-dependent signalling pathways in *Arabidopsis*. This discovery had opened new avenues of research in identifying the role of the receptor-dependent melatonin signalling in the physiological processes of plants. Although, a latest study contradicted and challenged some of the findings by Wei and colleagues such as the localisation of CAND2 and transcriptional levels of *CAND2* in the cand2 mutant, the weight of evidence provided is not satisfactory (Back and Lee, 2020). Firstly, the authors do not focus on the same experiments as that of Wei et al such as direct melatonin-CAND2 binding assays by yeast-two-hybrid and bimolecular fluorescence complementation, and stomatal assays. Secondly, the *cand2* mutant which is challenged for its authenticity has been previously reported to be a null mutant of *CAND2* as determined by reverse-transcriptase PCR (Jin et al., 2012). Without robust experiments, the contradiction provided by Back and Lee does not appear concrete. Overall, whether CAND2 is a melatonin receptor remains a question due to discrepancies between the two recent reports and more clarification studies are needed.

1.5.1 Melatonin-activated transcription factors

Transcription factors (TFs) are regulatory genes that play an integral role in plant stress responses and directly regulate the transcription of a set of downstream stress-responsive genes at a given time (Hoang et al., 2017). TFs are therefore potential candidates for genetic manipulation to enhance plant fitness (Century et al., 2008). Melatonin treatment in plants under stress has resulted in activation of several stress-related TFs. In

Arabidopsis, these include *Class A1 heat-shock factors* (*AtHSFA1a*), *Zinc finger of Arabidopsis thaliana* (*AtZAT6*) and *C-repeat/dehydration-responsive element binding factors* (*AtCBFs*) (Bajwa et al., 2014, Shi et al., 2015d, Shi et al., 2018). Briefly, expression levels of *class A1 heat-shock transcription factor (AtHSFA1a)*-activated genes, such as *heat-stress associated 32* (*HSA32*) and *heat-shock protein 90* (*HSP90*), are upregulated by melatonin (5 and 20 µM) and are shown to be essential for melatonin-mediated heat tolerance (Shi et al., 2015d).

In banana (*Musa acuminata* L.), melatonin (100 µM) upregulated the expression levels of *HSP90* to mediate disease resistance to *Fusarium* wilt (*Fusarium oxysporum* f. sp. cubense (Foc) (Wei et al., 2017). Differences due to monocotyledons (banana) and dicotyledonous plants (*Arabidopsis*) could explain the different melatonin functions in heat tolerance and disease resistance in both species concerning activation of HSP90 transcript levels (Wei et al., 2017). Moreover, the cysteine2/histidine2-type transcription factor *Zinc finger of Arabidopsis thaliana 6 AtZAT6*-activated cold-repeat binding factors (CBFs) in response to melatonin (20 µM) mediate cold and freezing tolerance (Bajwa et al., 2014, Shi and Chan, 2014). Apart from these transcription factors, genome-wide expression analyses have provided thousands of differentially expressed genes in response to melatonin that include many other transcription factors (Weeda et al., 2014, Zhang et al., 2014b, Wan et al., 2018)

1.5.1.1 RNA-Sequencing-based gene expression profiling in response to melatonin

RNA-Sequencing (RNA-Seq)-based global transcriptome profiling has shown that melatonin application regulates several differentially expressed genes (DEGs) in a range of plant species including *Arabidopsis*, rice (*Oryza sativa*), bermudagrass (*Cynodon dactylon* L. Pers.) and cucumber (*Cucumis sativum*) (Weeda et al., 2014, Zhang et al., 2014b, Shi et al., 2015b, Liang et al., 2017, Wan et al., 2018). Foliar spray application of melatonin at a high concentration (1000 µM) in *Arabidopsis* (ecotype Columbia (Col-0)) under normal growth conditions resulted in 1308 DEGs in the detached leaves amongst which the majority were annotated for their roles in plant defence to biotic and abiotic stress factors (Weeda et al., 2014). In another study, *Arabidopsis* (ecotype Columbia (Col-0)) seedlings grown on melatonin supplemented (10 µM and 50 µM) medium had a total

of 426 and 202 DEGs, respectively as compared to the untreated control. The DEGs included iron-deficiency responsive genes with marked induction (Wan et al., 2018). These are the only two studies (other than this author's publication) reporting the effects of exogenous melatonin on *Arabidopsis* transcriptome using RNA-Seq. Two additional studies are reported in the Chapter 2 (Zia et al., 2019) and Chapter 3 of this thesis. The effects of melatonin on transcriptome under abiotic stresses (drought and salt) have been reported in non-model plant species such as bermudagrass (*Cynodon dactylon* L. Pers.), cassava (*Manihot esculenta* Crantz) and soybean (*G. max*, SuiNong 28) (Shi et al., 2015b, Wei et al., 2015, Ding et al., 2019). Functional characterisation of these differentially expressed genes in response to melatonin are required to provide more details in understanding of the melatonin-mediated signalling under both normal and stressed growth conditions.

1.5.2 Crosstalk between melatonin and signalling molecule nitric oxide (NO)

The growth and defence responses conferred by melatonin involve many signalling molecules such as nitric oxide (NO) and sugars. Nitric oxide (NO) is an important gaseous small signalling molecule that is known for its numerous important functions such as plant growth and development, and plant defence (Hong et al., 2008). The interaction of melatonin and NO has shown to be both positive and antagonistic and is important for regulating growth and oxidative homeostasis (Mukherjee, 2019). Melatonin treatment has been shown to confer plant immunity to bacterial pathogen *Pseudomonas syringae* (pv.) tomato (*Pst*) DC3000 in *Arabidopsis* in a NO-dependent manner by activating sugars, glycerols and mitogen-activated protein kinases (MAPKs) cascades (Qian et al., 2015, Shi et al., 2015a). Melatonin-conferred tolerance to abiotic stresses such as cadmium, aluminium and heat has also shown to be NO-dependent (Jahan et al., 2019, Kaya et al., 2019, Zhang et al., 2019b). NO may thus act downstream of melatonin to contribute to plant defence against both biotic and abiotic stresses. The growth-related processes such as lateral root growth and delayed aging are also promoted by a functional cooperation between melatonin and NO (Wen et al., 2016). For example, NO is a downstream signal of melatonin-mediated adventitious root formation in tomato (*Solanum lycopersicum* L.) seedlings (Wen et al., 2016). An interesting aspect of melatonin and NO interaction is that melatonin can be nitrosated by NO at the nitrogen atom of indole ring in structure of melatonin (Williams, 2004). The nitrosated form of melatonin, referred to as *N*-

nitrosomelatonin (Nomela) is a unique signalling molecule that has potential to be associated in redox homeostasis and long-distance signalling (Mukherjee, 2019). Further research into Nomela can open new avenues in understanding melatonin-NO crosstalk for regulating growth and defence process in plants.

1.5.3 Melatonin interactions with plant hormones

Plant hormones are endogenous small organic compounds derived from metabolic pathways in response to specific cues to regulate developmental stages such as seed germination, dormancy, flowering and others and mediate tolerance to biotic and abiotic stresses. They also comprise the class of plant growth regulators (PGRs) consisting of both natural and synthetic compounds (Santner et al., 2009). The classical plant hormones jasmonic acid (JA), abscisic acid (ABA), indole-3-acetic acid (IAA or auxin), brassinosteroids (BR), ethylene (ET), salicylic acid (SA), cytokinin (CK) and gibberellins (GAs) have vital and specific roles in regulating numerous physiological processes in plants (Delaney et al., 1994, Wasternack, 2007, Mehrotra et al., 2014, Saini et al., 2015, Iqbal et al., 2017, Yang et al., 2017, Gujjar and Supaibulwatana, 2019, Wang et al., 2020c). The understanding of these plant hormones has progressed with the advances in identification of molecular mechanisms related to their biosynthesis, transport, perception and responses (Wasternack, 2007, Pluharova et al., 2019, Wang et al., 2020b). Along with specific functions of each plant hormone, the cooperative or antagonistic interaction among hormones occurs during plant growth and development (Yang et al., 2006, Berkowitz et al., 2016, Poupin et al., 2016). These interactions can result in changes in hormone levels and/or transcriptional responses (Stepanova et al., 2005, Wilkinson et al., 2012).

Melatonin has been shown to either enhance or decrease the endogenous content and/or the expression of hormonal biosynthetic, transport and response genes of hormones (e.g. IAA, CKs, ABA, SA and JA) to exert physiological effects in a range of plant species (Shi et al., 2015c, Arnao and Hernández-Ruiz, 2018, Liu et al., 2019a, Liu et al., 2019b). Interaction of melatonin with SA and JA has been shown to regulate plant biotic stress defence whereas the GAs and ABA have roles in melatonin-mediated plant development such as flowering and stomatal control. Though limited information exists in this area of plant melatonin, recent studies have provided basic insights into the relationship of

melatonin with plant hormones. These are summarized in Table 1.5. However, a current confusion in the literature pertains to the relationship between melatonin and auxin. This area of research has formed the basis of the study in Chapter 2 of this thesis.

Table 1.5: Interaction of melatonin (MT) with plant hormones

Plant	Growth conditions (normal/stress)	Melatonin	Melatonin application	Effect and mechanisms	References
Interaction with Jasmonic acid (JA)					
Tomato fruit (*Solanum lycopersicum* cv. La-bi)	Pathogenic Stress (*Botrytis cinerea*)	50 µM	Infiltration with MT solutions	▲Stress tolerance; MeJA content; expression of JA-signalling pathway genes (*AOC, LoxD; PI-II)*	Liu et al., 2019
Rice (*Oryza sativa*)	Pathogenic Stress (*Xanthomonas oryzae* pv. oryzae)	20 µg/mL	Root irrigation	▲Stress tolerance; expression of JA-induced defence marker gene (*PDF1.2*)	Xian et al., 2020
Thale cress (*Arabidopsis thaliana*)	normal	10-500 µM	MT-supplemented media	Inhibition of jasmonate-stimulated anthocyanin production	Ai and Zhu et al., 2018
Interaction with Salicylic acid (SA)					
Rice (*Oryza sativa*)	Pathogenic Stress (*Xanthomonas oryzae* pv. oryzae)	20 µg/mL	Root irrigation	▲Stress tolerance; expression of key regulator of SA-signalling pathway (*NPR1*)	Xian et al., 2020
Thale cress (*Arabidopsis thaliana*)	Pathogenic Stress (*Pseudomonas syringae* DC3000)	10 µM	Foliar spray	▲Stress tolerance; expression of SA-activated defence genes	Lee et al., 2014
Tobacco (*Nicotiana glutinosa*)	Pathogenic Stress (Tobacco mosaic virus (TMV)	100 µM	Root irrigation	▲Stress tolerance; SA content; expression of SA-activated pathogenesis-related genes (*PR1*, *PR5*)	Zhao et al., 2019

Table 1.5: contd.

Plant	Growth conditions (normal/stress)	Melatonin	Application method (MT)	MT effect and mechanisms	References
***Interaction with* Ethylene (*ET*)**					
Tomato fruits (*Solanum lycopersicum* var. Bmei)	Post-harvest	50 µM	Immersion in MT-solutions	▲Post-harvest quality; Ethylene production; expression of ET signal transduction-related genes (*NR, SIEIL1, SIETR4*)	(Sun et al., 2015)
Lupin (*Lupinus albus* L.)	normal	0.01-1 mM	Immersion in MT-solutions	▼Ethylene rate	(Arnao and Hernández-Ruiz, 2013)
Thale cress (*Arabidopsis thaliana)*	normal	1 mM	Foliar spray	▲ACC synthase genes; ▼expression of ethylene response factors (ERF)-transcription factors	(Weeda et al., 2014)
***Interaction with Abscisic acid* (*ABA*)**					
Barley (*Hordeum vulgare* L.)	Abiotic stress (Dark-induced senescence)	0.1-1 mM	MT-solutions	▼Senescence; ABA action	(Arnao and Hernández-Ruiz, 2009a)
Apple (*Malus prunifolia and Malus hupehensis)*	Abiotic stress (Drought)	100 µM	Immersion in MT-solutions	▲Drought tolerance;▼ABA content, biosynthesis genes;▲expression of ABA catabolism genes	(Li et al., 2015)
Cucumber (*Cucumis sativus L.*)	Abiotic stress (Salinity (NaCl)	1 µM	Seed treatment	▲Drought tolerance;▼ABA content, biosynthesis genes; ▲ABA catabolism genes	(Zhang et al., 2014a)

Table 1.5: contd.

Plant	Growth conditions (normal/stress)	Melatonin	Application method (MT)	MT effect and mechanisms	References
Interaction with auxin (Indole-3-acetic acid, IAA)-pathways					
Wild leaf mustard (*Brassica juncea)*	normal	0.01-0.5 µM	Incubated in MT-solutions	▲IAA content; root growth	(Chen et al., 2009)
Thale cress (*Arabidopsis thaliana*)	normal	1 mM	Foliar spray	Alteration of auxin-responsive and signalling genes (total 52; 29 down, 23 up)	(Weeda et al., 2014)
Thale cress (*Arabidopsis thaliana*)	Normal	600 µM	MT-supplemented media	▼Root meristem sizes; IAA content; expression of genes encoding auxin-carrier proteins (PIN)	(Wang et al., 2016)
Interaction with Cytokinin (CK), Gibberellins (GAs)and Strigolactones (SL)					
Perennial ryegrass leaves (*Lolium perenne*)	Abiotic stress (heat-induced senescence)	20 µM	Foliar spray	▼Senescence;▲CKs; expression of CK biosynthesis genes and transcription factors	(Zhang et al., 2017a)
Thale cress (*Arabidopsis thaliana*)	normal	N/A	N/A	SL application (5 µM) reduced MT content to induce floral transition	(Zhang et al., 2019c)
Pear (*Pyrus communis* L.)	normal	100 µM	Flower spray	▲GA content (GA_3 and GA_4) to induce parthenocarpy	(Liu et al., 2018)

MeJa = Methyl jasmonate;▲indicates 'enhanced' and ▼ indicates 'decreased'

1.6 Melatonin and microbes

1.6.1 Melatonin-producing microbes

To date, melatonin production has been reported for various genera of bacteria such as, *Bacillus* (0.53-0.87 ng/mL), *Agrobacterium* (0.22 ng/mL), *Pseudomonas* (1.32 ng/mL), *Rhodospirillum* (quantity not reported) and *Erythrobacter* (0.002 ng/g protein) (Manchester et al., 1995, Tilden et al., 1997, Jiao et al., 2016, Ma et al., 2016). Fungi have also been reported to produce melatonin, e.g. *Trichoderma* spp., (2758 – 1155 ng/g dry weight (DW) (Liu et al., 2016), yeast strains (*Saccharomyces uvarum*) and (*S. cerevisiae*) (0.001-1 ng/g protein) (Rodriguez-Naranjo et al., 2012) and *Agaricus bisporus* (4300-6400 ng/g DW) (Muszynska and Sulkowska-Ziaja, 2012). The melatonin biosynthetic pathway in microbes is not yet completely elucidated relative to animals and plants. Recently, the melatonin biosynthetic pathway associated with yeast (*S. cerevisiae*) has been reported (Germann et al., 2016).

1.6.2 Microbial responses to exogenous melatonin

Human pathogens

There are reports demonstrating anti-microbial properties of exogenously applied melatonin toward human pathogens (Atroshi et al., 1998, Oztürk et al., 2000). The biocidal activities of melatonin (130 – 530 µM) in vitro, reported as causing reduced number of colonies after a 48 hour incubation, have been reported toward a range of multidrug-resistant clinical isolates such as carbapenem-resistant strains, *Staphylococcus aureus*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Tekbas et al., 2008). In contrast, another finding revealed no antibacterial effects of melatonin (0.86 µM) against *P. aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli* (Wang et al., 2001). The differences in these studies could be interpreted as microbial responses to melatonin being concentration-dependent. The discrepancies could also arise from drug-resistant strains versus standard strains.

Plant pathogenic microbes

The in vitro effects of melatonin have also been studied in the context of agriculturally relevant plant pathogenic microbes. Melatonin treatment (0.86 µM) did not show anti-

fungal effect in terms of radial growth against *Botrytis cinerea* and *Mycosphaerella arachidicola* except for a weaker growth inhibition of the pear (*Pyrus pyrifolia*) pathogen, *Physalospora piricola* (Wang et al., 2001). Inhibitory growth effects by melatonin were observed towards *Alternaria* spp. at remarkably high concentrations (4000 µM) (Arnao and Hernández-Ruiz, 2015). The mycelial growth of the oomycete *Phytophthora capsici*, that causes fruit rot in watermelon, was inhibited by melatonin treatment (100 µM) in vitro (Mandal et al., 2018). Melatonin treatment (0.86 µM) has anti-bacterial effect against the rice (*Oryza sativa*) pathogen, *Xanthomonas oryzae* (pv.) *oryzae* and *X. o. oryzicola* by regulating cell-division and downregulating genes responsible for amino acid and carbohydrate metabolism in the bacteria (Chen et al., 2018a, Chen et al., 2019b). Overall, melatonin has shown to result in increased resistance in plants to bacterial, fungal and oomycete pathogens as discussed previously (Section 1.3.3 pg.19-22).

Host-beneficial microbial interactions

In terms of beneficial host-microbial interactions, melatonin (0.001 µM) resulted in increase in the growth rate and motility of the commensal human gut bacterium, *Enterobacter aerognenes*, that inhabits the human intestinal tract (Paulose et al., 2016). Authors of the study emphasized on the potential role of melatonin in host-commensal communication in the gut. This is an interesting field of study from plant perspective as both gut and root microbiota have commonalities such as the inhabitation of different microbes and variation in microbiota with factors including age and species (Ramirez-Puebla et al., 2013). It is probable that melatonin is utilised as a signal of communication between plants and microbes. Similarly, in plants, two studies have recently emerged showing production of melatonin by beneficial root-associated endophytic bacteria (*Bacillus amyloliquefaciens* SB-9 and *Pseudomonas fluorescens* RG11) which upon colonization resulted in improved health of grapevine (*V. vinifera*) host plant under abiotic stresses (salinity and drought). Interestingly, the bacterial colonization also resulted in increased melatonin biosynthetic gene expression in the host (Jiao et al., 2016, Ma et al., 2016).

1.6.3 Role of melatonin in microbes under stress

The potential role of melatonin in conferring microbes with stress tolerance was demonstrated in beneficial plant fungi, *Trichoderma* spp. including *Trichoderma*

asperellum, T. longibrachiatum, T. harzianum, T. koningii and T. viride which exhibited increased endogenous melatonin levels under abiotic stressors such as cadmium (3,000 µM $CdCl_2$) and salt (1% NaCl) compared to control conditions (Liu et al., 2016). This shows that melatonin production in *Trichoderma* spp. is one of the mechanisms for anti-stress response and the phenomenon further needs to be investigated in these fungi when growing in association with their host plants. Microbes have the capability to produce alcohols which serve as important sources of alternative energy. However, these alcohols can pose toxicity to the microbes themselves resulting in inhibited cell growth (Horinouchi et al., 2018). Certain stresses, e.g. pathogens, can also raise the endogenous ethanol levels in plants to a toxic level impairing aerobic respiration (Kelsey et al., 2013). The endogenous levels of melatonin have been reported to enhance in *S. cerevisiae* during the fermentation process which could be a way to enhance tolerance to ethanol during fermentation (Rodriguez-Naranjo et al., 2012). The role of melatonin in conferring oxidative tolerance in yeast has been described in section 1.1.

1.7 Overview

Environmental stresses negatively impact crop production and are the major constraints to global food security. The world-wide climatic change has exacerbated the environmental challenges such as extreme heat and drought (Tito et al., 2018, Raza et al., 2019). As a consequence, the concurrence of abiotic and biotic stress factors is becoming more prevalent (Pandey et al., 2017). These combined stresses affect agricultural productivity by directly targeting crops' cellular activities such as photosynthetic states, oxidative metabolism and membrane stability (Fahad et al., 2017, Dresselhaus and Hückelhoven, 2018).

A central mechanism in plants to resist environmental stresses is the modulation of their secondary metabolism (Austen et al., 2019). The secondary metabolites are not only important in stress adaptation but also in growth and developmental processes. Indolic tryptophan-derived secondary metabolites have been demonstrated to improve plant growth and developmental processes under stress (Ishihara et al., 2008, Erland et al., 2015, Smith et al., 2016). Of particular interest and potential among these secondary metabolites

is melatonin. With the promising features highlighted by studies on melatonin in plants as described in this chapter, it is envisaged that melatonin has a potential in agronomic improvement strategies (Tan et al., 2012). However, to evaluate the feasibility of melatonin in future agricultural practices, it is first important to understand the underlying phenotypic and molecular mechanisms governed by melatonin application in plant and soil microbes. This can be evaluated in the form of phenotype and transcriptomic responses to exogenous melatonin application.

Currently, there are three main distinct gaps in knowledge of action of melatonin. These are described below:

1.7.1 Research gaps

1) Melatonin and auxin are indole compounds and functionally similar (i.e. enhancing lateral root, fresh weight and root curvature at lower concentrations and inhibiting overall seedling growth at higher concentrations). However, the current understanding of crosstalk between melatonin and auxin is unclear with contrasting reports in the literature (Pelagio-Flores et al., 2012, Koyama et al., 2013, Weeda et al., 2014, Shi et al., 2015c, Kim et al., 2016, Wang et al., 2016, Wen et al., 2016, Wan et al., 2018). Most of these studies lack a direct comparison of melatonin and auxin treatments under identical set of experimental conditions. There are no studies that have compared the transcriptomic responses elicited by melatonin or auxin, which is crucial to test if melatonin acts through auxin signalling or has its independent mechanisms.

2) The endogenous levels of melatonin have shown to be remarkably divergent within plant species (Bhattacharjee et al., 2016, Hardeland, 2016, Meng et al., 2017). This suggests that melatonin is likely to play a role in stress adaptation in ecotypes adapted to specific environments. This is reasonable as melatonin has been previously reported to ameliorate negative impacts of a range of abiotic and biotic stresses (Arnao and Hernández-Ruiz, 2009a, Zhang et al., 2015, Moustafa-Farag et al., 2019). However, no data exist in terms of the phenotypic and transcriptional diversity in melatonin responses across geographically distinct natural variants (ecotypes) of a plant species. This is needed to decipher the variable key players induced by melatonin that would potentially enable local habitat adaptation within plant species. Moreover, the expression of core-conserved

genes among ecotypes in response to melatonin would enable to identify the role of melatonin as a conserved signalling molecule in different genotypes. The effect on transcriptome of disrupting the melatonin biosynthetic gene expression by mutagenesis has also not been evaluated. This is essential to identify the gene sets essential for melatonin signalling.

3) Microbes communicate with their hosts through an array of signals (Lareen et al., 2016). To date there are only two studies demonstrating the role of melatonin in plants and associated beneficial bacterial interactions (Jiao et al., 2016, Ma et al., 2016). More research is needed to first understand the responses of the plant beneficial bacteria to melatonin in vitro. Moreover, the role of melatonin in quorum-regulated motility behaviour in bacterium of agricultural interest is lacking.

1.7.2 Chapter outlines and hypotheses

This thesis comprises five chapters, with chapters 2-4 forming the three main research chapters. Chapter 2 is published as indicated and Chapters 3 and 4 are written as chapters to form the basis for preparing manuscript for submission to journals for publication.

1.7.2.1 Chapter 2 – Direct comparison of *Arabidopsis* gene expression reveals different responses to melatonin versus auxin

The aim of this chapter was to determine whether melatonin regulates gene expression similar to auxin by a direct comparison in *Arabidopsis*. This study used a direct comparative analysis of *Arabidopsis* to investigate whether melatonin and auxin share signalling pathways. Promoter-activation approach was utilised by employing promoter-reporter constructs, *Direct repeat 5 DR5*::GFP and *Alternative oxidase 1a AOX1a*::LUC, both of which are hallmarks of auxin action. Furthermore, transcriptome analyses (RNA-Sequencing) were conducted on rosette leaves, roots of which were treated with melatonin (5 and 100 µM) or auxin (4.5 µM) (1-naphthalene acetic acid, NAA). It was hypothesised that:

1. Melatonin does not affect the auxin-responsive marker lines in a similar fashion
2. Melatonin and auxin treatments would produce distinctly different gene expression responses in *Arabidopsis*
3. Melatonin has its independent mechanisms of signalling.

1.7.2.2 Chapter 3: Melatonin treatment reveals differences between the *serotonin-N-acetyltransferase1* mutant and wild-type and induces conserved and ecotype-dependent responses in *Arabidopsis thaliana*

The aim of this chapter was to determine core conserved and genotype-dependent responses to melatonin in *Arabidopsis*. Following from the investigation in Chapter 2, the transcriptome analysis was further extended to the melatonin biosynthetic gene mutant and natural variants (ecotypes) of *Arabidopsis.* These genotypes were selected based on their variability in phenotypic responses to exogenous melatonin at higher concentration (400 µM). The likely role of melatonin in stress adaptation was evaluated by identifying the genotype-specific gene expression responses. Moreover, core-conserved gene expression responses were also evaluated to assess the role of melatonin as a signalling molecule important among all genotypes. Briefly, it was hypothesised that:

1. Melatonin biosynthetic gene *AtSNAT1* expression disruption in the mutant results in differentially expressed genes compared to the wild-type under control and in response to melatonin.
2. Natural variation exists in phenotypic and transcriptomic response to melatonin in ecotypes.
3. Melatonin results in core-conserved transcriptional responses that are independent of ecotypes
4. Melatonin results in ecotype-specific transcriptional responses to melatonin among distinct ecotypes

1.7.2.3 Chapter 4: Melatonin restores swarming under ethanol treatment in *Pseudomonas aeruginosa* PAO1 but does not affect the growth of cadmium-tolerant plant growth-promoting rhizobacteria

The research in this chapter investigated the in vitro effects of melatonin on the growth rate of recently identified plant-growth promoting rhizobacteria (*Enterobacter ludwigii* NCR3, *Bacillus cereus* LCR12, *Rhodococcus erythropolis* NSX2). The effects of melatonin on the quorum-regulated swarming motility behaviour of *Pseudomonas aeruginosa* PAO1 was also investigated. It was hypothesised that:

1. Melatonin affects the growth rate of PGPRs in a concentration-dependent manner
2. Melatonin affects motility behaviour of PAO1 in a concentration-dependent manner

1.7.2.4 Chapter 5: General Discussion

This chapter discusses the overall research conducted in this thesis with links to major findings in the research chapters 2-4 and how they fit in the existing body of knowledge. The suggestions for future studies are provided.

1.8 Appendix

Criteria for Literature Selection

This review aims to detail the history and current key topics in melatonin in plants spanning from the year of its discovery in plants in 1995 to latest advancements up to June 2020. An introduction of melatonin as a molecule and functional evolution across kingdoms has also been provided. This research on melatonin in plants has followed the proceedings of a systematic review of the literature, based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Moher et al., 2009). The literature were sourced from databases, Scopus, PubMed, Google Scholar and Google Scholar Alerts. Scopus was the main database that was used. All content and papers selected for each phase of the review were imported in the EndNote Reference Management library version 9.0 (www.endnote.com).

The Boolean operators were used to connect search terms together and provide a narrow or broad set of results. The Boolean operator OR alone was not used to avoid excluding relevant works in the initial screening. The following search terms were used:

"Melatonin AND plants"; "Melatonin AND plants AND microbes"; "Melatonin AND plants OR microbes"; "Melatonin AND animals"; "Melatonin AND microbes".

The types of the literature included Article and Reviews. No filters were applied in the search unless otherwise stated.

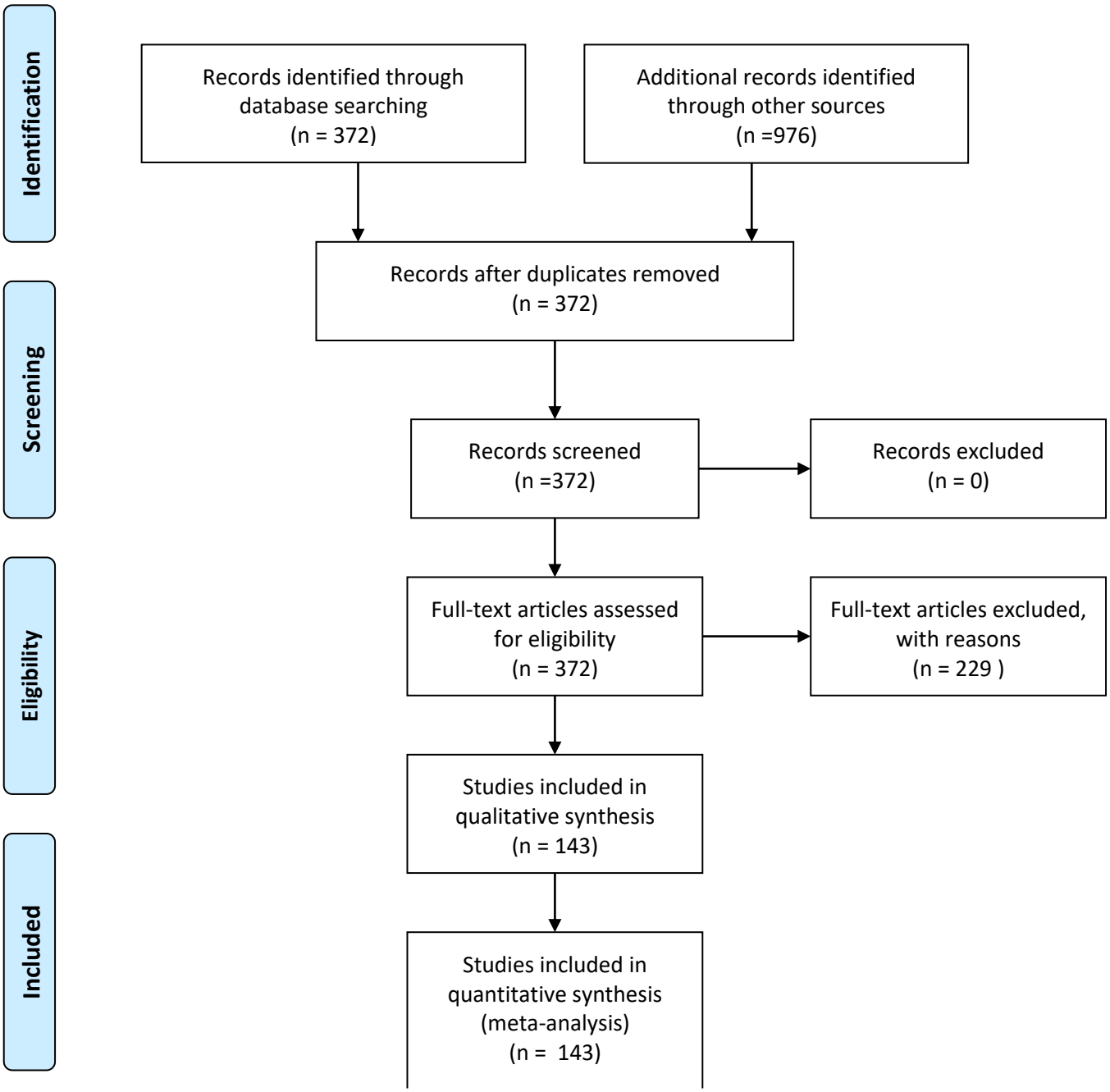
Rationale for inclusions/exclusion

Melatonin, while commonly known in animals and to medical researchers, is not well-known by plant scientists. The main reason for this is that most of the studies since the discovery of melatonin in plants are published in *Journal of Pineal Research*. Although the journal occasionally covers aspects of melatonin studies in plants and micro-organisms, it is largely a medical-based journal. Due to this, some of the pioneering work of melatonin in plants has not garnered much attention in the plant science community.

This is the reason why the systematic literature review in this chapter 1 needed to include an in-depth survey of historical events of introducing melatonin as a molecule (section 1.1). This required including some of the early pioneering work on melatonin in animals dating back to 1960's. However, the scope of the review is to align with the main body of this thesis and hence research on clinical aspects of melatonin in animals were excluded from the review. The PRISMA approach was applied to the main gist of the review, i.e.

melatonin in plants (PRISMA 2009 flow diagram). The first step was identification of the records which resulted in a total number of 372 records in Scopus and 976 in other sources such as PubMed and Google Scholar. In the further screening process of 1,348 records, the duplicates were removed using the de-duplication feature in EndNote reference management software package as outlined by (Kwon et al., 2015). This resulted in a total of 372 records. The full-text articles of these records were assessed for eligibility in terms of relevance and citations. This resulted in a total of 143 out of 372 papers on melatonin in plants (as identified in Scopus) to be included in qualitative as well as quantitative synthesis (sections 1.2-1.6). These articles include a detailed history of melatonin in plants with studies starting from 1995 when it was first discovered in plant species to all the highly cited key topics and latest advancements in the field up to April 2020. The screening of the full-text of the remaining 229 articles led to the identification of these as duplicated records of review articles and studies emphasising or over-emphasising the same or similar observation. These were subjected to a final exclusion to ensure a valid, relevant and reliable pool of studies for inclusion in the review .

PRISMA 2009 Flow Diagram



1.9 References

Aguilera, Y., Herrera, T., Benitez, V., Arribas, S. M., Lopez De Pablo, A. L., Esteban, R. M., et al. 2015. Estimation of scavenging capacity of melatonin and other antioxidants: contribution and evaluation in germinated seeds. *Food Chem,* 170**,** 203-11

Ahmad, S., Kamran, M., Ding, R., Meng, X., Wang, H., Ahmad, I., et al. 2019. Exogenous melatonin confers drought stress by promoting plant growth, photosynthetic capacity and antioxidant defense system of maize seedlings. *PeerJ,* 7**,** e7793

Ai, Y. & Zhu, Z. 2018. Melatonin Antagonizes Jasmonate-Triggered Anthocyanin Biosynthesis in *Arabidopsis thaliana*. *J Agric Food Chem,* 66**,** 5392-5400

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. & Walter, P. 2008. Molecular Biology of the Cell. *5th edn. Garland Science, New York,*

Alghamdi, B. S. 2018. The neuroprotective role of melatonin in neurological disorders. *J Neurosci Res,* 96**,** 1136-1149

Arendt, J. 2007. Aaron Lerner, who discovered melatonin. *J Pineal Res,* 43**,** 106-7

Arnao, M. B. & Hernández-Ruiz, J. 2009a. Chemical stress by different agents affects the melatonin content of barley roots. *J Pineal Res,* 46**,** 295-9

Arnao, M. B. & Hernández-Ruiz, J. 2009b. Protective effect of melatonin against chlorophyll degradation during the senescence of barley leaves. *J Pineal Res,* 46**,** 58-63

Arnao, M. B. & Hernández-Ruiz, J. 2013. Growth conditions determine different melatonin levels in *Lupinus albus* L. *J Pineal Res,* 55**,** 149-55

Arnao, M. B. & Hernández-Ruiz, J. 2015. Functions of melatonin in plants: a review. *J Pineal Res,* 59**,** 133-50

Arnao, M. B. & Hernández-Ruiz, J. 2018. Melatonin and its relationship to plant hormones. *Ann Bot,* 121**,** 195-207

Atroshi, F., Rizzo, A., Westermarck, T. & Ali-Vehmas, T. 1998. Effects of tamoxifen, melatonin, coenzyme Q10, and L-carnitine supplementation on bacterial growth in the presence of mycotoxins. *Pharmacol Res,* 38**,** 289-95

Axelrod, J. & Weissbach, H. 1960. Enzymatic *O*-methylation of *N*-acetylserotonin to melatonin. *Science,* 131**,** 1312

Back, K. & Lee, H. Y. 2020. The phytomelatonin receptor (PMRT1) *Arabidopsi*s Cand2 is not a bona fide G protein–coupled melatonin receptor. *Mel Res,* 3**,** 177-186

Back, K., Tan, D. X. & Reiter, R. J. 2016. Melatonin biosynthesis in plants: multiple pathways catalyze tryptophan to melatonin in the cytoplasm or chloroplasts. *J Pineal Res,* 61**,** 426-437

Bajwa, V. S., Shukla, M. R., Sherif, S. M., Murch, S. J. & Saxena, P. K. 2014. Role of melatonin in alleviating cold stress in *Arabidopsis thaliana*. *J Pineal Res,* 56**,** 238-45

Ben-Nathan, D., Maestroni, G. J. M., Lustig, S. & Conti, A. 1995. Protective effects of melatonin in mice infected with encephalitis viruses. *Arch Virol,* 140**,** 223-230

Berkowitz, O., De Clercq, I., Van Breusegem, F. & Whelan, J. 2016. Interaction between hormonal and mitochondrial signalling during growth, development and in plant defence responses. *Plant Cell Environ,* 39**,** 1127-39

Bhattacharjee, A., Shashidhara, S. C. & Saha, S. 2016. Overview of the Occurrence of Melatonin in Medicinal Plants with Special Reference to *Crataeva nurvala* Buch-Ham: Isolation, Purication, and Characterization. *Serotonin and Melatonin.* CRC Press.

Bisquert, R., Muniz-Calvo, S. & Guillamon, J. M. 2018. Protective Role of Intracellular Melatonin Against Oxidative Stress and UV Radiation in *Saccharomyces cerevisiae*. *Front Microbiol,* 9**,** 318

Blakely, L. M., Blakely, R. M., Colowit, P. M. & Elliott, D. S. 1988. Experimental Studies on Lateral Root Formation in Radish Seedling Roots: II. Analysis of the Dose-Response to Exogenous Auxin. *Plant Physiol,* 87**,** 414-9

Boga, J. A., Coto-Montes, A., Rosales-Corral, S. A., Tan, D. X. & Reiter, R. J. 2012. Beneficial actions of melatonin in the management of viral infections: a new use for this “molecular handyman”? *Rev Med Virol,* 22**,** 323-338

Bonomini, F., Favero, G., Rodella, L. F., Moghadasian, M. H. & Rezzani, R. 2018. Melatonin Modulation of Sirtuin-1 Attenuates Liver Injury in a Hypercholesterolemic Mouse Model. *Biomed Res Int,* 2018**,** 7968452

Byeon, Y. & Back, K. 2014. An increase in melatonin in transgenic rice causes pleiotropic phenotypes, including enhanced seedling growth, delayed flowering, and low grain yield. *J Pineal Res,* 56**,** 408-14

Byeon, Y. & Back, K. 2015. Molecular cloning of melatonin 2-hydroxylase responsible for 2-hydroxymelatonin production in rice (*Oryza sativa*). *J Pineal Res,* 58**,** 343-51

Byeon, Y., Lee, H. J., Lee, H. Y. & Back, K. 2016a. Cloning and functional characterization of the *Arabidopsis N*-acetylserotonin *O*-methyltransferase responsible for melatonin synthesis. *J Pineal Res,* 60**,** 65-73

Byeon, Y., Lee, H. Y. & Back, K. 2016b. Cloning and characterization of the serotonin *N*-acetyltransferase-2 gene (*SNAT2*) in rice (*Oryza sativa*). *J Pineal Res,* 61**,** 198-207

Byeon, Y., Lee, H. Y., Lee, K. & Back, K. 2014a. Caffeic acid *O*-methyltransferase is involved in the synthesis of melatonin by methylating *N*-acetylserotonin in *Arabidopsis*. *J Pineal Res,* 57**,** 219-27

Byeon, Y., Lee, H. Y., Lee, K., Park, S. & Back, K. 2014b. Cellular localization and kinetics of the rice melatonin biosynthetic enzymes SNAT and ASMT. *J Pineal Res,* 56**,** 107-14

Byeon, Y., Lee, K., Park, Y. I., Park, S. & Back, K. 2013. Molecular cloning and functional analysis of serotonin *N*-acetyltransferase from the cyanobacterium *Synechocystis* sp. PCC 6803. *J Pineal Res,* 55**,** 371-6

Byeon, Y., Tan, D. X., Reiter, R. J. & Back, K. 2015. Predominance of 2-hydroxymelatonin over melatonin in plants. *J Pineal Res,* 59**,** 448-54

Calvo, J. R., Gonzalez-Yanes, C. & Maldonado, M. D. 2013. The role of melatonin in the cells of the innate immunity: a review. *J Pineal Res,* 55**,** 103-20

Cao, Q., Li, G., Cui, Z., Yang, F., Jiang, X., Diallo, L., et al. 2019. Seed Priming with Melatonin Improves the Seed Germination of Waxy Maize under Chilling Stress via Promoting the Antioxidant System and Starch Metabolism. *Sci Rep,* 9**,** 15044

Cao, S., Shao, J., Shi, L., Xu, L., Shen, Z., Chen, W., et al. 2018. Melatonin increases chilling tolerance in postharvest peach fruit by alleviating oxidative damage. *Sci Rep,* 8**,** 806

Carrillo-Vico, A., Lardone, P. J., Alvarez-Sanchez, N., Rodriguez-Rodriguez, A. & Guerrero, J. M. 2013. Melatonin: buffering the immune system. *Int J Mol Sci,* 14**,** 8638-83

Carrillo-Vico, A., Lardone, P. J., Fernandez-Santos, J. M., Martin-Lacave, I., Calvo, J. R., Karasek, M., et al. 2005. Human lymphocyte-synthesized melatonin is involved in the regulation of the interleukin-2/interleukin-2 receptor system. *J Clin Endocrinol Metab,* 90**,** 992-1000

Catala, A. 2007. The ability of melatonin to counteract lipid peroxidation in biological membranes. *Curr Mol Med,* 7**,** 638-49

Century, K., Reuber, T. L. & Ratcliffe, O. J. 2008. Regulating the regulators: the future prospects for transcription-factor-based agricultural biotechnology products. *Plant Physiol,* 147**,** 20-9

Chang, A. M., Aeschbach, D., Duffy, J. F. & Czeisler, C. A. 2015. Evening use of light-emitting eReaders negatively affects sleep, circadian timing, and next-morning alertness. *Proc Natl Acad Sci U S A,* 112**,** 1232-7

Chang, H.-M., Wu, U.-I. & Lan, C.-T. 2009. Melatonin preserves longevity protein (sirtuin 1) expression in the hippocampus of total sleep-deprived rats. *J Pineal Res,* 47**,** 211-20

Chen, C. Q., Fichna, J., Bashashati, M., Li, Y. Y. & Storr, M. 2011. Distribution, function and physiological role of melatonin in the lower gut. *World J Gastroenterol,* 17**,** 3888-98

Chen, J., Li, H., Yang, K., Wang, Y., Yang, L., Hu, L., et al. 2019a. Melatonin facilitates lateral root development by coordinating PAO-derived hydrogen peroxide and Rboh-derived superoxide radical. *Free Radic Biol Med,* 143**,** 534-544

Chen, L., Liu, L., Lu, B., Ma, T., Jiang, D., Li, J., et al. 2020. Exogenous melatonin promotes seed germination and osmotic regulation under salt stress in cotton (*Gossypium hirsutum* L.). *PLoS One,* 15**,** e0228241

Chen, Q., Qi, W. B., Reiter, R. J., Wei, W. & Wang, B. M. 2009. Exogenously applied melatonin stimulates root growth and raises endogenous indoleacetic acid in roots of etiolated seedlings of *Brassica juncea*. *J Plant Physiol,* 166**,** 324-8

Chen, X., Sun, C., Laborda, P., He, Y., Zhao, Y., Li, C., et al. 2019b. Melatonin treatments reduce the pathogenicity and inhibit the growth of *Xanthomonas oryzae* pv. *oryzicola*. *Plant Pathol,* 68**,** 288-296

Chen, X., Sun, C., Laborda, P., Zhao, Y., Palmer, I., Fu, Z. Q., et al. 2018a. Melatonin Treatment Inhibits the Growth of *Xanthomonas oryzae* pv. *oryzae*. *Front Microbiol,* 9**,** 2280

Chen, Z., Gu, Q., Yu, X., Huang, L., Xu, S., Wang, R., et al. 2018b. Hydrogen peroxide acts downstream of melatonin to induce lateral root formation. *Ann Bot,* 121**,** 1127-1136

Chen, Z., Xie, Y., Gu, Q., Zhao, G., Zhang, Y., Cui, W., et al. 2017. The AtrbohF-dependent regulation of ROS signaling is required for melatonin-induced salinity tolerance in *Arabidopsis*. *Free Radic Biol Med,* 108**,** 465-477

Choi, G.-H. & Back, K. 2019. Cyclic 3-hydroxymelatonin exhibits diurnal rhythm and cyclic 3-hydroxymelatonin overproduction increases secondary tillers in rice by upregulating *MOC1* expression. *Melatonin Res,* 2**,** 120-138

Choi, G. H., Lee, H. Y. & Back, K. 2017. Chloroplast overexpression of rice caffeic acid *O*-methyltransferase increases melatonin production in chloroplasts via the 5-methoxytryptamine pathway in transgenic rice plants. *J Pineal Res,* 63

Delaney, T. P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., et al. 1994. A central role of salicylic Acid in plant disease resistance. *Science,* 266**,** 1247-50

Demidchik, V. 2015. Mechanisms of oxidative stress in plants: From classical chemistry to cell biology. *Environ Exp Bot,* 109**,** 212-228

Ding, Z., Wu, C., Tie, W., Yan, Y., He, G. & Hu, W. 2019. Strand-specific RNA-seq based identification and functional prediction of lncRNAs in response to melatonin and simulated drought stresses in cassava. *Plant Physiol Biochem,* 140**,** 96-104

Dresselhaus, T. & Hückelhoven, R. 2018. Biotic and Abiotic Stress Responses in Crop Plants. *Agronomy,* 8

Dubbels, R., Reiter, R. J., Klenke, E., Goebel, A., Schnakenberg, E., Ehlers, C., et al. 1995. Melatonin in edible plants identified by radioimmunoassay and by high performance liquid chromatography-mass spectrometry. *J Pineal Res,* 18**,** 28-31

Erland, L. A., Murch, S. J., Reiter, R. J. & Saxena, P. K. 2015. A new balancing act: The many roles of melatonin and serotonin in plant growth and development. *Plant Signal Behav,* 10**,** e1096469

Fahad, S., Bajwa, A. A., Nazir, U., Anjum, S. A., Farooq, A., Zohaib, A., et al. 2017. Crop Production under Drought and Heat Stress: Plant Responses and Management Options. *Front Plant Sci,* 8**,** 1147

Fathizadeh, H., Mirzaei, H. & Asemi, Z. 2019. Melatonin: an anti-tumor agent for osteosarcoma. *Cancer Cell Int,* 19**,** 319

Fujiwara, T., Maisonneuve, S., Isshiki, M., Mizutani, M., Chen, L., Wong, H. L., et al. 2010. Sekiguchi lesion gene encodes a cytochrome P450 monooxygenase that catalyzes conversion of tryptamine to serotonin in rice. *Journal of Biological Chemistry,* 285**,** 11308-11313

Ganguly, S., Mummaneni, P., Steinbach, P. J., Klein, D. C. & Coon, S. L. 2001. Characterization of the *Saccharomyces cerevisiae* homolog of the melatonin rhythm enzyme arylalkylamine *N*-acetyltransferase (EC 2.3.1.87). *J Biol Chem,* 276**,** 47239-47

Gao, H., Lu, Z., Yang, Y., Wang, D., Yang, T., Cao, M., et al. 2018. Melatonin treatment reduces chilling injury in peach fruit through its regulation of membrane fatty acid contents and phenolic metabolism. *Food Chem,* 245**,** 659-666

Germann, S. M., Baallal Jacobsen, S. A., Schneider, K., Harrison, S. J., Jensen, N. B., Chen, X., et al. 2016. Glucose-based microbial production of the hormone melatonin in yeast *Saccharomyces cerevisiae*. *Biotechnol J,* 11**,** 717-24

Golldack, D., Li, C., Mohan, H. & Probst, N. 2014. Tolerance to drought and salt stress in plants: Unraveling the signaling networks. *Front Plant Sci,* 5**,** 151

Goss, E. M., Tabima, J. F., Cooke, D. E., Restrepo, S., Fry, W. E., Forbes, G. A., et al. 2014. The Irish potato famine pathogen *Phytophthora infestans* originated in central Mexico rather than the Andes. *Proc Natl Acad Sci U S A,* 111**,** 8791-6

Grigg-Damberger, M. M. & Ianakieva, D. 2017. Poor quality control of over-the-counter melatonin: what they say is often not what you get. *J Clin Sleep Med,* 13**,** 163-165

Gujjar, R. S. & Supaibulwatana, K. 2019. The Mode of Cytokinin Functions Assisting Plant Adaptations to Osmotic Stresses. *Plants (Basel),* 8

Guo, Y., Sun, J., Li, T., Zhang, Q., Bu, S., Wang, Q., et al. 2017. Melatonin ameliorates restraint stress-induced oxidative stress and apoptosis in testicular cells via NF-kappaB/iNOS and Nrf2/ HO-1 signaling pathway. *Sci Rep,* 7**,** 9599

Hao, J., Fan, W., Li, Y., Tang, R., Tian, C., Yang, Q., et al. 2019. Melatonin synergizes BRAF-targeting agent vemurafenib in melanoma treatment by inhibiting iNOS/hTERT signaling and cancer-stem cell traits. *J Exp Clin Cancer Res,* 38**,** 48

Hardeland, R. 2013. Melatonin and the theories of aging: a critical appraisal of melatonin's role in antiaging mechanisms. *J Pineal Res,* 55**,** 325-56

Hardeland, R. 2016. Melatonin in Plants - Diversity of Levels and Multiplicity of Functions. *Front Plant Sci,* 7**,** 198

Hardeland, R. 2017. Taxon- and Site-Specific Melatonin Catabolism. *Molecules,* 22

Hattori, A., Migitaka, H., Iigo, M., Itoh, M., Yamamoto, K., Ohtani-Kaneko, R., et al. 1995a. Identification of melatonin in plants and its effects on plasma melatonin levels and binding to melatonin receptors in vertebrates. *Biochem Mol Biol Int,* 35**,** 627-34

Hattori, A., Migitaka, H., Iigo, M., Itoh, M., Yamamoto, K., Ohtani-Kaneko, R., et al. 1995b. Identification of melatonin in plants and its effects on plasma melatonin levels and binding to melatonin receptors in vertebrates

Biochem Mol Biol Int, 35**,** 627-34

Hattori, A., Wada, M., Majima, A., Tabata, M. & Itoh, M. 1999. Detection of melatonin and synthesizing enzyme activities in Cyanobacterium, *Spirulina platensis Proceedings-Japan Society for Comparative Endocrinology,* 14

Hernández-Ruiz, J. & Arnao, M. B. 2008. Melatonin stimulates the expansion of etiolated lupin cotyledons. *Plant Growth Regulation,* 55**,** 29-34

Hernández-Ruiz, J., Cano, A. & Arnao, M. B. 2004. Melatonin: a growth-stimulating compound present in lupin tissues. *Planta,* 220**,** 140-4

Hernández-Ruiz, J., Cano, A. & Arnao, M. B. 2005. Melatonin acts as a growth-stimulating compound in some monocot species. *J Pineal Res,* 39**,** 137-42

Hernández, I. G., Gomez, F. J., Cerutti, S., Arana, M. V. & Silva, M. F. 2015. Melatonin in *Arabidopsis thaliana* acts as plant growth regulator at low concentrations and preserves seed viability at high concentrations. *Plant Physiol Biochem,* 94**,** 191-6

Hevia, D., Gonzalez-Menendez, P., Quiros-Gonzalez, I., Miar, A., Rodriguez-Garcia, A., Tan, D. X., et al. 2015. Melatonin uptake through glucose transporters: a new target for melatonin inhibition of cancer. *J Pineal Res,* 58**,** 234-50

Hoang, X. L. T., Nhi, D. N. H., Thu, N. B. A., Thao, N. P. & Tran, L. P. 2017. Transcription Factors and Their Roles in Signal Transduction in Plants under Abiotic Stresses. *Curr Genomics,* 18**,** 483-497

Hong, J. K., Yun, B. W., Kang, J. G., Raja, M. U., Kwon, E., Sorhagen, K., et al. 2008. Nitric oxide function and signalling in plant disease resistance. *J Exp Bot,* 59**,** 147-54

Horinouchi, T., Maeda, T. & Furusawa, C. 2018. Understanding and engineering alcohol-tolerant bacteria using OMICS technology. *World J Microbiol Biotechnol,* 34**,** 157

Hu, W., Yang, H., Tie, W., Yan, Y., Ding, Z., Liu, Y., et al. 2017. Natural Variation in Banana Varieties Highlights the Role of Melatonin in Postharvest Ripening and Quality. *J Agric Food Chem,* 65**,** 9987-9994

Huang, B., Chen, Y. E., Zhao, Y. Q., Ding, C. B., Liao, J. Q., Hu, C., et al. 2019. Exogenous Melatonin Alleviates Oxidative Damages and Protects Photosystem II in Maize Seedlings Under Drought Stress. *Front Plant Sci,* 10**,** 677

Hwang, O. J. & Back, K. 2020. Simultaneous Suppression of Two Distinct Serotonin *N*-Acetyltransferase Isogenes by RNA Interference Leads to Severe Decreases in Melatonin and Accelerated Seed Deterioration in Rice. *Biomolecules,* 10

Inupakutika, M. A., Sengupta, S., Devireddy, A. R., Azad, R. K. & Mittler, R. 2016. The evolution of reactive oxygen species metabolism. *J Exp Bot,* 67**,** 5933-5943

Iqbal, N., Khan, N. A., Ferrante, A., Trivellini, A., Francini, A. & Khan, M. I. R. 2017. Ethylene Role in Plant Growth, Development and Senescence: Interaction with Other Phytohormones. *Front Plant Sci,* 8**,** 475

Ishihara, A., Hashimoto, Y., Tanaka, C., Dubouzet, J. G., Nakao, T., Matsuda, F., et al. 2008. The tryptophan pathway is involved in the defense responses of rice against pathogenic infection via serotonin production. *Plant J,* 54**,** 481-95

Jahan, M. S., Shu, S., Wang, Y., Chen, Z., He, M., Tao, M., et al. 2019. Melatonin alleviates heat-induced damage of tomato seedlings by balancing redox homeostasis and modulating polyamine and nitric oxide biosynthesis. *BMC Plant Biol,* 19**,** 414

Jan, J. E., Reiter, R. J., Wasdell, M. B. & Bax, M. 2009. The role of the thalamus in sleep, pineal melatonin production, and circadian rhythm sleep disorders. *J Pineal Res,* 46**,** 1-7

Janeczko, A. 2011. The significance of ethanol as a hormone solvent in experiments on the physiological activity of brassinosteroids. In: Hayat S., Ahmad A. (eds) Brassinosteroids: A Class of Plant Hormone. *Springer, Dodrecht,*

Jiao, J., Ma, Y., Chen, S., Liu, C., Song, Y., Qin, Y., et al. 2016. Melatonin-Producing Endophytic Bacteria from Grapevine Roots Promote the Abiotic Stress-Induced Production of Endogenous Melatonin in Their Hosts. *Front Plant Sci,* 7**,** 1387

Jin, G., Liu, F., Ma, H., Hao, S., Zhao, Q., Bian, Z., et al. 2012. Two G-protein-coupled-receptor candidates, Cand2 and Cand7, are involved in *Arabidopsis* root growth mediated by the bacterial quorum-sensing signals N-acyl-homoserine lactones. *Biochem Biophys Res Commun,* 417**,** 991-5

Jinying, W., Chuan, J., Shuke, L. & Jingui, Z. 2009. Study on analysis method of melatonin and melatonin content in corn and rice seeds. *Chinese Sci Bull,* 17**,** 6

Joshi, S., Choukimath, A., Isenegger, D., Panozzo, J., Spangenberg, G. & Kant, S. 2019. Improved Wheat Growth and Yield by Delayed Leaf Senescence Using Developmentally Regulated Expression of a Cytokinin Biosynthesis Gene. *Front Plant Sci,* 10**,** 1285

Kang, K., Lee, K., Park, S., Byeon, Y. & Back, K. 2013. Molecular cloning of rice serotonin *N*-acetyltransferase, the penultimate gene in plant melatonin biosynthesis. *J Pineal Res,* 55**,** 7-13

Kang, S., Kang, K., Lee, K. & Back, K. 2007. Characterization of rice tryptophan decarboxylases and their direct involvement in serotonin biosynthesis in transgenic rice. *Planta,* 227**,** 263-72

Kanjanaphachoat, P., Wei, B. Y., Lo, S. F., Wang, I. W., Wang, C. S., Yu, S. M., et al. 2012. Serotonin accumulation in transgenic rice by over-expressing tryptophan decarboxylase results in a dark brown phenotype and stunted growth. *Plant Mol Biol,* 78**,** 525-43

Karasek, M. 2004. Melatonin, human aging, and age-related diseases. *Exp Gerontol,* 39**,** 1723-9

Kaya, C., Okant, M., Ugurlar, F., Alyemeni, M. N., Ashraf, M. & Ahmad, P. 2019. Melatonin-mediated nitric oxide improves tolerance to cadmium toxicity by reducing oxidative stress in wheat plants. *Chemosphere,* 225**,** 627-638

Ke, Q., Ye, J., Wang, B., Ren, J., Yin, L., Deng, X., et al. 2018. Melatonin Mitigates Salt Stress in Wheat Seedlings by Modulating Polyamine Metabolism. *Front Plant Sci,* 9**,** 914

Kelsey, R. G., Beh, M. M., Shaw, D. C. & Manter, D. K. 2013. Ethanol attracts scolytid beetles to *Phytophthora ramorum* cankers on coast live oak. *J Chem Ecol,* 39**,** 494-506

Kim, M., Seo, H., Park, C. & Park, W. J. 2016. Examination of the auxin hypothesis of phytomelatonin action in classical auxin assay systems in maize. *J Plant Physiol,* 190**,** 67-71

Kocadagli, T., Yilmaz, C. & Gokmen, V. 2014. Determination of melatonin and its isomer in foods by liquid chromatography tandem mass spectrometry. *Food Chem,* 153**,** 151-6

Kolář, J., Johnson, C. H. & Machackova, I. 1999. Presence and possible role of melatonin in a short-day flowering plant, *Chenopodium rubrum*. *Adv Exp Med Biol,* 460**,** 391-3

Koskela, M. M., Brunje, A., Ivanauskaite, A., Grabsztunowicz, M., Lassowskat, I., Neumann, U., et al. 2018. Chloroplast Acetyltransferase NSI Is Required for State Transitions in *Arabidopsis thaliana*. *Plant Cell,* 30**,** 1695-1709

Koyama, F. C., Carvalho, T. L., Alves, E., Da Silva, H. B., De Azevedo, M. F., Hemerly, A. S., et al. 2013. The structurally related auxin and melatonin tryptophan-derivatives and their roles in

Arabidopsis thaliana and in the human malaria parasite *Plasmodium falciparum*. *J Eukaryot Microbiol,* 60**,** 646-51

Kriechbaumer, V., Botchway, S. W. & Hawes, C. 2016. Localization and interactions between *Arabidopsis* auxin biosynthetic enzymes in the TAA/YUC-dependent pathway. *J Exp Bot,* 67**,** 4195-207

Kwon, Y., Lemieux, M., Mctavish, J. & Wathen, N. 2015. Identifying and removing duplicate records from systematic review searches. *J Med Lib Assoc,* 103**,** 184-188

Lareen, A., Burton, F. & Schäfer, P. 2016. Plant root-microbe communication in shaping root microbiomes. *Plant Mol Biol,* 90**,** 575-87

Lee, H. J. & Back, K. 2016a. 2-Hydroxymelatonin promotes the resistance of rice plant to multiple simultaneous abiotic stresses (combined cold and drought). *J Pineal Res,* 61**,** 303-16

Lee, H. Y. & Back, K. 2016b. Mitogen-activated protein kinase pathways are required for melatonin-mediated defense responses in plants. *Journal of Pineal Research,* 60**,** 327-335

Lee, H. Y. & Back, K. 2018. Melatonin induction and its role in high light stress tolerance in *Arabidopsis thaliana*. *J Pineal Res,* 65**,** e12504

Lee, H. Y., Byeon, Y., Lee, K., Lee, H. J. & Back, K. 2014. Cloning of *Arabidopsis* serotonin *N*-acetyltransferase and its role with caffeic acid *O*-methyltransferase in the biosynthesis of melatonin in vitro despite their different subcellular localizations. *J Pineal Res,* 57**,** 418-26

Lee, H. Y., Byeon, Y., Tan, D. X., Reiter, R. J. & Back, K. 2015. *Arabidopsis* serotonin *N*-acetyltransferase knockout mutant plants exhibit decreased melatonin and salicylic acid levels resulting in susceptibility to an avirulent pathogen. *J Pineal Res,* 58**,** 291-9

Lee, H. Y., Lee, K. & Back, K. 2019. Knockout of *Arabidopsis* Serotonin *N*-Acetyltransferase-2 Reduces Melatonin Levels and Delays Flowering. *Biomolecules,* 9

Lee, K. & Back, K. 2017. Overexpression of rice *serotonin N-acetyltransferase 1* in transgenic rice plants confers resistance to cadmium and senescence and increases grain yield. *J Pineal Res,* 62

Lee, K. & Back, K. 2019. Melatonin-deficient rice plants show a common semidwarf phenotype either dependent or independent of brassinosteroid biosynthesis. *J Pineal Res,* 66**,** e12537

Lee, K., Lee, H. Y. & Back, K. 2018. Rice histone deacetylase 10 and *Arabidopsis* histone deacetylase 14 genes encode *N*-acetylserotonin deacetylase, which catalyzes conversion of *N*-

acetylserotonin into serotonin, a reverse reaction for melatonin biosynthesis in plants. *J Pineal Res,* 64

Lee, K., Zawadzka, A., Czarnocki, Z., Reiter, R. J. & Back, K. 2016. Molecular cloning of melatonin 3-hydroxylase and its production of cyclic 3-hydroxymelatonin in rice (*Oryza sativa*). *J Pineal Res,* 61**,** 470-478

Lei, Q., Wang, L., Tan, D. X., Zhao, Y., Zheng, X. D., Chen, H., et al. 2013. Identification of genes for melatonin synthetic enzymes in 'Red Fuji' apple (*Malus domestica* Borkh.cv.Red) and their expression and melatonin production during fruit development. *J Pineal Res,* 55**,** 443-51

Lerner, A. B., Case, J. D., Mori, W. & Wright, M. R. 1959. Melatonin in Peripheral Nerve. *Nature* 1821

Lerner, A. B., Case, J. D. & Takahashi, Y. 1960. Isolation of melatonin and 5-methoxyindole-3-acetic acid from bovine pineal glands. *J Biol Chem,* 235**,** 1992-7

Lerner, A. B., Case, J. D., Takahashi, Y., Lee, T. H. & Mori, W. 1958. Isolation of melatonin, the pineal gland factor that lightens melanocyte. *J Am Chem Soc,* 80**,** 2587-2587

Lewy, A. J., Wehr, T. A., Goodwin, F. K., Newsome, D. A. & Markey, S. P. 1980. Light suppresses melatonin secretion in humans. *Science,* 210**,** 1267-9

Li, C., Tan, D. X., Liang, D., Chang, C., Jia, D. & Ma, F. 2015. Melatonin mediates the regulation of ABA metabolism, free-radical scavenging, and stomatal behaviour in two *Malus* species under drought stress. *J Exp Bot,* 66**,** 669-80

Li, C., Zhao, Q., Gao, T., Wang, H., Zhang, Z., Liang, B., et al. 2018. The mitigation effects of exogenous melatonin on replant disease in apple. *J Pineal Res,* 65**,** e12523

Li, D., Wei, J., Peng, Z., Ma, W., Yang, Q., Song, Z., et al. 2020. Daily rhythms of phytomelatonin signaling modulate diurnal stomatal closure via regulating reactive oxygen species dynamics in *Arabidopsis*. *J Pineal Res,* 68**,** e12640

Liang, C., Li, A., Yu, H., Li, W., Liang, C., Guo, S., et al. 2017. Melatonin Regulates Root Architecture by Modulating Auxin Response in Rice. *Front Plant Sci,* 8**,** 134

Liang, C., Zheng, G., Li, W., Wang, Y., Hu, B., Wang, H., et al. 2015. Melatonin delays leaf senescence and enhances salt stress tolerance in rice. *J Pineal Res,* 59**,** 91-101

Liang, D., Shen, Y., Ni, Z., Wang, Q., Lei, Z., Xu, N., et al. 2018. Exogenous Melatonin Application Delays Senescence of Kiwifruit Leaves by Regulating the Antioxidant Capacity and Biosynthesis of Flavonoids. *Front Plant Sci,* 9**,** 426

Liu, C., Chen, L., Zhao, R., Li, R., Zhang, S., Yu, W., et al. 2019a. Melatonin Induces Disease Resistance to *Botrytis cinerea* in Tomato Fruit by Activating Jasmonic Acid Signaling Pathway. *J Agric Food Chem,* 67**,** 6116-6124

Liu, J., Yang, J., Zhang, H., Cong, L., Zhai, R., Yang, C., et al. 2019b. Melatonin Inhibits Ethylene Synthesis via Nitric Oxide Regulation To Delay Postharvest Senescence in Pears. *J Agric Food Chem,* 67**,** 2279-2288

Liu, J., Zhai, R., Liu, F., Zhao, Y., Wang, H., Liu, L., et al. 2018. Melatonin Induces Parthenocarpy by Regulating Genes in Gibberellin Pathways of 'Starkrimson' Pear (*Pyrus communis* L.). *Front Plant Sci,* 9**,** 946

Liu, T., Zhao, F., Liu, Z., Zuo, Y., Hou, J. & Wang, Y. 2016. Identification of melatonin in *Trichoderma* spp. and detection of melatonin content under controlled-stress growth conditions from *T. asperellum*. *J Basic Microbiol,* 56**,** 838-43

López-Burillo, S., Tan, D. X., Rodriguez-Gallego, V., Manchester, L. C., Mayo, J. C., Sainz, R. M., et al. 2003. Melatonin and its derivatives cyclic 3-hydroxymelatonin, N1-acetylN2-formyl-5-methoxykynuramine and 6-methoxymelatonin reduce oxidative DNA damage induced by Fenton reagents. *J Pineal Res,* 34**,** 178-184

Ma, Y., Jiao, J., Fan, X., Sun, H., Zhang, Y., Jiang, J., et al. 2016. Endophytic Bacterium *Pseudomonas fluorescens* RG11 May Transform Tryptophan to Melatonin and Promote Endogenous Melatonin Levels in the Roots of Four Grape Cultivars. *Front Plant Sci,* 7**,** 2068

Magnanou, E., Attia, J., Fons, R., Boeuf, G. & Falcon, J. 2009. The timing of the shrew: continuous melatonin treatment maintains youthful rhythmic activity in aging *Crocidura russula*. *PLoS One,* 4**,** e5904

Mahlberg, R., Tilmann, A., Salewski, L. & Kunz, D. 2006. Normative data on the daily profile of urinary 6-sulfatoxymelatonin in healthy subjects between the ages of 20 and 84. *Psychoneuroendocrinology,* 31**,** 634-41

Manchester, L. C., Poeggeler, B., Alvares, F. L., Ogden, G. B. & Reiter, R. J. 1995. Melatonin immunoreactivity in the photosynthetic prokaryote *Rhodospirillum rubrum*: implications for an ancient antioxidant system. *Cell Mol Biol Res,* 41**,** 391-5

Manchester, L. C., Tan, D. X., Reiter, R. J., Park, W., Monis, K. & Qi, W. 2000. High levels of melatonin in the seeds of edible plants: possible function in germ tissue protection. *Life Sci,* 67**,** 3023-9

Mandal, M. K., Suren, H., Ward, B., Boroujerdi, A. & Kousik, C. 2018. Differential roles of melatonin in plant-host resistance and pathogen suppression in cucurbits. *J Pineal Res,* 65**,** e12505

Mayo, J. C., Sainz, R. M., Gonzalez Menendez, P., Cepas, V., Tan, D. X. & Reiter, R. J. 2017. Melatonin and sirtuins: A "not-so unexpected" relationship. *J Pineal Res,* 62

Mcisaac, W. M. & Page, I. H. 1958. The Metabolism of Serotonin (5-Hydroxytryptamine). *J Biol Chem,* 234**,** 858-864

Mehrotra, R., Bhalothia, P., Bansal, P., Basantani, M. K., Bharti, V. & Mehrotra, S. 2014. Abscisic acid and abiotic stress tolerance - different tiers of regulation. *J Plant Physiol,* 171**,** 486-96

Meng, X., Li, Y., Li, S., Zhou, Y., Gan, R. Y., Xu, D. P., et al. 2017. Dietary Sources and Bioactivities of Melatonin. *Nutrients,* 9

Mercolini, L., Mandrioli, R. & Raggi, M. A. 2012. Content of melatonin and other antioxidants in grape-related foodstuffs: measurement using a MEPS-HPLC-F method. *J Pineal Res,* 53**,** 21-8

Moher, D., Liberati, A., Tetzlaff, J., Altman, D. G. & Group, P. 2009. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLoS Med,* 6**,** e1000097

Mosa, K. A., Ismail, A. & Helmy, M. 2017. Introduction to Plant Stresses. *Plant Stress Tolerance: An Integrated Omics Approach.* Cham: Springer International Publishing.

Moustafa-Farag, M., Almoneafy, A., Mahmoud, A., Elkelish, A., Arnao, M. B., Li, L., et al. 2019. Melatonin and Its Protective Role against Biotic Stress Impacts on Plants. *Biomolecules,* 10

Mukherjee, S. 2019. Insights into nitric oxide-melatonin crosstalk and *N*-nitrosomelatonin functioning in plants. *J Exp Bot,* 70**,** 6035-6047

Muniz-Calvo, S., Bisquert, R., Fernandez-Cruz, E., Garcia-Parrilla, M. C. & Guillamon, J. M. 2019. Deciphering the melatonin metabolism in *Saccharomyces cerevisiae* by the bioconversion of related metabolites. *J Pineal Res,* 66**,** e12554

Murch, S. J., Simmons, C. B. & Saxena, P. K. 1997. Melatonin in feverfew and other medicinal plants. *Lancet,* 350**,** 1598-9

Muszynska, B., Kala, K., Sulkowska-Ziaja, K., Krakowska, A. & Opoka, W. 2016. *Agaricus bisporus* and its in vitro culture as a source of indole compounds released into artificial digestive juices. *Food Chem,* 199**,** 509-15

Muszynska, B. & Sulkowska-Ziaja, K. 2012. Analysis of indole compounds in edible *Basidiomycota* species after thermal processing. *Food Chem,* 132**,** 455-9

Nagajyoti, P. C., Lee, K. D. & Sreekanth, T. V. M. 2010. Heavy metals, occurrence and toxicity for plants: a review. *Environ Chem Lett,* 8**,** 199-216

Nelson, R. J. & Demas, G. E. 1996. Seasonal changes in immune function. *Q Rev Biol,* 71**,** 511-48

Nelson, R. J. & Drazen, D. L. 1999. Melatonin mediates seasonal adjustments in immune function. *Reprod Nutr Dev,* 39**,** 383-98

Noir, S., Bomer, M., Takahashi, N., Ishida, T., Tsui, T. L., Balbi, V., et al. 2013. Jasmonate controls leaf growth by repressing cell proliferation and the onset of endoreduplication while maintaining a potential stand-by mode. *Plant Physiol,* 161**,** 1930-51

Oladi, E., Mohamadi, M., Shamspur, T. & Mostafavi, A. 2014. Spectrofluorimetric determination of melatonin in kernels of four different *Pistacia* varieties after ultrasound-assisted solid-liquid extraction. *Spectrochim Acta A Mol Biomol Spectrosc,* 132**,** 326-9

Oztürk, A. I., Yilmaz, O., Kirbag, S. & Arslan, M. 2000. Antimicrobial and biological effects of ipemphos and amphos on bacterial and yeast strains. *Cell Biochem Funct,* 18**,** 117-26

Pandey, P., Irulappan, V., Bagavathiannan, M. V. & Senthil-Kumar, M. 2017. Impact of Combined Abiotic and Biotic Stresses on Plant Growth and Avenues for Crop Improvement by Exploiting Physio-morphological Traits. *Front Plant Sci,* 8**,** 537

Park, S. & Back, K. 2012. Melatonin promotes seminal root elongation and root growth in transgenic rice after germination. *J Pineal Res,* 53**,** 385-9

Park, S., Byeon, Y., Lee, H. Y., Kim, Y. S., Ahn, T. & Back, K. 2014. Cloning and characterization of a serotonin *N*-acetyltransferase from a gymnosperm, loblolly pine (*Pinus taeda*). *J Pineal Res,* 57**,** 348-55

Patke, A., Young, M. W. & Axelrod, S. 2020. Molecular mechanisms and physiological importance of circadian rhythms. *Nat Rev Mol Cell Biol,* 21**,** 67-84

Paul, M. A., Love, R. J., Hawton, A. & Arendt, J. 2015. Sleep and the endogenous melatonin rhythm of high arctic residents during the summer and winter. *Physiol Behav,* 141**,** 199-206

Paulose, J. K., Wright, J. M., Patel, A. G. & Cassone, V. M. 2016. Human Gut Bacteria Are Sensitive to Melatonin and Express Endogenous Circadian Rhythmicity. *PLoS One,* 11**,** e0146643

Pelagio-Flores, R., Munoz-Parra, E., Ortiz-Castro, R. & Lopez-Bucio, J. 2012. Melatonin regulates *Arabidopsis* root system architecture likely acting independently of auxin signaling. *J Pineal Res,* 53**,** 279-88

Pierpaoli, W. & Regelson, W. 1994. Pineal control of aging: effect of melatonin and pineal grafting on aging mice. *Proc Natl Acad Sci U S A,* 91**,** 787-91

Pluharova, K., Leontovycova, H., Stoudkova, V., Pospichalova, R., Marsik, P., Kloucek, P., et al. 2019. "Salicylic Acid Mutant Collection" as a Tool to Explore the Role of Salicylic Acid in Regulation of Plant Growth under a Changing Environment. *Int J Mol Sci,* 20

Poeggeler, B., Reiter, R. J., Tan, D. X., Chen, L. D. & Manchester, L. C. 1993. Melatonin, hydroxyl radical-mediated oxidative damage, and aging: a hypothesis. *J Pineal Res,* 14**,** 151-68

Poeggeler, B., Saarela, S., Reiter, R. J., Tan, D. X., Chen, L. D., Manchester, L. C., et al. 1994. Melatonin--a highly potent endogenous radical scavenger and electron donor: new aspects of the oxidation chemistry of this indole accessed in vitro. *Ann N Y Acad Sci,* 738**,** 419-20

Poupin, M. J., Greve, M., Carmona, V. & Pinedo, I. 2016. A Complex Molecular Interplay of Auxin and Ethylene Signaling Pathways Is Involved in *Arabidopsis* Growth Promotion by *Burkholderia phytofirmans* PsJN. *Front Plant Sci,* 7**,** 492

Qian, Y., Tan, D. X., Reiter, R. J. & Shi, H. 2015. Comparative metabolomic analysis highlights the involvement of sugars and glycerol in melatonin-mediated innate immunity against bacterial pathogen in *Arabidopsis*. *Sci Rep,* 5**,** 15815

Rademacher, W. 2015. Plant Growth Regulators: Backgrounds and Uses in Plant Production. *J Plant Growth Regul,* 34**,** 845-872

Rahman, A., Bannigan, A., Sulaman, W., Pechter, P., Blancaflor, E. B. & Baskin, T. I. 2007. Auxin, actin and growth of the *Arabidopsis thaliana* primary root. *Plant J,* 50**,** 514-28

Ramakrishna, A., Giridhar, P., Sankar, K. U. & Ravishankar, G. A. 2012. Melatonin and serotonin profiles in beans of *Coffea* species. *J Pineal Res,* 52**,** 470-6

Ramirez-Puebla, S. T., Servin-Garciduenas, L. E., Jimenez-Marin, B., Bolanos, L. M., Rosenblueth, M., Martinez, J., et al. 2013. Gut and root microbiota commonalities. *Appl Environ Microbiol,* 79**,** 2-9

Rath, M. F., Coon, S. L., Amaral, F. G., Weller, J. L., Moller, M. & Klein, D. C. 2016. Melatonin Synthesis: Acetylserotonin O-Methyltransferase (ASMT) Is Strongly Expressed in a Subpopulation of Pinealocytes in the Male Rat Pineal Gland. *Endocrinology,* 157**,** 2028-40

Raza, A., Razzaq, A., Mehmood, S. S., Zou, X., Zhang, X., Lv, Y., et al. 2019. Impact of Climate Change on Crops Adaptation and Strategies to Tackle Its Outcome: A Review. *Plants (Basel),* 8

Reiter, R. J. 1986. Normal patterns of melatonin levels in the pineal gland and body fluids of humans and experimental animals. *J Neural Transm Suppl,* 21**,** 35-54

Reiter, R. J. 1991. Melatonin: the chemical expression of darkness. *Mol Cell Endocrinol,* 79**,** C153-8

Reiter, R. J., Manchester, L. C. & Tan, D. X. 2005. Melatonin in walnuts: influence on levels of melatonin and total antioxidant capacity of blood. *Nutrition,* 21**,** 920-4

Reiter, R. J., Mayo, J. C., Tan, D. X., Sainz, R. M., Alatorre-Jimenez, M. & Qin, L. 2016. Melatonin as an antioxidant: under promises but over delivers. *J Pineal Res,* 61**,** 253-78

Reiter, R. J., Paredes, S. D., Korkmaz, A., Manchester, L. C. & Tan, D. X. 2008. Melatonin in relation to the "strong" and "weak" versions of the free radical theory of aging. *Adv Med Sci,* 53**,** 119-29

Reiter, R. J., Richardson, B. A., Johnson, L. Y., Ferguson, B. N. & Dinh, D. T. 1980. Pineal melatonin rhythm: reduction in aging Syrian hamsters. *Science,* 210**,** 1372-3

Reiter, R. J., Rosales-Corral, S., Tan, D. X., Jou, M. J., Galano, A. & Xu, B. 2017. Melatonin as a mitochondria-targeted antioxidant: one of evolution's best ideas. *Cell Mol Life Sci,* 74**,** 3863-3881

Reiter, R. J., Rosales-Corral, S. A., Manchester, L. C., Xiaoyan, L. & Tan, D. X. 2014. Melatonin in the biliary tract and liver: health implications. *Curr Pharm Des,* 20**,** 4788-4801

Ren, S., Jiang, G. & Rutto, L. 2019a. Melatonin priming enhances symbiotic nitrogen fixation in soybean, *Glycine max* L. *J Biotech Res,* 10**,** 136-144

Ren, S., Rutto, L. & Katuuramu, D. 2019b. Melatonin acts synergistically with auxin to promote lateral root development through fine tuning auxin transport in *Arabidopsis thaliana*. *PLoS One,* 14**,** e0221687

Ressmeyer, A. R., Mayo, J. C., Zelosko, V., Sainz, R. M., Tan, D. X., Poeggeler, B., et al. 2003. Antioxidant properties of the melatonin metabolite N1-acetyl-5-methoxykynuramine (AMK): scavenging of free radicals and prevention of protein destruction. *Redox Rep,* 8**,** 205-13

Rodriguez-Naranjo, M. I., Gil-Izquierdo, A., Troncoso, A. M., Cantos, E. & Garcia-Parrilla, M. C. 2011. Melatonin: a new bioactive compound in wine. *J Food Compos Anal,* 24**,** 603-608

Rodriguez-Naranjo, M. I., Torija, M. J., Mas, A., Cantos-Villar, E., Garcia-Parrilla Mdel, C., Tilden, A. R., et al. 2012. Production of melatonin by *Saccharomyces* strains under growth and fermentation conditions.

. *J Pineal Res,* 53**,** 219-24

Rosales-Corral, S. A., Acuna-Castroviejo, D., Coto-Montes, A., Boga, J. A., Manchester, L. C., Fuentes-Broto, L., et al. 2012. Alzheimer's disease: pathological mechanisms and the beneficial role of melatonin. *J Pineal Res,* 52**,** 167-202

Saini, S., Sharma, I. & Pati, P. K. 2015. Versatile roles of brassinosteroid in plants in the context of its homoeostasis, signaling and crosstalks. *Front Plant Sci,* 6**,** 950

Salehi, B., Sharopov, F., Fokou, P. V. T., Kobylinska, A., Jonge, L., Tadio, K., et al. 2019. Melatonin in Medicinal and Food Plants: Occurrence, Bioavailability, and Health Potential for Humans. *Cells,* 8

Santner, A., Calderon-Villalobos, L. I. & Estelle, M. 2009. Plant hormones are versatile chemical regulators of plant growth. *Nat Chem Biol,* 5**,** 301-7

Sarropoulou, V., Dimassi-Theriou, K., Therios, I. & Koukourikou-Petridou, M. 2012. Melatonin enhances root regeneration, photosynthetic pigments, biomass, total carbohydrates and proline content in the cherry rootstock PHL-C (*Prunus avium* x *Prunus cerasus*). *Plant Physiol Biochem,* 61**,** 162-8

Savary, S., Ficke, A., Aubertot, J.-N. & Hollier, C. 2012. Crop losses due to diseases and their implications for global food production losses and food security. *Food Security,* 4**,** 519-537

Shah, A. A., Ahmed, S., Ali, A. & Yasin, N. A. 2020a. 2-Hydroxymelatonin mitigates cadmium stress in *Cucumis sativus* seedlings: Modulation of antioxidant enzymes and polyamines. *Chemosphere,* 243

Shah, A. A., Ahmed, S. & Yasin, N. A. 2020b. 2-Hydroxymelatonin induced nutritional orchestration in *Cucumis sativus* under cadmium toxicity: modulation of non-enzymatic antioxidants and gene expression. *Int J Phytoremediat,* 22**,** 497-507

Shi, H. & Chan, Z. 2014. The cysteine2/histidine2-type transcription factor *ZINC FINGER OF ARABIDOPSIS THALIANA* 6-activated *C-REPEAT-BINDING FACTOR* pathway is essential for melatonin-mediated freezing stress resistance in *Arabidopsis*. *J Pineal Res,* 57**,** 185-91

Shi, H., Chen, Y., Tan, D. X., Reiter, R. J., Chan, Z. & He, C. 2015a. Melatonin induces nitric oxide and the potential mechanisms relate to innate immunity against bacterial pathogen infection in *Arabidopsis*. *J Pineal Res,* 59**,** 102-8

Shi, H., Jiang, C., Ye, T., Tan, D. X., Reiter, R. J., Zhang, H., et al. 2015b. Comparative physiological, metabolomic, and transcriptomic analyses reveal mechanisms of improved abiotic stress resistance in bermudagrass [*Cynodon dactylon* (L). Pers.] by exogenous melatonin. *J Exp Bot,* 66**,** 681-94

Shi, H., Reiter, R. J., Tan, D. X. & Chan, Z. 2015c. *INDOLE-3-ACETIC ACID INDUCIBLE* 17 positively modulates natural leaf senescence through melatonin-mediated pathway in *Arabidopsis*. *J Pineal Res,* 58**,** 26-33

Shi, H., Tan, D. X., Reiter, R. J., Ye, T., Yang, F. & Chan, Z. 2015d. Melatonin induces *class A1 heat-shock factors (HSFA1s)* and their possible involvement of thermotolerance in *Arabidopsis*. *J Pineal Res,* 58**,** 335-42

Shi, H., Wei, Y. & He, C. 2016a. Melatonin-induced *CBF/DREB1s* are essential for diurnal change of disease resistance and *CCA1* expression in *Arabidopsis*. *Plant Physiol Biochem,* 100**,** 150-155

Shi, H., Wei, Y., Wang, Q., Reiter, R. J. & He, C. 2016b. Melatonin mediates the stabilization of DELLA proteins to repress the floral transition in *Arabidopsis*. *J Pineal Res,* 60**,** 373-9

Shi, H., Zhang, S., Lin, D., Wei, Y., Yan, Y., Liu, G., et al. 2018. Zinc finger of *Arabidopsis thaliana 6* is involved in melatonin-mediated auxin signaling through interacting INDETERMINATE DOMAIN15 and INDOLE-3-ACETIC ACID 17. *J Pineal Res,* 65**,** e12494

Shida, C. S., Castrucci, A. M. & Lamy-Freund, M. T. 1994. High melatonin solubility in aqueous medium. *J Pineal Res,* 16**,** 198-201

Smith, J. D., Woldemariam, M. G., Mescher, M. C., Jander, G. & De Moraes, C. M. 2016. Glucosinolates from Host Plants Influence Growth of the Parasitic Plant *Cuscuta gronovii* and Its Susceptibility to Aphid Feeding. *Plant Physiol,* 172**,** 181-97

Sprenger J, H. R., Fuhrberg B, Han S.-Z. 1999. Melatonin and other 5-methoxylated indoles in yeast: presence in high concentrations and and dependence on tryptophan availability. *Cytologia,* 64**,** 209-213

Stepanova, A. N., Hoyt, J. M., Hamilton, A. A. & Alonso, J. M. 2005. A Link between ethylene and auxin uncovered by the characterization of two root-specific ethylene-insensitive mutants in *Arabidopsis*. *Plant Cell,* 17**,** 2230-42

Stevens, L. H., Blom, T. J. & Verpoorte, R. 1993. Subcellular localization of tryptophan decarboxylase, strictosidine synthase and strictosidine glucosidase in suspension cultured cells of *Catharanthus roseus* and *Tabernaemontana divaricata*. *Plant Cell Rep,* 12**,** 573-6

Sturtz, M., Cerezo, A. B., Cantos-Villar, E. & Garcia-Parrilla, M. C. 2011. Determination of the melatonin content of different varieties of tomatoes (*Lycopersicon esculentum*) and strawberries (*Fragaria ananassa*). *Food Chem,* 127**,** 1329-34

Sun, Q., Zhang, N., Wang, J., Zhang, H., Li, D., Shi, J., et al. 2015. Melatonin promotes ripening and improves quality of tomato fruit during postharvest life. *J Exp Bot,* 66**,** 657-68

Sung, D. Y., Kaplan, F., Lee, K. J. & Guy, C. L. 2003. Acquired tolerance to temperature extremes. *Trends Plant Sci,* 8**,** 179-87

Szewczyk-Golec, K., Rajewski, P., Gackowski, M., Mila-Kierzenkowska, C., Wesolowski, R., Sutkowy, P., et al. 2017. Melatonin Supplementation Lowers Oxidative Stress and Regulates Adipokines in Obese Patients on a Calorie-Restricted Diet. *Oxid Med Cell Longev,* 2017**,** 8494107

Tamura, H., Jozaki, M., Tanabe, M., Shirafuta, Y., Mihara, Y., Shinagawa, M., et al. 2020. Importance of Melatonin in Assisted Reproductive Technology and Ovarian Aging. *Int J Mol Sci,* 21**,** 1135

Tan, D. X., Chen, L. D., Poeggeler, B., Manchester, L. C., Reiter, R. J. & Poeggler, B. 1993. Melatonin a potent endogenous hydroxyl radical scavenger. *Endocr J,* 1**,** 57-60

Tan, D. X., Hardeland, R., Back, K., Manchester, L. C., Alatorre-Jimenez, M. A. & Reiter, R. J. 2016. On the significance of an alternate pathway of melatonin synthesis via 5-methoxytryptamine: comparisons across species. *J Pineal Res,* 61**,** 27-40

Tan, D. X., Hardeland, R., Manchester, L. C., Korkmaz, A., Ma, S., Rosales-Corral, S., et al. 2012. Functional roles of melatonin in plants, and perspectives in nutritional and agricultural science. *J Exp Bot,* 63**,** 577-97

Tan, D. X., Hardeland, R., Manchester, L. C., Paredes, S. D., Korkmaz, A., Sainz, R. M., et al. 2010a. The changing biological roles of melatonin during evolution: from an antioxidant to signals of darkness, sexual selection and fitness. *Biol Rev Camb Philos Soc,* 85**,** 607-23

Tan, D. X., Hardeland, R., Manchester, L. C., Poeggeler, B., Lopez-Burillo, S., Mayo, J. C., et al. 2003. Mechanistic and comparative studies of melatonin and classic antioxidants in terms of their interactions with the ABTS cation radical. *J Pineal Res,* 34**,** 249-59

Tan, D. X., Manchester, L. C., Di Mascio, P., Martinez, G. R., Prado, F. M. & Reiter, R. J. 2007. Novel rhythms of N^1-acetyl-N^2-formyl-5-methoxykynuramine and its precursor melatonin in water hyacinth: importance for phytoremediation. *FASEB J,* 21**,** 1724-9

Tan, D. X., Manchester, L. C., Esteban-Zubero, E., Zhou, Z. & Reiter, R. J. 2015. Melatonin as a Potent and Inducible Endogenous Antioxidant: Synthesis and Metabolism. *Molecules,* 20**,** 18886-906

Tan, D. X., Manchester, L. C., Sainz, R. M., Mayo, J. C., Leon, J., Hardeland, R., et al. 2005. Interactions between melatonin and nicotinamide nucleotide: NADH preservation in cells and in cell-free systems by melatonin. *J Pineal Res,* 39**,** 185-94

Tan, D. X., Manchester, L. C., Sanchez-Barcelo, E., Mediavilla, M. D. & Reiter, R. J. 2010b. Significance of high levels of endogenous melatonin in Mammalian cerebrospinal fluid and in the central nervous system. *Curr Neuropharmacol,* 8**,** 162-7

Tan, D. X., Reiter, R. J., Manchester, L. C., Yan, M. T., El-Sawi, M., Sainz, R. M., et al. 2002. Chemical and physical properties and potential mechanisms: melatonin as a broad spectrum antioxidant and free radical scavenger. *Curr Top Med Chem,* 2**,** 181-97

Tan, D. X., Xu, B., Zhou, X. & Reiter, R. J. 2018. Pineal Calcification, Melatonin Production, Aging, Associated Health Consequences and Rejuvenation of the Pineal Gland. *Molecules,* 23

Tan, D. X., Zanghi, B. M., Manchester, L. C. & Reiter, R. J. 2014. Melatonin identified in meats and other food stuffs: potentially nutritional impact. *J Pineal Res,* 57**,** 213-8

Tapia, M. I., Sánchez-Morgado, J. R., García-Parra, J., Ramírez, R., Hernández, T., González-Gómez, D. J. J. O. F. C., et al. 2013. Comparative study of the nutritional and bioactive compounds content of four walnut (*Juglans regia* L.) cultivars. *J Food Compost Anal,* 31**,** 232-237

Tekbas, O. F., Ogur, R., Korkmaz, A., Kilic, A. & Reiter, R. J. 2008. Melatonin as an antibiotic: new insights into the actions of this ubiquitous molecule. *J Pineal Res,* 44**,** 222-6

Tian, H., De Smet, I. & Ding, Z. 2014. Shaping a root system: regulating lateral versus primary root growth. *Trends Plant Sci,* 19**,** 426-31

Tilden, A. R., Becker, M. A., Amma, L. L., Arciniega, J. & Mcgaw, A. K. 1997. Melatonin production in an aerobic photosynthetic bacterium: an evolutionarily early association with darkness. *J Pineal Res,* 22**,** 102-6

Tito, R., Vasconcelos, H. L. & Feeley, K. J. 2018. Global climate change increases risk of crop yield losses and food insecurity in the tropical Andes. *Glob Chang Biol,* 24**,** e592-e602

Tuzcu, M. & Baydas, G. 2006. Effect of melatonin and vitamin E on diabetes-induced learning and memory impairment in rats. *Eur J Pharmacol,* 537**,** 106-10

Van Tassel, L., Roberts, N., Lewy, A. & O'neill, S. D. 2001. Melatonin in plant organs. *J Pineal Res,* 31**,** 8-15

Vazquez, J., Grillitsch, K., Daum, G., Mas, A., Torija, M. J. & Beltran, G. 2018. Melatonin Minimizes the Impact of Oxidative Stress Induced by Hydrogen Peroxide in *Saccharomyces* and Non-conventional Yeast. *Front Microbiol,* 9**,** 1933

Venegas, C., Garcia, J. A., Escames, G., Ortiz, F., Lopez, A., Doerrier, C., et al. 2012. Extrapineal melatonin: analysis of its subcellular distribution and daily fluctuations. *J Pineal Res,* 52**,** 217-27

Viglione, G. 2020. Tens of thousands of scientists are redeploying to fight coronavirus. *Nature,*

Vitalini, S., Gardana, C., Zanzotto, A., Simonetti, P., Faoro, F., Fico, G., et al. 2011. The presence of melatonin in grapevine (*Vitis vinifera* L.) berry tissues. *J Pineal Res,* 51**,** 331-7

Wan, J., Zhang, P., Wang, R., Sun, L., Ju, Q. & Xu, J. 2018. Comparative physiological responses and transcriptome analysis reveal the roles of melatonin and serotonin in regulating growth and metabolism in *Arabidopsis*. *BMC Plant Biol.,* 18**,** 362

Wang, H. X., Liu, F. & Ng, T. B. 2001. Examination of pineal indoles and 6-methoxy-2-benzoxazolinone for antioxidant and antimicrobial effects. *Comp Biochem Physiol C Toxicol Pharmacol,* 130**,** 379-88

Wang, L., Zhao, Y., Reiter, R. J., He, C., Liu, G., Lei, Q., et al. 2014. Changes in melatonin levels in transgenic 'Micro-Tom' tomato overexpressing ovine *AANAT* and ovine *HIOMT* genes. *J Pineal Res,* 56**,** 134-42

Wang, M., Duan, S., Zhou, Z., Chen, S. & Wang, D. 2019. Foliar spraying of melatonin confers cadmium tolerance in *Nicotiana tabacum* L. *Ecotoxicol Environ* 170**,** 68-76

Wang, M., Zhang, S. & Ding, F. 2020a. Melatonin Mitigates Chilling-Induced Oxidative Stress and Photosynthesis Inhibition in Tomato Plants. *Antioxidants (Basel),* 9

Wang, P., Yin, L., Liang, D., Li, C., Ma, F. & Yue, Z. 2012. Delayed senescence of apple leaves by exogenous melatonin treatment: toward regulating the ascorbate-glutathione cycle. *J Pineal Res,* 53**,** 11-20

Wang, Q., An, B., Shi, H., Luo, H. & He, C. 2017. High Concentration of Melatonin Regulates Leaf Development by Suppressing Cell Proliferation and Endoreduplication in *Arabidopsis*. *Int J Mol Sci,* 18

Wang, Q., An, B., Wei, Y., Reiter, R. J., Shi, H., Luo, H., et al. 2016. Melatonin Regulates Root Meristem by Repressing Auxin Synthesis and Polar Auxin Transport in *Arabidopsis*. *Front Plant Sci,* 7**,** 1882

Wang, Q., Qin, G., Cao, M., Chen, R., He, Y., Yang, L., et al. 2020b. A phosphorylation-based switch controls TAA1-mediated auxin biosynthesis in plants. *Nat Commun,* 11**,** 679

Wang, Y., Yao, Q., Zhang, Y., Zhang, Y., Xing, J., Yang, B., et al. 2020c. The Role of Gibberellins in Regulation of Nitrogen Uptake and Physiological Traits in Maize Responding to Nitrogen Availability. *Int J Mol Sci,* 21

Wasternack, C. 2007. Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. *Ann Bot,* 100**,** 681-97

Weeda, S., Zhang, N., Zhao, X., Ndip, G., Guo, Y., Buck, G. A., et al. 2014. *Arabidopsis* transcriptome analysis reveals key roles of melatonin in plant defense systems. *PLoS One,* 9**,** e93462

Wei, J., Li, D. X., Zhang, J. R., Shan, C., Rengel, Z., Song, Z. B., et al. 2018a. Phytomelatonin receptor PMTR1-mediated signaling regulates stomatal closure in *Arabidopsis thaliana*. *J Pineal Res,* 65**,** e12500

Wei, W., Li, Q. T., Chu, Y. N., Reiter, R. J., Yu, X. M., Zhu, D. H., et al. 2015. Melatonin enhances plant growth and abiotic stress tolerance in soybean plants. *J Exp Bot,* 66**,** 695-707

Wei, Y., Chang, Y., Zeng, H., Liu, G., He, C. & Shi, H. 2018b. RAV transcription factors are essential for disease resistance against cassava bacterial blight via activation of melatonin biosynthesis genes. *J Pineal Res,* 64

Wei, Y., Hu, W., Wang, Q., Zeng, H., Li, X., Yan, Y., et al. 2017. Identification, transcriptional and functional analysis of heat-shock protein 90s in banana (*Musa acuminata* L.) highlight their novel role in melatonin-mediated plant response to Fusarium wilt. *J Pineal Res,* 62

Wen, D., Gong, B., Sun, S., Liu, S., Wang, X., Wei, M., et al. 2016. Promoting Roles of Melatonin in Adventitious Root Development of *Solanum lycopersicum* L. by Regulating Auxin and Nitric Oxide Signaling. *Front Plant Sci,* 7**,** 718

Wierman, M. B. & Smith, J. S. 2014. Yeast sirtuins and the regulation of aging. *FEMS Yeast Res,* 14**,** 73-88

Wilkinson, S., Kudoyarova, G. R., Veselov, D. S., Arkhipova, T. N. & Davies, W. J. 2012. Plant hormone interactions: innovative targets for crop breeding and management. *J Exp Bot,* 63**,** 3499-509

Williams, D. L. H. 2004. *Nitrosation reactions and the chemistry of nitric oxide*, Elsevier.

Xia, H., Shen, Y., Shen, T., Wang, X., Zhang, X., Hu, P., et al. 2020. Melatonin Accumulation in Sweet Cherry and Its Influence on Fruit Quality and Antioxidant Properties. *Molecules,* 25

Xiao, S., Liu, L., Wang, H., Li, D., Bai, Z., Zhang, Y., et al. 2019. Exogenous melatonin accelerates seed germination in cotton (*Gossypium hirsutum* L.). *PLoS One,* 14**,** e0216575

Xu, L., Yue, Q., Xiang, G., Bian, F. & Yao, Y. 2018. Melatonin promotes ripening of grape berry via increasing the levels of ABA, H2O2, and particularly ethylene. *Hortic Res,* 5**,** 41

Xu, T., Chen, Y. & Kang, H. 2019. Melatonin Is a Potential Target for Improving Post-Harvest Preservation of Fruits and Vegetables. *Front Plant Sci,* 10**,** 1388

Yakhin, O. I., Lubyanov, A. A., Yakhin, I. A. & Brown, P. H. 2016. Biostimulants in Plant Science: A Global Perspective. *Front Plant Sci,* 7**,** 2049

Yang, J., Yuan, Z., Meng, Q., Huang, G., Perin, C., Bureau, C., et al. 2017. Dynamic Regulation of Auxin Response during Rice Development Revealed by Newly Established Hormone Biosensor Markers. *Front Plant Sci,* 8**,** 256

Yang, J., Zhang, J., Liu, K., Wang, Z. & Liu, L. 2006. Abscisic acid and ethylene interact in wheat grains in response to soil drying during grain filling. *New Phytol,* 171**,** 293-303

Yang, W. J., Du, Y. T., Zhou, Y. B., Chen, J., Xu, Z. S., Ma, Y. Z., et al. 2019. Overexpression of TaCOMT Improves Melatonin Production and Enhances Drought Tolerance in Transgenic *Arabidopsis*. *Int J Mol Sci,* 20

Ye, T., Yin, X., Yu, L., Zheng, S. J., Cai, W. J., Wu, Y., et al. 2019. Metabolic analysis of the melatonin biosynthesis pathway using chemical labeling coupled with liquid chromatography-mass spectrometry. *J Pineal Res,* 66**,** e12531

Yilmaz, T., Celebi, S. & Kukner, A. S. 2002. The protective effects of melatonin, vitamin E and octreotide on retinal edema during ischemia-reperfusion in the guinea pig retina. *Eur J Ophthalmol,* 12**,** 443-9

Yu, G. M., Kubota, H., Okita, M. & Maeda, T. 2017. The anti-inflammatory and antioxidant effects of melatonin on LPS-stimulated bovine mammary epithelial cells. *PLoS One,* 12**,** e0178525

Yu, H., Dickson, E. J., Jung, S. R., Koh, D. S. & Hille, B. 2016. High membrane permeability for melatonin. *J Gen Physiol,* 147**,** 63-76

Yu, Y., Bian, L., Jiao, Z., Yu, K., Wan, Y., Zhang, G., et al. 2019. Molecular cloning and characterization of a grapevine (*Vitis vinifera* L.) serotonin *N*-acetyltransferase (*VvSNAT2*) gene involved in plant defense. *BMC Genomics,* 20**,** 880

Yu, Y., Lv, Y., Shi, Y., Li, T., Chen, Y., Zhao, D., et al. 2018. The Role of Phyto-Melatonin and Related Metabolites in Response to Stress. *Molecules,* 23

Zampol, M. A. & Barros, M. H. 2018. Melatonin improves survival and respiratory activity of yeast cells challenged by alpha-synuclein and menadione. *Yeast,* 35**,** 281-290

Zhang, H., Wang, L., Shi, K., Shan, D., Zhu, Y., Wang, C., et al. 2019a. Apple tree flowering is mediated by low level of melatonin under the regulation of seasonal light signal. *J Pineal Res,* 66**,** e12551

Zhang, H. J., Zhang, N., Yang, R. C., Wang, L., Sun, Q. Q., Li, D. B., et al. 2014a. Melatonin promotes seed germination under high salinity by regulating antioxidant systems, ABA and GA(4) interaction in cucumber (*Cucumis sativus* L.). *J Pineal Res,* 57**,** 269-79

Zhang, H. M. & Zhang, Y. 2014. Melatonin: a well-documented antioxidant with conditional pro-oxidant actions. *J Pineal Res,* 57**,** 131-46

Zhang, J., Li, D., Wei, J., Ma, W., Kong, X., Rengel, Z., et al. 2019b. Melatonin alleviates aluminum-induced root growth inhibition by interfering with nitric oxide production in *Arabidopsis*. *Environ Exp Bot,* 161**,** 157-165

Zhang, J., Shi, Y., Zhang, X., Du, H., Xu, B. & Huang, B. 2017a. Melatonin suppression of heat-induced leaf senescence involves changes in abscisic acid and cytokinin biosynthesis and signaling pathways in perennial ryegrass (*Lolium perenne* L.). *Environ Exp Bot,* 138**,** 36-45

Zhang, N., Sun, Q., Zhang, H., Cao, Y., Weeda, S., Ren, S., et al. 2015. Roles of melatonin in abiotic stress resistance in plants. *J Exp Bot,* 66**,** 647-56

Zhang, N., Zhang, H. J., Sun, Q. Q., Cao, Y. Y., Li, X., Zhao, B., et al. 2017b. Proteomic analysis reveals a role of melatonin in promoting cucumber seed germination under high salinity by regulating energy production. *Sci Rep,* 7**,** 503

Zhang, N., Zhang, H. J., Zhao, B., Sun, Q. Q., Cao, Y. Y., Li, R., et al. 2014b. The RNA-seq approach to discriminate gene expression profiles in response to melatonin on cucumber lateral root formation. *J Pineal Res,* 56**,** 39-50

Zhang, N., Zhao, B., Zhang, H. J., Weeda, S., Yang, C., Yang, Z. C., et al. 2013. Melatonin promotes water-stress tolerance, lateral root formation, and seed germination in cucumber (*Cucumis sativus* L.). *J Pineal Res,* 54**,** 15-23

Zhang, Z., Hu, Q., Liu, Y., Cheng, P., Cheng, H., Liu, W., et al. 2019c. Strigolactone represses the synthesis of melatonin, thereby inducing floral transition in *Arabidopsis thaliana* in an FLC-dependent manner. *J Pineal Res,* 67**,** e12582

Zhao, D., Yu, Y., Shen, Y., Liu, Q., Zhao, Z., Sharma, R., et al. 2019a. Melatonin Synthesis and Function: Evolutionary History in Animals and Plants. *Front Endocrinol (Lausanne),* 10**,** 249

Zhao, G., Yu, X., Lou, W., Wei, S., Wang, R., Wan, Q., et al. 2019b. Transgenic *Arabidopsis* overexpressing *MsSNAT* enhances salt tolerance via the increase in autophagy, and the reestablishment of redox and ion homeostasis. *Environ Exp Bot,* 164**,** 20-28

Zheng, X., Tan, D. X., Allan, A. C., Zuo, B., Zhao, Y., Reiter, R. J., et al. 2017. Chloroplastic biosynthesis of melatonin and its involvement in protection of plants from salt stress. *Sci Rep,* 7**,** 41236

Zhou, L., Chen, X., Liu, T., Gong, Y., Chen, S., Pan, G., et al. 2015. Melatonin reverses H2O2-induced premature senescence in mesenchymal stem cells via the SIRT1-dependent pathway. *J Pineal Res,* 59**,** 190-205

Zhou, Y., Hou, Y., Shen, J., Huang, Y., Martin, W. & Cheng, F. 2020. Network-based drug repurposing for novel coronavirus 2019-nCoV/SARS-CoV-2. *Cell Discov,* 6**,** 14

Zia, S. F., Berkowitz, O., Bedon, F., Whelan, J., Franks, A. E. & Plummer, K. M. 2019. Direct comparison of *Arabidopsis* gene expression reveals different responses to melatonin versus auxin. *BMC Plant Biol,* 19**,** 567

Zuo, B., Zheng, X., He, P., Wang, L., Lei, Q., Feng, C., et al. 2014. Overexpression of *MzASMT* improves melatonin production and enhances drought tolerance in transgenic *Arabidopsis thaliana* plants. *J Pineal Res,* 57**,** 408-17

Chapter 2: Direct comparison of *Arabidopsis* gene expression reveals different responses to melatonin versus auxin

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Direct comparison of *Arabidopsis* gene expression reveals different responses to melatonin versus auxin

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Abstract

Background: Melatonin (*N*-acetyl-5-methoxytryptamine) in plants, regulates shoot and root growth and alleviates environmental stresses. Melatonin and the phyto-hormone auxin are tryptophan-derived compounds. However, it largely remains controversial as to whether melatonin and auxin act through similar or overlapping signalling and regulatory pathways.

Results: Here, we have used a promoter-activation study to demonstrate that, unlike auxin (1-naphthalene acetic acid, NAA), melatonin neither induces *Direct repeat 5 DR5* expression in *Arabidopsis thaliana* roots under normal growth conditions nor suppresses the induction of *Alternative oxidase 1a AOX1a* in leaves upon Antimycin A treatment, both of which are the hallmarks of auxin action. Additionally, comparative global transcriptome analysis conducted on Arabidopsis treated with melatonin or NAA revealed differences in the number and types of differentially expressed genes. Auxin (4.5 μM) altered the expression of a diverse and large number of genes whereas melatonin at 5 μM had no significant effect but melatonin at 100 μM had a modest effect on transcriptome compared to solvent-treated control. Interestingly, the prominent category of genes differentially expressed upon exposure to melatonin trended towards biotic stress defence pathways while downregulation of key genes related to photosynthesis was observed.

Conclusion: Together these findings indicate that though they are both indolic compounds, melatonin and auxin act through different pathways to alter gene expression in *Arabidopsis thaliana*. Furthermore, it appears that effects of melatonin enable *Arabidopsis thaliana* to prioritize biotic stress defence signalling rather than growth. These findings clear the current confusion in the literature regarding the relationship of melatonin and auxin and also have greater implications of utilizing melatonin for improved plant protection.

Keywords: Melatonin, Auxin, Promoter activation, Transcriptome, *Arabidopsis thaliana*

Background

Plant hormones are considered as major determinants of plant's overall growth and development. Multiple plant hormones such as auxin, cytokinin (CK), gibberellins (GA) and brassinosteroids (BR) have been shown to exert key functions in regulating various developmental processes such as, seed and fruit development, shoot and root architecture [1]. With the advancement in forward and reverse genetics, there is now a good understanding of how these hormones are perceived and the key players identified in their signalling pathways. For this purpose, plant hormones and their signalling transduction networks have been widely studied and employed to improve sustainable agriculture such as stem elongation, flowering time and processes like nitrogen use efficiency [2]. Interestingly, these effects on growth regulation are controlled by interacting signalling pathways among plant hormones. This interaction is either antagonistic, synergistic or occurs in parallel [3]. For example, processes such as seed germination, shoot and root growth and grain-filling are governed by antagonistic relationship between abscisic acid (ABA) and ethylene (ET) in maize [4]. Moreover, auxin and cytokinin exhibit antagonism during formation of root

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apical meristem but act in synergy during shoot apical meristem formation [5].

Melatonin (MT) is an indolic molecule ubiquitously present in all living organisms. Melatonin in plants is associated with growth and development, such as leaf and root organogenesis, senescence and flowering [6–9]. Moreover, melatonin also mitigates a variety of abiotic and biotic stresses in plants such as cold, drought, heat and infections by the fungal pathogen *Diplocarpon mali*, biotrophic and hemibiotrophic bacteria *Xanthomonas oryzae* and *Pseudomonas syringae* DC3000, respectively [10–15]. To date, melatonin is considered as a growth-regulating secondary metabolite in plants but recent studies suggest that it also has the potential to be a plant hormone [16]. In order to be considered as potential plant hormone, there are certain fundamental characteristics that a candidate molecule needs to exhibit. These include knowledge about biosynthetic pathway, receptor and physiological effects. Studies on biosynthetic pathway of melatonin in plants have made considerable progress. These show tryptophan (Trp) as the precursor followed by four sequential reactions with enzymes Tryptophan decarboxylase (TDC), Tryptophan-5-hydroxylase (T5H), Serotonin-*N*-acetyltransferase (SNAT) and Acetyl serotonin methyltransferase (ASMT) [17, 18]. This has been proposed to be the standard biosynthetic route in plants such as *Arabidopsis thaliana* and Rice *(Zea mays)* under normal growth conditions, however, an alternate pathway has also been proposed to exist under conditions such as senescence in which the key enzymes SNAT and ASMT switch in their order to produce melatonin. This has also been proposed to be the most prevailing biosynthetic route in *Arabidopsis thaliana* and Rice *(Zea mays)* as compared to the classic route [19]. Recently, a reverse biosynthetic reaction involving an enzyme *N*-acetylserotonin deacetylase (ASDAC) has been identified in *Arabidopsis thaliana* and rice in which melatonin intermediate *N*-acetylserotonin is rapidly converted to serotonin. This reaction restricts synthesis of melatonin thereby maintaining optimal levels of melatonin for balanced plant growth and development [20]. Like other plant hormones, melatonin exerts multiple physiological effects in plants such as regulation of stomatal opening/closing, photosynthesis, tropism, changes in metabolism of carbohydrates and nitrogen and cellular effects like changing the intracellular calcium (Ca^{2+}) content and membrane permeability [21–24]. Recently, the first melatonin receptor CAND2/PMTR1, a G-protein coupled receptor has been identified in *Arabidopsis thaliana* which was shown to regulate stomatal closure mediated by melatonin [25]. This was long-sought because a lack of melatonin receptor in plants was impeding the full understanding of melatonin-mediated signalling. Without an identified receptor, it was also a challenge to view melatonin as a potential plant hormone.

Recent studies have investigated the crosstalk of melatonin with the well-known plant hormones such as salicylic acid (SA), abscisic acid (ABA), and ethylene [26]. Of particular interest has been the comparison between melatonin and the widely studied hormone, auxin, because of their common biosynthetic precursor, tryptophan, which leads to structural similarities such as having an indole core. These similarities have led to the hypothesis that melatonin could also share auxin-like activities, in terms of regulating growth in a concentration-dependent manner. However, the current understanding of the relationship between melatonin and auxin remains unclear. Previous studies using promoter-reporter constructs, gene expression and physiological responses both support and contradict similar modes of action or overlapping signalling pathway between auxin and melatonin. In support of similar functions, it has been reported that melatonin stimulated plant growth at low concentrations (10^{-4} M,10^{-7} M, 0.01 M) similar to auxin by increasing root growth, lateral and adventitious root formation in a variety of plant species [6, 27, 28]. Similarly, in roots of *Brassica juncea*, melatonin treatment (0.1 μM) increased the concentration of indole-acetic acid (IAA) and enhanced root growth [13, 29]. In, transgenic tomato plants over-expressing the ovine melatonin biosynthetic pathway gene, *Serotonin-N-acetyltransferase* (*SNAT),* led to a decrease in IAA levels and loss of apical dominance [15, 30]. Similarly, melatonin decreased the transcript abundance of *YUCCA (YUC)* (*YUC1, YUC2, YUC5, YUC6* and *TAR2*) auxin biosynthetic genes upon 600 μM treatment in Arabidopsis roots [31]. Auxin-responsive marker lines such as *Direct Repeat 5, DR5,* have been used to investigate the response and distribution of auxin in many plant species such as Arabidopsis and soybean [32]. *DR5* is a synthetic promoter containing auxin responsive elements (AuxREs) and is widely utilized as an indirect marker of endogenous auxin distribution, signalling and responses [33, 34]. Wang and colleagues showed that exogenous melatonin treatment at an inhibitory concentration (600 μM) to Arabidopsis roots enhanced *GFP* and *GUS* expression of *DR5* lines [31]. Moreover, RNA-sequencing analysis from 10 and 20 μM melatonin-treated roots of 2-week old rice seedlings showed that auxin-signalling genes were significantly increased in abundance [35]. In contradiction to above studies, Kim et al. (2016) reported the inability of melatonin (10^{-7} M–10^{-4} M) to stimulate the plant responses in maize in the classical bioassays that are specifically used to demonstrate auxin responsiveness i.e. elongation of coleoptiles, inhibition of roots in young seedlings and induction of ethylene biosynthetic gene, *1-aminocyclopropane-1-carboxylate (ACC) synthase* [36]. Gene expression studies in Arabidopsis plants treated with 100 pM and 1 mM melatonin revealed that auxin biosynthetic and related genes were not changed in transcript abundance except for one auxin-responsive gene *IAA-amino synthase,* that was

increased in abundance upon melatonin treatment [37]. It has been shown that melatonin treatment (5, 100, 450 and 500 μM) was unable to induce expression of auxin-responsive marker line *DR5:GUS* in *Arabidopsis* seedlings [38, 39]. The data from these studies point to the contrasting findings between and within plant species. A common confounding factor especially for transcriptomic analysis, has been the lack of direct comparisons between melatonin and auxin treatments under identical set of experimental conditions.

Interplay of melatonin and mitochondria has been extensively studied in mammals but just recently begun to be investigated in plants [40, 41]. Mitochondria are the powerhouses of cells and play a key role in growth and development of plants by providing necessary metabolites, enzyme cofactors and energy (ATP). Recent studies have shown that mitochondria play integral role in cellular signalling. Mitochondrial signalling, or mitochondrial retrograde signalling results when mitochondria functioning is perturbed by stimuli and this leads to transmission of signals to alter nuclear gene expression [42]. This shows that mitochondria are not only crucial for plant's growth and development but also for driving responses to biotic and abiotic stresses. It is thus not a surprise that there exists an interaction between mitochondrial and hormone signalling pathways as hormones are strongly linked with processes of growth and stress defence [43]. Nuclear genes encoding mitochondrial proteins have been shown to be responsive to a variety of hormone treatments based on a meta-analysis study. The main regulators of mitochondrial function identified were plant hormones auxin, cytokinin (CK), jasmonic acid (JA), and salicylic acid (SA) [43]. More direct targeted approaches have shown an interaction between these hormones and mitochondrial signalling. For example, ABA-induced signalling of guard cells in response to drought stress is negatively regulated by a pyruvate carrier of mitochondria termed as *Negative Regulator of Guard Cell ABA Signalling 1,* (*NRGA1)* in *Arabidopsis thaliana* [44]. Salicylic acid (SA) treatment has been shown to uncouple and inhibit mitochondrial electron transport in *Nicotiana tabacum* [45]. Auxin and mitochondrial respiration has been long hypothesized to have a connection [46]. Moreover, multiple studies have shown a link between auxin responses and mitochondrial function [47, 48]. The mutants of genes encoding proteins that are involved in the synthesis of the inner mitochondrial membrane such as Filamentation Temperature Sensitive H 4 (FTSH4) and PROHIBITIN3 were shown to inhibit auxin response [49, 50]. Additionally, auxin was oxidatively degraded in the *ftsh4* mutant through hydrogen peroxide (H_2O_2)-mediation which is suggested to be a strategy to prioritize processes such as stress defence over growth-related processes [51]. Antimycin A is a chemical stimulus of mitochondrial stress that acts by blocking complex III of the mitochondrial respiratory chain. Treatment by antimycin A also led to decreased auxin (IAA) levels and down-regulation of auxin receptors and transporters such as auxin efflux transporters PIN1/3/4/7 in *Arabidopsis thaliana* [52, 53]. Additionally, auxin homeostasis was defective in mutants of the gene *IAA-alanine Resistant 4 (IAR4)* which encodes a putative mitochondrial pyruvate dehydrogenase E1 alpha-subunit suggesting its integral role in maintaining auxin homeostasis [54].

Alternative oxidase (AOX) is a terminal oxidase which is part of the plant mitochondrial electron transport chain and acts to uncouple respiration by bypassing proton-pumping complexes III and IV, thereby reducing excessive burst of reactive oxygen species (ROS). This activity is particularly dominant under stressful environmental conditions such as drought, low temperature and bacterial infection by *Pseudomonas syringae* where studies have shown a remarkable increase in AOX transcript and/or protein [55]. This indicates that a wide array of pathways can trigger AOX and hence it is considered as a marker for mitochondrial retrograde signalling. While a range of plant hormones can trigger/induce AOX such as SA and ET [45, 56] others such as auxin are antagonistic to the induction of AOX [52]. Auxin (4.5 μM NAA) application was shown to inhibit the Antimycin A-mediated induction of promoter-reporter *Alternative oxidase1a (AOX1a::LUC)* in Arabidopsis [52]. The antagonistic relationship of auxin and mitochondrial retrograde signalling plays a central role in balancing growth and stress responses. Mitochondria along with chloroplasts have been hypothesized to be the original sites of synthesis of melatonin. This relates to the endosymbiotic theory where these organelles are considered to be the descendants of endosymbiotic bacteria which produced melatonin [57]. Very recently, synthesis of melatonin in mitochondria, as well as chloroplasts has been reported in leaves of apple *Malus zumi* and Arabidopsis. Moreover, apple melatonin biosynthetic genes *Serotonin N-acetyltransferase SNAT* and *Acetylserotonin O-methyltransferase ASMT* were found to be localized to mitochondria and chloroplasts, respectively in both apple and Arabidopsis [41, 58]. However, there is lack of understanding in how melatonin functions with mitochondrial retrograde signalling and its relatedness with auxin. Thus, alternative oxidase is an ideal marker to test the interaction between auxin and melatonin.

In this study, the effects of melatonin were compared directly to auxin treatments. Two different transgenic Arabidopsis lines carrying inducible promoter-reporter constructs that are responsive to auxin were used to compare the response of plants to melatonin versus auxin. *Direct repeat 5 (green fluorescent protein) DR5::GFP* as a marker for auxin response and *Alternative oxidase1a (luciferase)*

AOX1a::LUC as a marker for mitochondrial retrograde signalling was used [52, 59]. Furthermore, the potential molecular crosstalk between melatonin and auxin was investigated using global transcriptome analysis of Arabidopsis rosette leaves of seedlings whose roots were treated with either melatonin or auxin.

Results

Effect of melatonin on *DR5::GFP* expression in primary root tips

The *DR5::GFP* auxin-responsive marker line was used to assess whether melatonin (MT) and NAA treatments have similar effects on *DR5* transactivation in primary root tips of 5-day old *Arabidopsis thaliana* seedlings. Arabidopsis *DR5::GFP* root tips grown on half strength MS media or solvent control (0.1% v/v ethanol) for 5 days, showed basal GFP fluorescence indicative of *DR5* expression at the columella cells of the primary root tip (Fig. 1). When roots were treated with 0.1 μM NAA (used as a positive control), the GFP expression was more intense compared to basal expression as expected and reported by previous studies [32, 60]. Addition of melatonin to concentrations of 0.1, 5 and 50 μM in the growth media did not affect the expression of *DR5::GFP* in primary root tips in terms of intensity of location, neither enhancing nor suppressing the GFP expression. GFP fluorescence intensity was also quantified by calculating Integrated Density (sum of pixel) (Additional file 1: Figure S1). NAA (0.1 μM) treatment enhanced *DR5* transactivation by 2.5-fold compared to solvent control (0.1% v/v ethanol) whereas melatonin treatments up to 50 μM were similar to the basal GFP level expression (Additional file 1: Figure S1). Cellular localization of *DR5* at the columella cells of the root cap was however same regardless of all treatments.

Effect of melatonin on *AOX1a::LUC* expression in leaves treated with Antimycin A

An *Arabidopsis thaliana* transgenic line carrying a promoter-reporter *AOX1a::LUC* construct [61, 62] was used to assess whether melatonin could, like auxin, negatively regulate the expression of *AOX1a* under 50 μM Antimycin A spray treatment. The enhanced expression of *AOX1a::LUC* induced by Antimycin A was significantly suppressed when plants were pre-treated with NAA treatment, bringing LUC luminescence down to the basal expression level (Fig. 2) as previously reported [52]. The melatonin treatments (5 μM, 10 μM, 20 μM, 50 μM, 100 μM and 200 μM) were chosen based on previous literature studies where 10–100 μM has been reported to affect gene expression in Arabidopsis [10, 12] and an approximate equimolar concentration of melatonin (5 μM) and NAA (4.5 μM) were also used. None of the melatonin treatments suppressed *AOX1a* expression under Antimycin A treatment compared to NAA treatment. By also quantifying luminescence intensity through the integrated density method (ImageJ), it was observed that melatonin (5 μM, 20 μM, 50 μM, and 200 μM) did not significantly suppress *AOX1a* expression as compared to the solvent control (0.1% v/v ethanol) (Additional file 1: Figure S2A). Melatonin (at 10 and 100 μM) slightly reduced *AOX1a* transactivation compared to control treatment but expression level was still significantly higher (3-fold) than the levels at NAA treatment (Additional file 1: Figure S2A). Melatonin or NAA treatments alone were not statistically different to the solvent-only control (0.1% v/v ethanol)

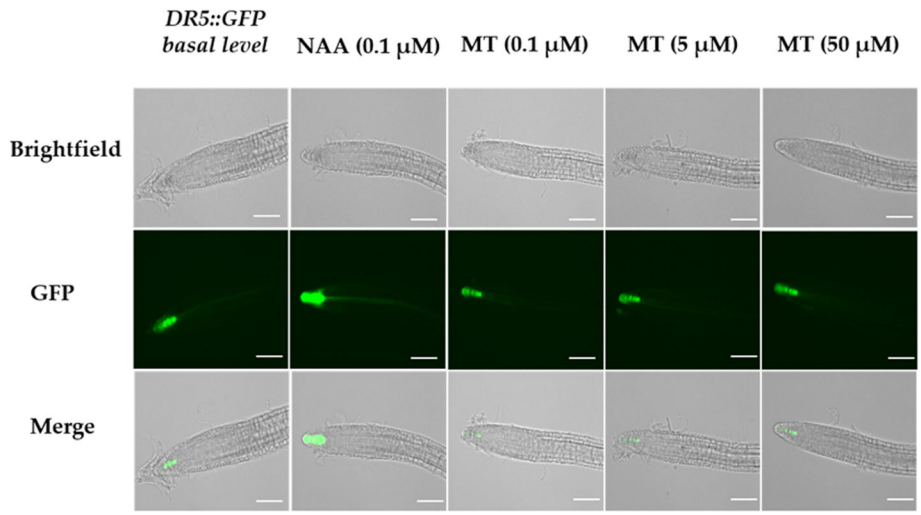

Fig. 1 Effect of MT on *DR5::GFP* expression in 5-day old primary root tip of *Arabidopsis thaliana*. The solvent control (0.1% v/v ethanol) represents the basal level of *DR5::GFP* expression. Figure shows representative images of three biological repeats conducted on independent days with ten plants per treatment per replicate. Scale bar = 100 μm, exposure level = 3.5 ms and magnification = 20X

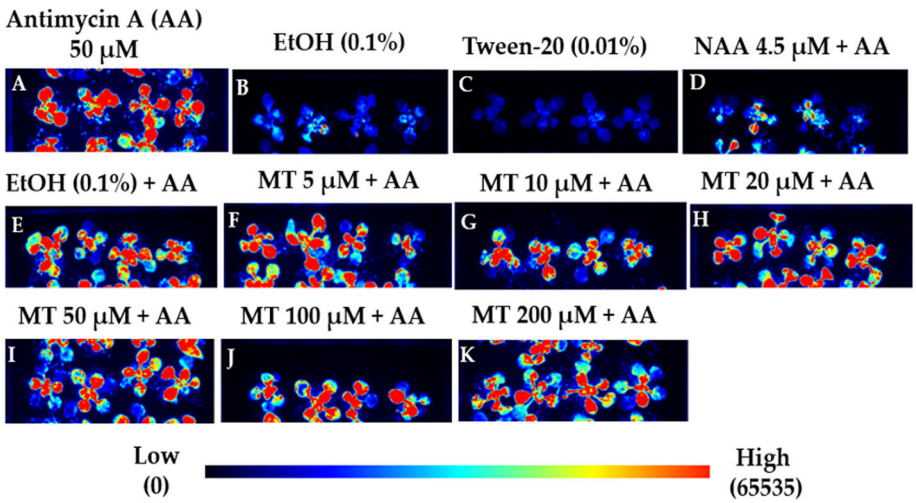

Fig. 2 Effect of MT on *AOX1a::LUC* reporter expression in rosette leaves treated with Antimycin A (AA). Bioluminescence images of *AOX1a::LUC* reporter (Col-0) plants grown on +/− MT or NAA containing media for 3 days and sprayed with +/− AA and visualized for *LUC* activity after 6 h in ChemiDoc (BioRad). (**a**) AA spray (**b**) Solvent control (ethanol 0.1% v/v) for AA spray (**c**) Spray surfactant control (0.01% tween-20 v/v) (**d**) NAA-supplemented media and plants sprayed with AA (**e**) media supplemented with solvent control (0.1% ethanol v/v) for MT and NAA and plants sprayed with AA (**f**–**k**) MT-supplemented media and plants sprayed with AA. Figure shows representative images of three biological repeats conducted on independent days with 12 plants per treatment per replicate. All the images correspond to the *AOX1a::LUC* reporter

(Additional file 1: Figure S2 B, C). Overall, it can be concluded that melatonin does not suppress the induction of *AOX1a* by Antimycin A, in contrast to NAA which suppresses this induction.

Analysis of differentially expressed genes (DEGs) in leaves of melatonin and NAA treated seedlings

Given the differential responses of melatonin and NAA toward auxin-signalling pathways, we compared the transcriptome responses of Arabidopsis leaves from seedlings supplemented with melatonin (5 μM or 100 μM) or NAA (4.5 μM) to gain further insights into the modes of action of these indoles. Differentially expressed genes (DEGs) in melatonin (5 or 100 μM) or NAA (4.5 μM) treated samples were obtained by comparison with their respective untreated solvent controls (0.1% v/v ethanol). A false discovery rate of FDR < 0.05 and $\log_2$ fold change ($\log_2$ FC) ≥1.2 were utilised to call transcripts as significantly differentially expressed. No statistically significantly different gene expression was observed upon treatment with melatonin at 5 μM. However, there were remarkable differences observed in the number of genes significantly expressed between melatonin (100 μM) and NAA (4.5 μM) treatment as compared to the untreated solvent control (0.1% v/v ethanol). NAA had a greater impact on gene expression, with a total of 1065 genes significantly differentially expressed, whereas 100 μM melatonin had a modest effect on transcriptome, with only 49 genes differentially expressed (Fig. 3a). Comparison of DEGs differentially expressed upon exposure to either NAA (specifically auxin-responsive genes) or melatonin (100 μM) in our study with other published transcriptome data sets revealed approximately 30–40% overlap [37, 63, 64]. It is important to note that there were differences between our study and previous transcriptome studies in terms of type of auxin used, concentrations, tissue, exposure time and development stage analysed (Additional file 1: Table S1 and S2).

Both treatments shared a set of 21 differentially expressed genes (DEGs). To further obtain statistical insight, a hypergeometric test was employed with 'dhyper' command in R, to analyse the significance of overlap, relative to the genomic background between two gene lists and thus assesses the likelihood that any overlap between DEGs of gene lists is non-random [65–67]. It was found that the overlap between DEGs upon exposure to melatonin and auxin was statistically significant at a *p*-value of 2.2×10^{-17}.

Transcript levels of a total of 709 genes were significantly enhanced in abundance, and 356 genes significantly decreased in abundance, in response to NAA treatment (Fig. 3b). In contrast, transcript abundance of only 37 genes were significantly increased, and 12 genes significantly decreased in abundance, in response to melatonin (100 μM) treatment. Among these, the expression of 28 genes was uniquely altered by melatonin, 18 of which were enhanced, and 10 decreased in expression (Table 1). In contrast, a total of 1044 genes were exclusively differentially expressed by NAA, with 690 genes showing increased expression and 354 genes with decreased expression. Among the 28 DEGs exclusive to melatonin treatment, 18 genes having increased

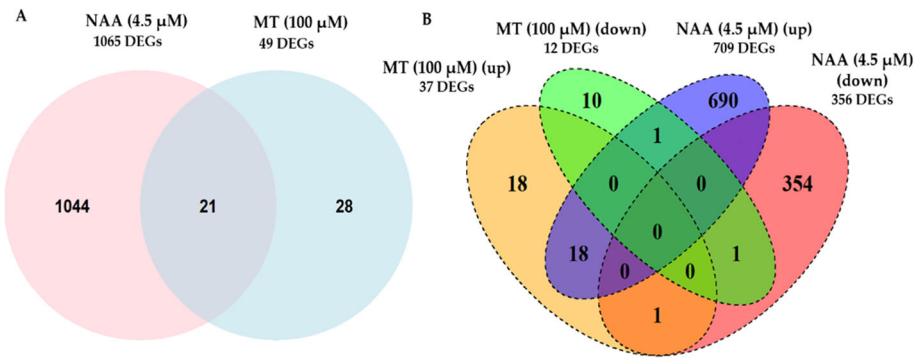

Fig. 3 Expression patterns of DEGs in 15-days old rosette leaves in response to MT or NAA (**a**) Venn diagram indicating the common and exclusive DEGs in response to MT (100 μM) or NAA (4.5 μM) as compared to the untreated control (0.1% v/v ethanol). (**b**) Quad Venn diagram showing the number of up- and down-regulated genes in exclusive and common DEGs. The numbers shown in each set indicate the number of genes regulated in response to MT and/or NAA. Genes were called as differentially expressed with FDR < 0.05 and |log2fold change| ≥ 1.2. Three independent biological experiments were conducted for the analysis

expression are annotated as being defensins or defensin-like (five), transcription factors (four) and being involved in post-translational protein modification (three), while most of the 12 genes with decreased expression were assigned to being involved in photosynthesis (six) (Table 1). Transcript levels of genes exclusively altered by auxin are listed in detail in Additional file 2: Table S4. These included pathways reported by other studies such as root development and auxin-signalling genes such as transport, biosynthesis and conjugation [63, 64, 68].

The differentially expressed genes (DEGs) with increased transcript abundance in the melatonin (100 μM) treatment included two plant defensin genes *AT2G26010* (*PDF 1.3*) and *AT2G26020* (*PDF 1.2 b*) which were 25-fold and 20-fold higher as compared to the control. Moreover, three *defensin-like* (DEFL) gene transcript levels were also significantly enhanced, i.e. *AT3G59930, AT5G33355* and *AT1G34047* were 30-fold, 30-fold and 29-fold higher than the control. Expression levels of genes encoding transcription factors that were significantly induced upon melatonin (100 μM) treatment included *AT1G06160* (*Ethylene responsive factor AP2/ERF59*), *AT3G51910* (*Heat shock transcription factor A7A HSFA7A* and *AT5G07100* (*WRKY DNA-binding protein26*, *WRKY26*) which were 5.5-fold, 2.9-fold and 2.3-fold higher than the control. Interestingly, among the twelve genes with decreased expression upon melatonin treatment (100 μM), seven of them were involved in the light-dependent reactions of photosynthesis such as *ATCG00330* (*Chloroplast ribosomal protein S14*, *RPS14*), ATCG00020 (*Photosystem II reaction center protein A*, *PSBA*), *ATCG00340* (*Photosystem I, PsaA/PsaB protein*, *PSAB*) with fold change of 0.3, 0.4, 0.4 and *ATCG00490* (*Ribulose-biphosphate carboxylases*, *RBCL*) with fold change of 0.4 as compared to the control, respectively. Among the DEGs with expression altered by melatonin, 21 genes were commonly expressed by both melatonin (100 μM) and NAA. Eighteen of these co-expressed genes were increased in abundance and were mainly classified as kinases (3) and lipid metabolic process (3) among others, while only one *AT4G04840* (*methionine-sulfoxide reductase*, *MSRB6*), was decreased in abundance in both melatonin (100 μM) and NAA treatments (Table 2). Whereas, *AT5G13170* (*senescence-associated gene*, *SAG12*) was significantly enhanced in expression by NAA but showed a decreased expression upon melatonin (100 μM) treatment and *AT2G44130* (*Kelch-domain containing F Box protein*, *KFB39*) had enhanced expression in melatonin (100 μM) treatment but decreased expression in NAA treatment (Fig. 3b and Table 2).

Gene ontology (GO) enrichment analysis of DEGs

Gene Ontology (GO) functional and enrichment analysis was conducted to obtain further insights into the functions of DEGs expressed by melatonin (100 μM) and NAA. DEGs that were enriched in NAA treatment were assigned 33 parent GO terms ($p < 0.05$; Bonferroni corrected). These GO terms were related to the biological processes (BP). It was evident that NAA treatment resulted in enrichment encompassing a diverse set of GO terms, based on the larger number of DEGs, while melatonin treatment led to a specific enrichment of three parent GO terms for the corresponding DEGs. Among these were "response to salicylic acid (GO:0009751)", "defence response to other organisms (GO:0006952)" and "photosynthesis, light reaction (GO:0019864)" (Fig. 4). DEGs related to GO term "response to salicylic acid (GO:0009751)" included, among others, *AT1G75040* (*pathogenesis-related protein* 5, *PR5*), AT1G21250 (*Wall-associated receptor kinase 1*, *WAK1*),

Table 1 DEGs exclusively regulated by MT (100 μM) as compared to control

Gene ID	Annotation	MT (100 μM)	
		FDR[a]	Log2 (Fold Change)
Defensins (5)			
AT3G59930	*Defensin-like (DEFL) family protein*	6.0E-04	4.92
AT5G33355	*Defensin-like (DEFL) family protein*	6.0E-04	4.92
AT1G34047	*Defensin-like (DEFL) family protein*	7.7E-03	4.88
AT2G26010	*Plant defensin PDF1.3*	1.2E-02	4.62
AT2G26020	*Plant defensin PDF1.2b*	2.7E-02	4.33
Transcription factors (5)			
AT2G47950	*Myelin-transcription factor like protein*	1.7E-02	4.11
AT1G06160	*Ethylene responsive factor AP2/ERF59*	7.7E-03	2.47
AT3G51910	*Heat shock transcription factor HSFA7A*	2.4E-02	1.52
AT5G07100	*WRKY DNA binding protein WRKY26*	2.5E-03	1.23
AT5G17300	*Myb-like transcription factor RVE1*	3.3E-02	−1.42
Photosynthesis (7)			
ATCG00330	*Chloroplast ribosomal protein RPS14*	5.9E-06	−1.55
ATCG00020	*Photosystem II reaction center A PSBA*	1.9E-03	−1.49
ATCG00350	*Photosystem I, PsaA/PsaB*	8.9E-10	−1.47
ATCG00280	*Photosystem II reaction center C, PSBC*	1.9E-04	−1.43
ATCG00340	*Photosystem I, PsaA/PsaB*	1.8E-13	−1.41
ATCG00270	*Photosystem II reaction center D PSBD*	1.1E-03	−1.40
ATCG00490	*Ribulose-biphosphate carboxylases RBCL*	3.8E-08	−1.25
Post-translational protein modification (3)			
AT3G13310	*Chaperone DnaJ-domain superfamily DJC66*	6.3E-85	3.80
AT2G15310	*ADP-ribosylation factor ARFB1A*	5.5E-07	2.02
AT5G10770	*Eukaryotic aspartyl protease family protein*	1.9E-02	1.26
Response to iron ion and indole glucosinolate metabolic process (1)			
AT4G31940	*Cytochrome P450 CYP82C4*	7.7E-03	4.46
Systemic acquired resistance (1)			
AT1G75040	*Pathogenesis-related gene 5, PR5*	1.3E-03	3.69
Response to abscisic acid (1)			
AT5G27420	*Carbon/nitrogen insensitive ubiquitin ligase CNI1*	2.9E-02	2.22
Auxin response, polar transport and activated-signaling (1)			
AT1G29460	*SAUR-like auxin responsive SAUR65*	4.3E-02	−1.26
Miscellaneous (4)			
AT1G10140	*Uncharacterized conserved protein*	9.2E-03	1.50
AT1G78450	*SOUL-heme binding protein*	5.9E-04	1.43
AT2G40095	*Alpha/beta hydrolase related protein*	4.2E-02	1.36
AT2G07706	*Hypothetical protein*	9.2E-05	−1.53

[a]*FDR* False Discovery Rate

AT1G32960 (*Subtilisin-like protease, SBT3.3*), *AT2G16720* (*R2R3-MYB Transcription factor, MYB7*), *AT3G50480* (*RPW8-like protein 4*). Moreover, some of the DEGs associated with enriched GO term "defence response to other organisms (GO:0006952)" included along with defensins and defensin-like genes, AT5G25250 (*Flotillin-like protein 1, FLOT1*) and *AT1G15520* (*ABC transporter G family member 40, ABCG40*). Further GO functional annotation of DEGs in melatonin treatment (100 μM) are detailed in Additional file 3: Table S5. GO term analysis provide further weight to the finding that while NAA exhibited a diverse response in

Table 2 DEGs influenced by both MT (100 μM) and NAA (4.5 μM) treatment, as compared to control

Gene ID	Annotation	MT		NAA	
		FDR[a]	Log2 (Fold Change)	FDR[a]	Log2 (Fold Change)
Kinases (3)					
AT1G21240	*Wall-associated kinase 3, WAK3*	1.1E-02	3.55	9.3E-05	3.91
AT4G18250	*Receptor serine/threonine kinase-like*	7.7E-03	3.43	1.5E-03	3.07
AT1G21250	*Wall-associated kinase 1, WAK1*	3.7E-02	1.55	1.1E-12	3.18
Transcription factors (1)					
AT2G16720	*Myb domain protein 7, MYB7*	1.9E-03	1.81	5.0E-03	1.36
Senescence (1)					
AT5G13170	*Senescence-associated gene, SAG12*	3.9E-02	−1.46	2.7E-10	2.70
Transporter (1)					
AT1G15520	*ABC transporter family, ABCG40*	5.8E-03	3.01	2.2E-04	3.00
Oxidation-reduction process (1)					
AT4G04840	*Methionine sulfoxide reductase, MSRB6*	1.0E-02	−1.36	2.5E-18	3.05
Lipid metabolic process (3)					
AT3G48080	*Alpha-beta hydrolases ABH*	1.0E-05	2.68	5.8E-10	3.16
AT2G26400	*Acireductone dioxygenase, ARD3*	4.4E-02	2.62	2.2E-03	2.62
AT3G22231	*Pathogen circadian controlled PCC1*	2.9E-02	2.95	4.6E-09	4.98
Plant type hypersensitive response (1)					
AT3G50480	*Homolog of RPW8, HR4*	1.9E-03	3.03	4.3E-02	1.70
Induced systemic resistance (1)					
AT1G32960	*Subtilase family protein, ATSBT3.3*	1.9E-02	2.90	1.7E-02	2.21
Response to other organisms (2)					
AT2G14560	*Late upregulated in response to downy mildew LURP1*	2.4E-02	2.90	3.0E-10	5.13
AT5G25250	*Flotillin-like protein 1, FLOT1*	1.9E-03	2.61	1.1E-3	2.18
Regulation of phenylpropanoid metabolic process (1)					
AT2G44130	*Galactose/oxidase kelch repeat protein*	4.6E-05	3.40	3.4E-2	3.06
Response to abiotic stresses (2)					
AT1G14880	*Plant cadmium resistance1, PCR1*	5.5E-03	6.55	2.4E-2	4.33
AT4G02520	*Glutathione-S transferase GSTF2*	1.3E-03	1.82	4.9E-5	1.80
Miscellaneous (4)					
AT1G10340	*Ankyrin repeat family protein*	1.0E-02	2.37	2.1E-2	1.7
AT2G44480	*Beta glucosidase 17, BGLU17*	3.3E-02	1.81	1.2E-3	1.84
AT5G55450	*Bifunctional inhibitor/lipid transfer, ATLTP4.4*	3.9E-04	1.47	1.0E-02	3.01
AT5G53830	*VQ-motif containing protein, MVQ3*	1.9E-03	1.25	4.3E-06	1.41

[a] FDR = False Discovery Rate

transcriptome, the most prominent gene category induced and enriched by melatonin (100 μM) was involved in biotic stress defence and responses to hormone salicylic acid.

Effect of melatonin treatment on expression of auxin-signalling genes

Differentially expressed genes (DEGs) in response to melatonin-treated samples that had a GO annotation with auxin-response were analysed. Unlike NAA treatment, no significant alteration on the transcript levels of genes was observed with melatonin treatment (100 μM) in the "auxin homeostasis (GO:0010252)", "indole-3-acetic acid amido synthetase activity (GO:0010279)", "basipetal auxin transport (GO:0010540)", "auxin efflux transmembrane transporter activity (GO:0010329)", "auxin: proton symporter activity (GO:0009672)", "auxin efflux (GO:0010315)", "auxin influx (GO:0060919)" and "cellular response to auxin stimulus (GO: 0071365)" (Fig. 5a). However, the gene expression of *AT1G29460* (*Small Auxin Up Regulated SAUR65*) was markedly

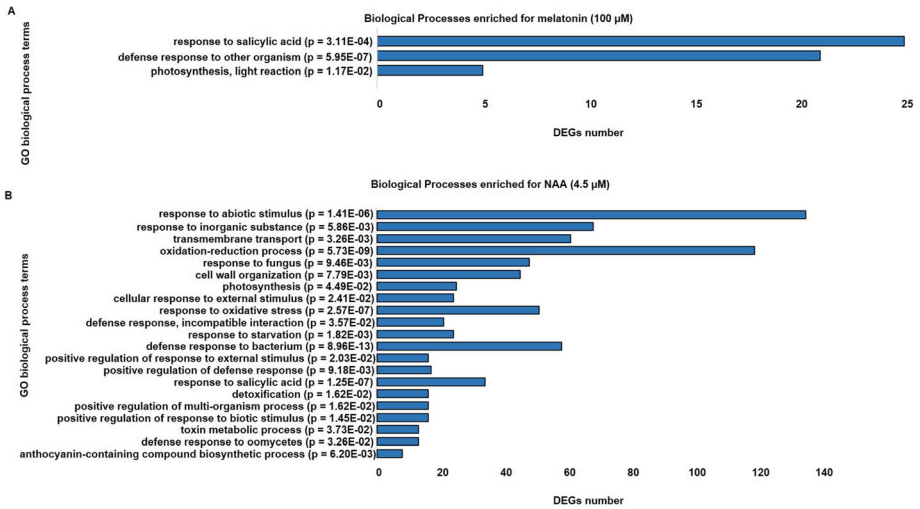

Fig. 4 Differential GO terms in response to MT or NAA compared to untreated control. Significantly enriched GO biological process terms of (**a**) DEGs in response to MT (100 µM) and (**b**) NAA (4.5 µM) as compared to the set of all protein-coding genes in the Arabidopsis genome derived from the functional annotation classification tool in publicly available database (www.geneontology.org) [69] with *p*-values < 0.05 classified as statistically significantly different by a Fisher's exact test with Bonferroni correction for multiple testing

decreased 0.4-fold by melatonin compared to control. *AT1G29460* has assigned GO terms of "response to auxin (GO:0009733)", "auxin polar transport (GO:0060918)" and "auxin-activated signalling pathway (GO:0009734)". Additionally, expression of *AT5G17300* (*Myb-like transcription factor*, *RVE1*) was also significantly decreased by melatonin. This gene is involved in growth of hypocotyls by regulating levels of auxin dependent on circadian rhythm. It is also involved in "regulation of auxin biosynthetic process (GO: 0010600)" and "auxin-activated signalling pathway (GO: 0009734)" (Fig. 5). However, expression of both genes was not significantly altered by NAA, but other auxin-signalling genes were altered (Fig. 5b). Taken together, this further shows that melatonin does not affect expression of any of the known-auxin responsive genes except for *AT1G29460* and *AT5G17300* which trend toward decreased expression.

Expression profiling of the DEGs exclusively and commonly regulated by melatonin using publicly available microarray-based expression data

In order to obtain additional insight into the functions of DEGs regulated by melatonin we explored publicly available microarray data for Arabidopsis [70] Bio-Analytic Resource, BAR, https://bar.utoronto.ca. We focussed on selected microarray data from different organs and in response to a variety of biotic and abiotic stresses (Fig. 6 and Additional file 1: Figure S4). The data in BAR have been sourced from the previous studies (Schmid et al., 2005; Kilian et al., 2007). The genes belonged to two clusters. *AT5G33355* (*DEFL*), *AT3G59930* (*DEFL*), *AT3G51910* (*HSFA*), *AT1G06160* (*ERF59*) and *AT4G31940* (*CYP82C4*) are melatonin-unique genes that have increased transcript levels that formed a part of cluster 1(a) based on their high expression in roots (Fig. 6). Four out of these five genes are also expressed in rosettes but not to a significant level as in roots and *AT3G51910* (*HSFA*) is decreased in abundance in rosettes. Interestingly, 14 genes forming a cluster had lower expression in rosettes (cluster 2b). These included among others, *AT5G27420 (CNI1)*, *AT5G07100 (WRKY26)*, *AT1G14880 (PCR1)* and *AT2G15310 (ARFB1A)*. Expression of 13 out of these 14 genes were shown to be highly induced in rosettes by melatonin treatment in our RNA-Seq data. Expression levels of four out of these genes have been exclusively altered by melatonin. The fact that melatonin was applied via roots in the growth medium in our experiment and is able to induce gene expression in rosettes indicates that melatonin is likely to have a systemic effect on plants.

Discussion

Melatonin shares its biosynthetic precursor, tryptophan, with the plant hormone, auxin (indole-3-acetic acid, IAA). Owing to this, many studies have investigated if melatonin exhibits auxin-like activities. Some reports show that melatonin indeed acts in a similar manner to auxin, whilst other equally convincing reports contradict the notion [23, 36]. This has rendered the current understanding of relation between melatonin and auxin quite enigmatic. In the current study, we determined whether melatonin regulates auxin-signalling pathways in a similar fashion to auxin to get a clearer picture of

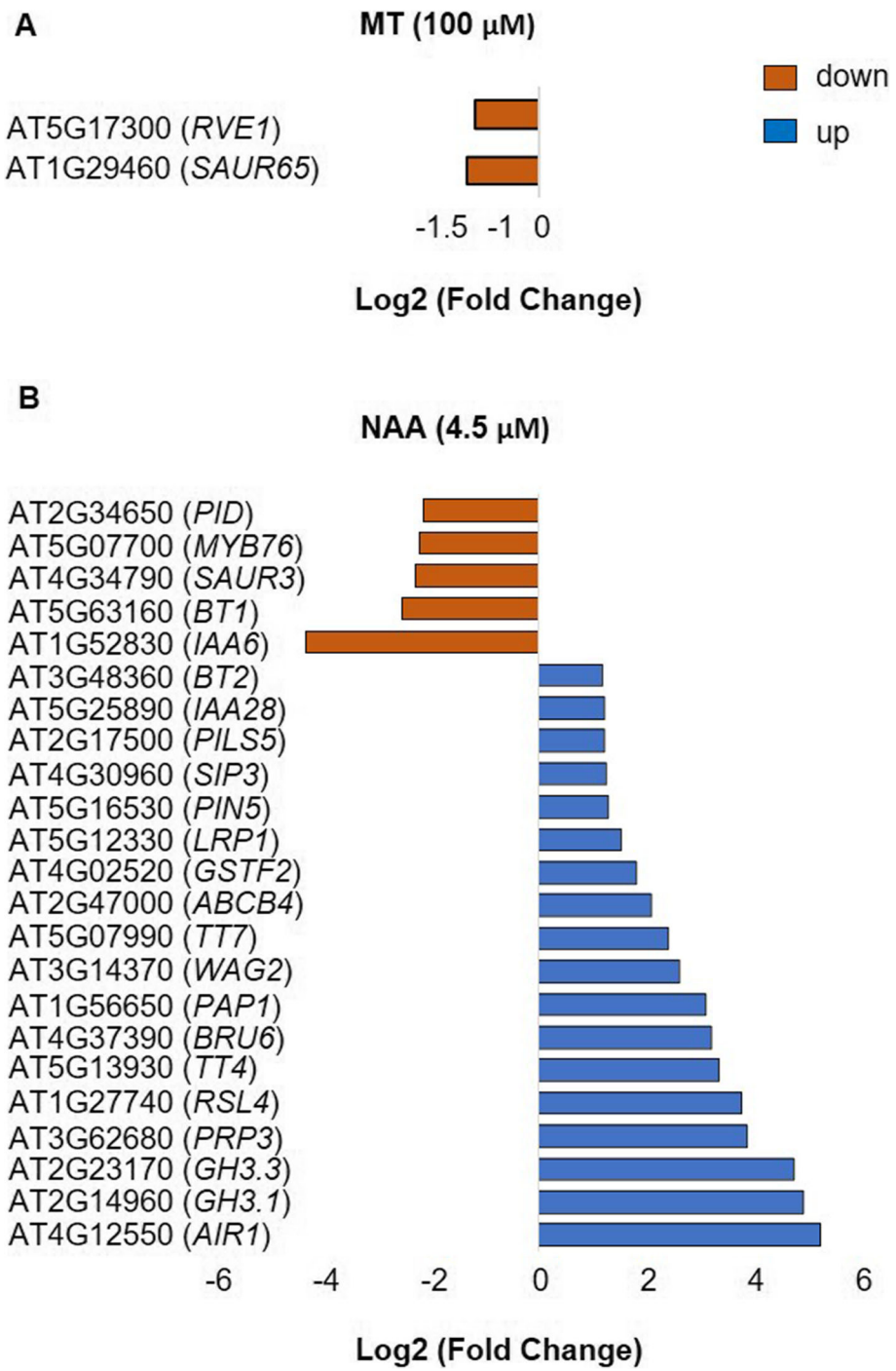

Fig. 5 Effect of MT or NAA on DEGs involved in auxin biosynthesis, response, transport and signalling pathway. **a** DEGs of MT (100 μM) vs control involved in response to auxin, regulation of auxin biosynthetic process, polar transport and auxin activated signalling pathway. **b** DEGs of NAA (4.5 μM) vs control involved in response to auxin, auxin homeostasis, IAA amido synthetase activity, auxin polar transport, basipetal auxin transport, auxin efflux transmembrane transporter activity, auxin:proton symporter activity, auxin-activated signalling pathway, auxin biosynthetic process, auxin efflux, auxin influx and cellular response to auxin stimulus

the potential crosstalk between melatonin and auxin. We have found that melatonin does not act in a manner similar to auxin in *Arabidopsis thaliana* and has its own separate mode of action.

Auxin distribution and perception is not affected by melatonin

It is known that endogenous auxin (IAA) levels are reduced by melatonin (1, 50, 100 and 200 μM) when

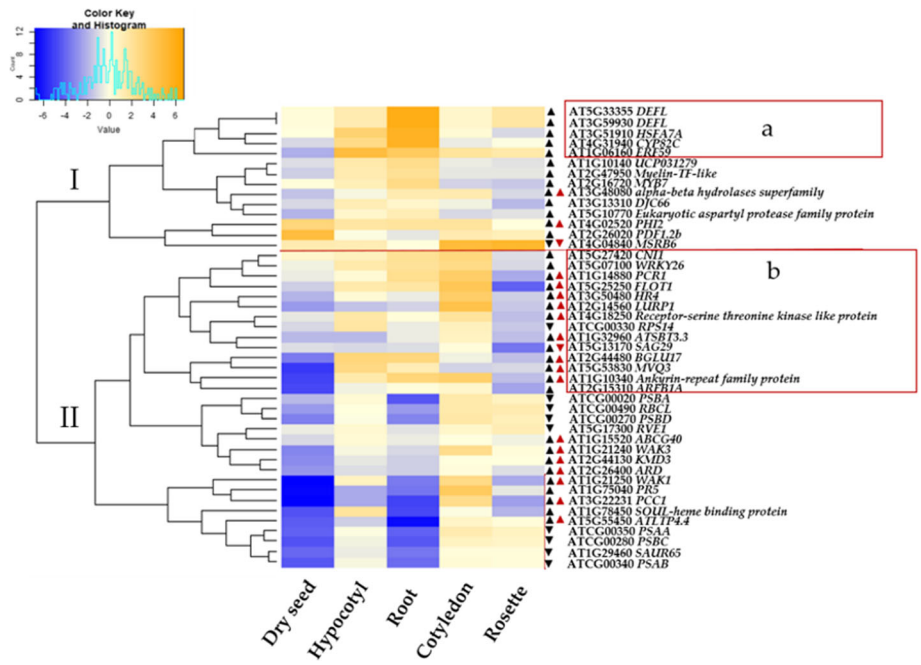

Fig. 6 Microarray-based expression pattern of DEGs regulated by MT or commonly regulated with NAA in organs. Data were sourced from Bio-Analytic Resource (BAR). Age of *Arabidopsis* plants was 7 days cotyledon (7 days), hypocotyl (7 days), root (17 days), rosette (17 days) [71]. Arrows indicate trend of regulation (up or down) in our RNA-Seq data in response to melatonin (100 μM) (black arrows) and/or NAA (red arrows). The heat map represents hierarchical clustering of log2 transformed fold change of signal values as compared to control. All the values have been standardized prior to hierarchical clustering by z-scoring method in function 'scale' in R (version 3.5.2). The colour key and histogram represent the scale for relative expression with trace levels. Blue colour represents low expression levels, light represent medium expression and orange represents highest expression level. Different clusters are denoted by I and II. Boxed in red (**a** and **b**) are the DEGs forming part of each cluster

supplemented in the growth medium in Arabidopsis by enhancing expression of Zinc Finger transcription factor *ZAT6* [72]. Interestingly in the same study, *ZAT6* showed no direct binding to TACAAT motifs in the promoter of the auxin biosynthetic gene *YUC2* as analysed by ChIP-PCR. Based on this, it is also likely that melatonin does not share AuxRE cis-regulatory elements with auxin in its signalling pathway. Our result is in accordance with previous similar studies that showed that melatonin treatment (5, 50, 100, 450 and 500 μM) was unable to affect expression of auxin-responsive marker lines *DR5: GUS* or *DII-VENUS* in *Arabidopsis* seedlings as compared to auxin (5, 10 μM IAA, 5 μM NAA) [38, 39, 73].

Unlike auxin, melatonin does not affect mitochondrial retrograde signalling

Plants repress auxin signalling upon mitochondrial dysfunction to allocate cellular resources from growth (mediated by auxin) to stress defence response (mediated by ROS burst as a result of mitochondrial perturbation) [74]. We observed that, unlike auxin, melatonin did not suppress the induction of *AOX1a::LUC* upon Antimycin A treatment, suggesting that melatonin does not negatively regulate mitochondrial retrograde signalling and hence does not function like auxin in this pathway (Fig. 2). Moreover, melatonin also did not induce the expression of *AOX1a*. It was recently shown that rhizospheric application of melatonin (10 μM) to *Medicago sativa* plants resulted in enhanced drought tolerance in leaves by reduced H_2O_2 and (nitric oxide) NO contents regulating antioxidant enzymes compounds related to redox and their transcripts. Transcript levels of *AOX* were not enhanced by melatonin, implying that melatonin did not affect the electron transport chain in mitochondria under drought stress. Rather, homeostasis was maintained by regulating other enzymes involved in ROS detoxification [75]. It is thus unsurprising that melatonin also did not further enhance *AOX* expression in our study. AOX has been shown to be activated at post-translation level by certain organic acids and redox-related mechanisms [76, 77]. Intermediates of TCA (Tricarboxylic acid cycle) such as 2-oxoglutarate (2-OGs) activate AOX [78]. Interestingly, 2-ODD (2-oxoglutarate-dependent dioxygenases) or ROS-related reactions transform melatonin into its catabolites [79]. It is, thus, likely that melatonin regulates AOX post-translationally, either through ROS scavenging or via its metabolites such as 2-hydroxymelatonin and cyclic-3-hydroxy melatonin. Another possibility is that melatonin directly scavenges free radicals in mitochondria under stress very

efficiently and without having to alter *AOX* gene expression or activity. This could be achieved by melatonin already present in mitochondria [41] or by entry of melatonin via mitochondrial channels such as the ABC oligopeptide transporters as has been demonstrated in mammals [80].

Differential number of genes expressed by melatonin or auxin

Though previous studies have assessed the effect of melatonin on transcriptome of Arabidopsis, there is no report of direct comparisons of melatonin with NAA (or auxin)-treated transcriptome under identical experimental conditions. Previous transcriptome analysis of melatonin-treated Arabidopsis plants (10, 50 μM and 1 mM) have resulted in a large number of differentially expressed genes (81 to 1308 genes) in [37] and (202 to 426 genes) in [73]. However, the type of tissues analysed, method and duration of melatonin exposure, could be the cause of differences in the actual number of DEGs expressed. Tissue-specific effects of melatonin action and concentration in a variety of plant species such as *Arabidopsis thaliana, Hypericum perforatum L.* and *Oryza sativa L.* have been widely studied [81, 82]. Nevertheless, in our data, the number of melatonin-treated DEGs revealed about 40% match (i.e. out of 49 genes, 18 genes matched) with other reported studies (Additional file 1: Table S1 and Table S2). RNA-Seq data revealed that while NAA (4.5 μM) had a large and a diverse effect on transcriptome, as expected, approximately equimolar concentration of melatonin (5 μM) did not result in any significant DEGs as compared to the control. However, melatonin (100 μM) had a modest effect with expression of 49 genes significantly altered (Fig. 3). Hence, the effect of melatonin at 100 μM will be discussed in detail.

Altered expression of genes related to biotic stress defence by melatonin

The role of melatonin in conferring enhanced tolerance to a variety of plant pathogens in diverse plant species has been documented in the past few years. These include hemibiotrophic bacterium *Pseudomonas syringae*, biotrophic bacterium *Xanthomonas oryzae* pv. *oryzae* and necrotrophic oomycete *Phytophthora infestans* [13, 14, 83, 84]. Spray application of melatonin (1 mM) and transgenic watermelon plants overexpressing melatonin biosynthetic gene *SNAT* resulted in resistance against infection by obligate biotrophic fungus powdery mildew *Podosphaera xanthii.* This was achieved by reduction in growth of hyphae and development of conidia. Further transcriptomic analysis on watermelon leaves sprayed with melatonin (1 mM) revealed alteration in gene expression related to plant defences of both types, PAMP and effector-triggered immunity [85]. Melatonin results in plant innate immunity against *Pseudomonas syringae* PstDC3000 via mitogen-activated protein kinases (MAPK) cascades in Arabidopsis [86, 87].

Melatonin is likely to have a systemic resistance response, as in our experiment, melatonin was applied to the roots through growth medium and effect on gene expression observed in rosettes. In fact, most of the DEGs regulated by melatonin are lowly expressed in rosettes based on publicly available microarray data (Fig. 5) and showed high induction upon melatonin in our experiment. It also suggests melatonin to be a mobile signal. This has been recently tested in watermelon, where melatonin results in cold-tolerance via long distance transport [88]. Moreover, melatonin has been shown to accumulate in the leaves when applied to the roots of maize seedlings [89]. Direct uptake and translocation of melatonin or serotonin from axenic roots to shoot has been demonstrated in *St. John's wort.* Melatonin is absorbed through the root hairs and is mainly shown to be concentrated in pericycle and endodermal tissues which is distinct to the localized distribution of auxin in vasculature [90].

DEGs regulated exclusively by melatonin treatment included pathogenesis-related (*AT1G75040 PR5),* defensins (*AT2G26010 PDF1.3, AT2G26020 PDF1.2b)* and defensin-like genes (*AT3G59930, AT5G33355, AT1G34047*) (Table 1). Melatonin has been shown to act upstream of salicylic acid [15, 91]. Our result complements previous study where exogenous melatonin (10 μM) leads to a rapid induction (within 30 min) of a variety of pathogenesis-related (PR) genes and defence genes (*PR1, PDF1.2, ICS1, ACS6, GST1, APX1,* and *VSP1)* that are activated upon treatment with SA and ethylene. This results in enhanced resistance to *P. syringae Pst* DC3000 in Arabidopsis [15]. Plant defensins are antimicrobial peptides that lead to microcidal activity by interacting with and disrupting microbial membrane [92, 93]. *PDF1.2* is decreased in expression upon attack by *P. syringae* (Cluster IIA in Additional file 1: Figure S4A). The fact that it shows higher expression by melatonin treatment in our RNA-Seq data further sheds light onto the role of melatonin in inducing defensins for biotic defence. SA is a mobile signalling molecule in defence that upregulates a variety of defence-related compounds such as defensins and PR proteins [94]. Thus, it is also likely that melatonin triggers SA in order to induce systemic acquired resistance. In our data, only one of the defensin-like genes, *AT5G44973*, was exclusively expressed by auxin, and it was shown to be have decreased expression (Additional file 2: Table S4). Unlike melatonin, auxin has been shown to promote diseases caused by biotrophic bacterium *P. syringae* in Arabidopsis [95]. Interestingly, one of the components of basal and SA-driven defence responses is repressing auxin signalling [96]. Apart from *P. syringae*, DEGs regulated by melatonin have also been shown to be responsive to a variety of pathogens such as *Botrytis cinerea* and *P. infestans* (Fig. 5b). This is, in agreement with previous studies where melatonin leads to disease resistance to these pathogens [84,

97]. This supports the idea that melatonin elicits broad-spectrum disease resistance. However, it is also important to note that defensins are also stimulated by environmental stresses such as osmotic stress and UV [98]. Moreover, many other defence-related genes were enhanced in expression in microarray data upon abiotic stresses (Additional file 1: Figure S4B). In this regard, it is also likely that melatonin induces defensin and other defence-related genes to confer tolerance to abiotic stresses as well. Another gene, *AT4G31940* encoding for a cytochrome P450 enzyme CYP82C4, represents a key biosynthetic enzyme of a redox-active metabolite sideretin and was strongly induced by melatonin (22-fold). Its transcript level is increased during iron-deficiency conditions [99]. Interestingly, the role of melatonin in enhancing tolerance under iron-deficient condition has been recently shown [73, 100]. Sideretin is exuded by the roots into the rhizosphere upon iron-limiting conditions. It plays a role in efficient iron acquisition by mobilizing and reducing insoluble form of iron making it readily available to the plant. Targeting the iron homeostasis is an effective strategy in plant-pathogen interactions [101, 102]. It is probable that melatonin induces *AT4G31940* CYPB2C4 as part of the defence strategy against microorganisms by enhancing iron acquisition processes such as increasing iron levels locally to activate oxidative burst upon infection site or by iron sequestration resulting in deprivation of iron to microorganism.

Among the transcription factors with altered transcript levels exclusively by melatonin that were involved in biotic defence were *AT1G06160* Ethylene responsive factor *AP2/ERF59*, *AT3G51910* Heat shock transcription factor *A7A* and AT5G07100 WRKY DNA-binding protein26 *WRKY26*. Interestingly, *PDF1.2* is transcriptionally activated by AP2/ERF59 through a crosstalk between jasmonic acid and ethylene [103]. Moreover, transcription factors belonging to AP2/ERF gene family directly activate transcription of melatonin biosynthetic genes in Cassava resulting in enhanced disease resistance against *Xanthomonas* [14]. In our data, all the mentioned genes whose expression levels were exclusively altered by melatonin were also enriched in response to salicylic acid. However, some genes whose transcript levels were commonly altered by melatonin and auxin were also enriched in response to salicylic acid (Table 1). Among these were included *AT1G21250* (*Wall-associated kinase 1*, *WAK1)*, *AT2G14560* (*Late upregulated in response to oomycete downy mildew*, *LURP1)*, *AT2G16720* (*Myb domain protein 7*, *MYB7)*, *AT3G50480* (*Homolog of RPW8, HR4)* and *AT1G15520* (*ABC transporter family*, *ABCG40)*. Cell-wall-associated kinase 1 has been shown to be induced upon salicylic acid treatment and involved in plant defence against *P. syringae* in Arabidopsis [64]. *AT2G14560 Late upregulated in response to oomycete downy mildew (LURP1)*, plays a role in basal defence against pathogenic oomycete, *Hyaloperonospora parasitica* and is dependent on recognition that is resistance (R) protein-mediated [104]. It is likely that melatonin and auxin regulate certain common defence-related processes by regulating a few similar pathways via salicylic acid. A partial crosstalk between melatonin and auxin (IAA) was observed in a study where melatonin increased IAA levels upon infection by Fusarium wilt (Foc4) in banana [83].

Influence of melatonin on auxin-responsive gene expression signatures

The *Small-auxin-up RNA* (*SAUR*) family is among the three gene families that are known to show a rapid and transient induction upon auxin treatment [105]. They are known to have distinct expression patterns and responsiveness to auxin. Based on this, they have been classified into two sub-clades. *SAUR65* belongs to clade-I, where all *SAUR* genes show increased expression upon auxin (IAA, 5 and 10 μM) treatment [63]. *SAUR65*, however, did not show differential expression by NAA in our experiment. This is likely to be due to differences between the type of auxin, tissue studied and duration of exposure. The exact function of *SAURs* has not been widely explored. However, some studies demonstrate *SAURs* to play a role in elongating tissues mediated by auxin in Arabidopsis and also negatively affecting auxin synthesis and polar auxin transport in rice [106]. Auxins are known to induce cell wall expansion [107]. It is likely that melatonin decreases the transcript levels of a member of *SAUR* gene family to reduce cell wall expansion to limit opportunity for pathogen invasion. *RVE1* (*AT5G17300*) is a gene encoding a Myb-like transcription factor that regulates free auxin levels in a circadian-rhythm dependent way to control hypocotyl growth [108]. Effects of melatonin in promoting hypocotyl growth in lupin have been studied previously [27]. Even though hypocotyl elongation is an 'auxin-like' effect, our result gives a hint that melatonin is likely to induce hypocotyl growth with its own mechanism. Additionally, expression levels of the genes involved in auxin transport such as *PIN-FORMED (PIN)* were not altered by melatonin. Auxin transport positively regulates many aspects of plant growth and disease development [109]. This further corroborates that effects of melatonin on transcriptome are direct and do not require the establishment of local auxin maxima. Our result is in line with a recent similar study where melatonin or serotonin treatment (10 or 50 μM) did not affect auxin transport in *AUX1-YFP* and *PIN1/2/4/7-GFP* marker lines in Arabidopsis. However, auxin treatment as control or direct comparison for this experiment was not used [73]. However, inhibitory concentration of melatonin (600 μM) has been shown to reduce root growth and meristem by

reducing the expression of auxin polar transport marker lines such as *PIN1/2/4/7-GFP.* This suggests that inhibitory effect of melatonin on root growth in Arabidopsis partially requires auxin polar transport [31]. Interestingly, melatonin has been shown to travel laterally rather than in a polar manner across cells of *St. John's wort* which further explains why it does not regulate auxin polar transport genes [90].

In other study, RNA-Seq analysis revealed that melatonin was able to induce the expression of auxin-responsive and related genes in rice roots [35]. Similarly, melatonin application to roots in tomato also leads to higher expression of auxin-related signalling genes mediated by Nitric oxide (NO) in hypocotyl [110]. While it is likely that melatonin exhibits its effects species-dependently, it is also important to note that differences exist with regards to specificity of auxin biosynthetic pathway between plant species [111]. Moreover, melatonin has been shown to possess auxinic activity compared with auxin in a variety of monocotyledons such as wheat, barley and oats [28]. However, no report of direct comparison of monocots and dicots together in response to melatonin or auxin has been described. Therefore, it is crucial to consider direct comparison of melatonin with auxin in a study looking to decipher how both indoles work under exact same set of experimental conditions.

Decreased expression of photosynthetic genes by melatonin to enable switch between growth and defence

Melatonin treatment exclusively decreased expression of photosynthesis-related genes such as *ATCG00490 Ribulose-biphosphate carboxylases RBCL, ATCG00270 Photosystem II reaction center protein D PSBD* and *ATCG00330 Chloroplast ribosomal protein S14; RPS14* (Table 1; Fig. 4). Our result is in agreement with previous finding on melatonin (1 mM)-treated transcriptome in Arabidopsis where photosynthesis related genes trended toward decreased expression [37]. A possible explanation could be that there is a trade-off between growth and defence. One of the many components of mounting a plant defence response, is the downregulation of photosynthesis, especially genes related to light reactions. This is done in order to avoid any superfluous costs with the general growth and carefully allocate resources to activating plant defences [112]. Interestingly, a cluster of seven photosynthetic genes with decreased expression exclusively by melatonin also showed decreased expression by *P. infestans* in public microarray data (Cluster II b, Additional file 1: Figure S4A). Upon treatment by SA and bacterial peptide, elf18, the transcription factor *AT4G36990* (Heat Shock factor protein HSF4/*TFB1*) downregulates expression of genes encoding chloroplast proteins [113]. *TFB1* is involved in pre-invasive immunity as it has been shown to be a major molecular switch in regulating transition from growth-defence. It also binds to the *cis*-element *TL1* that is enriched in the promoters of genes related to plant defence [114, 115]. Interestingly, in our study, the transcription factor highly induced (17-fold) by melatonin (100 μM) treatment is *AT2G47950* (myelin transcription factor-like protein) (Table 1). At the moment, no information exists regarding the function of this gene. However, co-expression analysis reveals that *AT4G36990* (Heat Shock factor protein HSF4/ *TFB1*) is strongly co-expressed with *AT2G47950* [116] (Additional file 1: Figure S5). Myelin-transcription factor-like protein *(AT2G47950)* and its co-expressed gene *AT4G36990 (*HSF4, Heat-shock factor-like transcription factor) may thus be important for orchestrating the response of melatonin in terms of regulating the growth-to-defence transition and are interesting candidates for further investigation.

Conclusion

In this study, we show by a direct comparison, that melatonin does not behave like an auxin, as it does not influence auxin-specific gene expression similarly to exert its functions in Arabidopsis. The predominant effect of melatonin on transcriptome of plants is systemically affecting biotic defence-signalling and response genes. Moreover, unlike auxin, melatonin does not affect retrograde signalling in mitochondria but rather affects photosynthesis-related genes potentially as a trade-off between growth and defence. This distinction between melatonin and auxin provides clarity to the current confusion in the scientific literature regarding the role/s of phytomelatonin. It would, therefore, be interesting to determine any common regulation mechanisms of these genes in melatonin-mediated biotic stress defence. The findings in this study support the role of melatonin in plant defence and this is worthy of further investigation for improving plant protection in agriculture.

Materials and methods

Plant materials and chemical treatments

AOX1a::*LUC* in *Arabidopsis thaliana* (Col-0) was previously constructed by fusing 2Kb of promoter of *AOX1a* (accession no. *AT3G22370*) to firefly luciferase *LUC* reporter as described in detail by previous study [61]. All seeds were sterilized by vapour-phase method of chlorine gas for 3 h and then stratified immersed in 0.1% agarose filled Eppendorf tubes at 4 °C for 2 days to synchronize germination. Post-stratification seeds were directly sown on either Gamborg's B5 media (G398, PhytoTechnology, Kansas, USA) supplemented with 3% (w/v) Sucrose and 0.75% (w/v) agar (pH 5.7) in square plates (100 X 100 mm, LabServ) or on +/− melatonin (M5250, Sigma, Castle Hill, NSW) and NAA containing

(N0640, Sigma, Castle Hill, NSW) ½ Murashige and Skoog (MS) media (M0404, Sigma, Castle Hill, NSW) which was then placed in controlled environment room at 23 °C, 16 h/8 h light-dark cycle and 56% humidity with 120 μmol/m^2/s^2 light intensity which was provided by cool fluorescent tubes.

Melatonin or auxin (NAA) were dissolved in 100% ethanol (v/v) to obtain stock concentrations which were further diluted in the medium to give the desired final concentrations as indicated for every experiment separately. Final concentration of ethanol as a solvent was 0.1% (v/v) for all treatments with same concentration and volume of ethanol to control for any potential effect arising from the solvent. Treatments were added into autoclaved medium (cooled to 55 °C), and equal amounts of media (50 mL) were added into each plate. Antimycin A (A8674, Sigma, Castle Hill NSW) was dissolved in 100% ethanol to prepare the stock solution (50 mM) and diluted with autoclaved MilliQ water to give final concentration of 50 μM in 0.1% ethanol (v/v). D-Luciferin (LUCK, GoldBio, St Louis MO) was dissolved in autoclaved MilliQ water to give final concentration of 2.5 mM. Melatonin, Antimycin A and D-luciferin treatments were covered in aluminium foil as they are light-sensitive. All solutions were prepared fresh on the day of the experiment and those intended for spraying on plants were also supplemented with 0.01% (v/v) tween-20 (P9416, Sigma, Castle Hill, NSW) to act as a spray surfactant.

Fluorescence microscopy

Auxin-responsive marker line (*DR5::GFP*) was assessed for fluorescence in response to melatonin (0 μM (ethanol control), 0.1 μM, 5 μM and 50 μM) and NAA (0.1 μM) which was used as a positive control. 5-day old seedlings growing vertically on +/− melatonin or NAA-supplemented ½ MS agar media were assessed for GFP fluorescence in primary root tips. The seedlings were gently pulled off the agar plates and place on the microscopic slide with 100 μL of ½ MS liquid media. The samples were sealed with cover slip and quickly assessed under the upright epi-fluorescence microscope (Olympus BX53, DP80). GFP filter (unit name U-FGFP) was utilised with wavelengths of excitation filter at 460–480 (nm) and an emission filter at 495–540 (nm). GFP fluorescence from 8-bit converted processed images was quantified as sum of pixels in a region of fluorescence after background subtraction by integrated density method in the publicly available Java software ImageJ version 1.52a (https://imagej.nih.gov/ij/).

Luciferase reporter bioluminescence imaging

12-day old *AOX1a::LUC* seedlings growing on Gamborg's B5 media were transferred to +/− melatonin (0, 5, 10, 20, 50, 100 and 200 μM) or +/− NAA 4.5 μM containing media for a further 3 days. Following that, 15-day old plants were sprayed with +/− Antimycin A 50 μM. Six hours post application of Antimycin A, plants were sprayed with D-luciferin (2.5 mM GoldBio) and dark adapted for 30 min as previously described [21]. Luminescence was measured by ChemiDoc (Bio-Rad, MP). Quantification of luminescence was conducted by integrated density method in ImageJ.

RNA-isolation

Rosettes from 15-day old *AOX1a::LUC* Arabidopsis seedlings were flash-frozen and homogenized in liquid nitrogen. Five individual rosettes were pooled per treatment with three independent biological replicates for each treatment. Total RNA was extracted using the Spectrum™ Plant Total RNA kit (Sigma, NSW, Australia) following manufacturer's guidelines. Prior to elution, On-column DNase I digestion (Sigma, NSW, Australia) was performed on the extracted total RNA according to manufacturer's instructions. Spectrophotometric analysis using Nanodrop™ ND-1000 (Analytical Technologies, Australia) was conducted to check RNA quality and quantity.

RNA-sequencing and analysis

The TruSeq stranded mRNA library kit (Illumina) was used following manufacturer's protocol to construct twelve RNA libraries which comprised three biological replicates for each treatment (0.1% ethanol, 4.5 μM NAA, 5 μM melatonin and 100 μM melatonin). The constructed libraries were sequenced using NextSeq550 system (Illumina) as 75 bp single-end reads with an average read number of 20 million per sample. The Kallisto program was used to determine transcript abundance as transcripts per million (TPM) by pseudoalignment of reads to the Araport11 model transcriptome with a k-mer length of 31 [117, 118]. For differential gene expression analysis, the sleuth program was used which utilizes a Wald test to determine differential gene expression [117]. Genes with a false discovery rate (FDR) of < 0.05 and $\log_2$ fold change of at least 1.2 were classified as differentially expressed. For functional analysis, gene ontology (GO) term enrichment of differentially expressed genes was conducted from a publicly available database (www.geneontology.org) and functional GO annotations for each gene was obtained from the bulk data retrieval tool in TAIR (www.arabidopsis.org).

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12870-019-2158-3.

Additional file 1. Description of data: **Figure S1**: Effect of melatonin on expression of auxin-responsive marker line DR5::GFP in *Arabidopsis thaliana* primary root **Figure S2**: Effect of melatonin on expression of auxin-responsive gene *AOX1a::LUC* in rosette leaves of *Arabidopsis thaliana* **Figure S3**: Differential seed development response of wild type *AOX1a::LUC* (Col-0) toward melatonin or auxin **Table S1**. Overlap analysis of

differentially expressed genes (DEGs) related to auxin-responsive GO terms **Table S2**. Overlap analysis of DEGs with previous transcriptome data sets on melatonin in Arabidopsis. **Table S3**. Summary statistics of RNA-Seq data Figure S4. Microarray-based expression pattern of unique or commonly regulated DEGs by MT under stresses.

Additional file 2: Table S4. DEGs regulated by NAA as compared to the solvent control (0.1% v/v ethanol).

Additional file 3: Table S5: GO functional annotation and additional details of genes regulated by melatonin (100 μM) as comapred to the solvent control (0.1% v/v ethanol).

Abbreviations

AOX: Alternative oxidase; DEGs: Differentially expressed genes; DR5: Direct repeat 5; FC: Fold change; FDR: False discovery rate; GO: Gene ontology; MT: Melatonin; NAA: 1-naphthalene acetic acid; RNA-Seq: RNA sequencing; SA: Salicylic acid

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Authors' contributions

SFZ, FB, JW, AEF, KMP: contributed toward conceptualization. SFZ: performed the experiments; SFZ and OB: contributed toward data curation and statistical analysis; SFZ: conducted the formal analysis, visualization and writing-original draft; SFZ, FB, OB, JW, AEF, KMP: reviewed and edited the draft; KMP and FB: supervised the project and KMP, AEF and FB: acquired funding. All the authors have read and approved the final manuscript.

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Availability of data and materials

Microarray expression data used for hierarchical clustering and heatmap are available in the Bio-Analytic Resource, BAR, (https://bar.utoronto.ca) public database. The RNA-seq data from our study were deposited in the Gene Expression Omnibus (GEO) database at NCBI (https://www.ncbi.nlm.nih.gov/geo/) with accession number GSE134079. All other datasets supporting the conclusions of this article are included within the article (and its additional files).

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Santner A, Calderon-Villalobos LI, Estelle M. Plant hormones are versatile chemical regulators of plant growth. Nat Chem Biol. 2009;5(5):301–7.
2. Wen CK, Zhao Y, Ruan YL. Editorial: hormonal control of important agronomic traits. Front Plant Sci. 2018;9:1504.
3. Wilkinson S, Kudoyarova GR, Veselov DS, Arkhipova TN, Davies WJ. Plant hormone interactions: innovative targets for crop breeding and management. J Exp Bot. 2012;63(9):3499–509.
4. Yang J, Zhang J, Liu K, Wang Z, Liu L. Abscisic acid and ethylene interact in wheat grains in response to soil drying during grain filling. New Phytol. 2006;171(2):293–303.
5. Zhao Z, Andersen SU, Ljung K, Dolezal K, Miotk A, Schultheiss SJ, et al. Hormonal control of the shoot stem-cell niche. Nature. 2010;465(7301):1089–92.
6. Hernandez IG, Gomez FJ, Cerutti S, Arana MV, Silva MF. Melatonin in Arabidopsis thaliana acts as plant growth regulator at low concentrations and preserves seed viability at high concentrations. Plant Physiol Biochem. 2015;94:191–6.
7. Arnao MB, Hernandez-Ruiz J. Protective effect of melatonin against chlorophyll degradation during the senescence of barley leaves. J Pineal Res. 2009;46(1):58–63.
8. Zhang H, Wang L, Shi K, Shan D, Zhu Y, Wang C, et al. Apple tree flowering is mediated by low level of melatonin under the regulation of seasonal light signal. J Pineal Res. 2019;66(2):e12551.
9. Liang D, Shen Y, Ni Z, Wang Q, Lei Z, Xu N, et al. Exogenous melatonin application delays senescence of kiwifruit leaves by regulating the antioxidant capacity and biosynthesis of flavonoids. Front Plant Sci. 2018;9:426.
10. Bajwa VS, Shukla MR, Sherif SM, Murch SJ, Saxena PK. Role of melatonin in alleviating cold stress in *Arabidopsis thaliana*. J Pineal Res. 2014;56(3):238–45.
11. Huang B, Chen YE, Zhao YQ, Ding CB, Liao JQ, Hu C, et al. Exogenous melatonin alleviates oxidative damages and protects photosystem II in maize seedlings under drought stress. Front Plant Sci. 2019;10:677.
12. Shi H, Tan DX, Reiter RJ, Ye T, Yang F, Chan Z. Melatonin induces class A1 heat-shock factors (HSFA1s) and their possible involvement of thermotolerance in *Arabidopsis*. J Pineal Res. 2015;58(3):335–42.
13. Yin L, Wang P, Li M, Ke X, Li C, Liang D, et al. Exogenous melatonin improves *Malus* resistance to Marssonina apple blotch. J Pineal Res. 2013;54(4):426–34.
14. Wei Y, Chang Y, Zeng H, Liu G, He C, Shi H. RAV transcription factors are essential for disease resistance against cassava bacterial blight via activation of melatonin biosynthesis genes. J Pineal Res. 2018;64(1). https://doi.org/10.1111/jpi.12454
15. Lee HY, Byeon Y, Back K. Melatonin as a signal molecule triggering defense responses against pathogen attack in Arabidopsis and tobacco. J Pineal Res. 2014;57(3):262–8.
16. Arnao MB, Hernandez-Ruiz J. Melatonin: a new plant hormone and/or a plant master regulator? Trends Plant Sci. 2019;24(1):38–48.
17. Lee HY, Byeon Y, Lee K, Lee HJ, Back K. Cloning of *Arabidopsis* serotonin *N*-acetyltransferase and its role with caffeic acid *O*-methyltransferase in the biosynthesis of melatonin in vitro despite their different subcellular localizations. J Pineal Res. 2014;57(4):418–26.
18. Byeon Y, Lee HJ, Lee HY, Back K. Cloning and functional characterization of the *Arabidopsis N*-acetylserotonin *O*-methyltransferase responsible for melatonin synthesis. J Pineal Res. 2016;60(1):65–73.
19. Back K, Tan DX, Reiter RJ. Melatonin biosynthesis in plants: multiple pathways catalyze tryptophan to melatonin in the cytoplasm or chloroplasts. J Pineal Res. 2016;61(4):426–37.
20. Lee K, Lee HY, Back K. Rice histone deacetylase 10 and Arabidopsis histone deacetylase 14 genes encode *N*-acetylserotonin deacetylase, which catalyzes conversion of *N*-acetylserotonin into serotonin, a reverse reaction for melatonin biosynthesis in plants. J Pineal Res. 2018;64(2). https://doi.org/10.1111/jpi.12460
21. Shi H, Jiang C, Ye T, Tan DX, Reiter RJ, Zhang H, et al. Comparative physiological, metabolomic, and transcriptomic analyses reveal mechanisms of improved abiotic stress resistance in bermudagrass [*Cynodon dactylon* (L). Pers.] by exogenous melatonin. J Exp Bot. 2015;66(3):681–94.
22. Li C, Liang B, Chang C, Wei Z, Zhou S, Ma F. Exogenous melatonin improved potassium content in *Malus* under different stress conditions. J Pineal Res. 2016;61(2):218–29.

23. Arnao MB, Hernández-Ruiz J. Growth activity, rooting capacity, and tropism: three auxinic precepts fulfilled by melatonin. Acta Physiol Plant. 2017;39(6). https://doi.org/10.1007/s11738-017-2428-3
24. Li H, Chang J, Chen H, Wang Z, Gu X, Wei C, et al. Exogenous melatonin confers salt stress tolerance to watermelon by improving photosynthesis and redox homeostasis. Front Plant Sci. 2017;8:295.
25. Wei J, Li DX, Zhang JR, Shan C, Rengel Z, Song ZB, et al. Phytomelatonin receptor PMTR1-mediated signaling regulates stomatal closure in *Arabidopsis thaliana*. J Pineal Res. 2018;65(2):e12500.
26. Arnao MB, Hernandez-Ruiz J. Melatonin and its relationship to plant hormones. Ann Bot. 2018;121(2):195–207.
27. Hernandez-Ruiz J, Cano A, Arnao MB. Melatonin: a growth-stimulating compound present in lupin tissues. Planta. 2004;220(1):140–4.
28. Hernandez-Ruiz J, Cano A, Arnao MB. Melatonin acts as a growth-stimulating compound in some monocot species. J Pineal Res. 2005;39(2):137–42.
29. Chen Q, Qi WB, Reiter RJ, Wei W, Wang BM. Exogenously applied melatonin stimulates root growth and raises endogenous indoleacetic acid in roots of etiolated seedlings of *Brassica juncea*. J Plant Physiol. 2009;166(3):324–8.
30. Wang L, Zhao Y, Reiter RJ, He C, Liu G, Lei Q, et al. Changes in melatonin levels in transgenic 'Micro-Tom' tomato overexpressing ovine *AANAT* and ovine *HIOMT* genes. J Pineal Res. 2014;56(2):134–42.
31. Wang Q, An B, Wei Y, Reiter RJ, Shi H, Luo H, et al. Melatonin regulates root meristem by repressing Auxin synthesis and polar Auxin transport in *Arabidopsis*. Front Plant Sci. 2016;7:1882.
32. Chen Y, Yordanov YS, Ma C, Strauss S, Busov VB. DR5 as a reporter system to study auxin response in *Populus*. Plant Cell Rep. 2013;32(3):453–63.
33. Ulmasov T, Murfett J, Hagen G, Guilfoyle TJ. Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell. 1997;9(11):1963–71.
34. Casimiro I, Marchant A, Bhalerao RP, Beeckman T, Dhooge S, Swarup R, et al. Auxin transport promotes *Arabidopsis* lateral root initiation. Plant Cell. 2001;13(4):843–52.
35. Liang C, Li A, Yu H, Li W, Liang C, Guo S, et al. Melatonin regulates root architecture by modulating Auxin response in Rice. Front Plant Sci. 2017;8:134.
36. Kim M, Seo H, Park C, Park WJ. Examination of the auxin hypothesis of phytomelatonin action in classical auxin assay systems in maize. J Plant Physiol. 2016;190:67–71.
37. Weeda S, Zhang N, Zhao X, Ndip G, Guo Y, Buck GA, et al. Arabidopsis transcriptome analysis reveals key roles of melatonin in plant defense systems. PLoS One. 2014;9(3):e93462.
38. Pelagio-Flores R, Munoz-Parra E, Ortiz-Castro R, Lopez-Bucio J. Melatonin regulates *Arabidopsis* root system architecture likely acting independently of auxin signaling. J Pineal Res. 2012;53(3):279–88.
39. Koyama FC, Carvalho TL, Alves E, da Silva HB, de Azevedo MF, Hemerly AS, et al. The structurally related auxin and melatonin tryptophan-derivatives and their roles in *Arabidopsis thaliana* and in the human malaria parasite *Plasmodium falciparum*. J Eukaryot Microbiol. 2013;60(6):646–51.
40. Tan DX, Manchester LC, Qin L, Reiter RJ. Melatonin: A Mitochondrial Targeting Molecule Involving Mitochondrial Protection and Dynamics. Int J Mol Sci. 2016;17(12). https://doi.org/10.3390/ijms17122124
41. Wang L, Feng C, Zheng X, Guo Y, Zhou F, Shan D, et al. Plant mitochondria synthesize melatonin and enhance the tolerance of plants to drought stress. J Pineal Res. 2017;63(3). https://doi.org/10.1111/jpi.12429
42. Ng S, De Clercq I, Van Aken O, Law SR, Ivanova A, Willems P, et al. Anterograde and retrograde regulation of nuclear genes encoding mitochondrial proteins during growth, development, and stress. Mol Plant. 2014;7(7):1075–93.
43. Berkowitz O, De Clercq I, Van Breusegem F, Whelan J. Interaction between hormonal and mitochondrial signalling during growth, development and in plant defence responses. Plant Cell Environ. 2016;39(5):1127–39.
44. Li CL, Wang M, Ma XY, Zhang W. NRGA1, a putative mitochondrial pyruvate carrier, mediates ABA regulation of guard cell ion channels and drought stress responses in *Arabidopsis*. Mol Plant. 2014;7(10):1508–21.
45. Norman C, Howell KA, Millar AH, Whelan JM, Day DA. Salicylic acid is an uncoupler and inhibitor of mitochondrial electron transport. Plant Physiol. 2004;134(1):492–501.
46. Leonova LA, Gamburg KZ, Vojnikov VK, Varakina NN. Promotion of respiration by auxin in the induction of cell division in suspension culture. J Plant Growth Regul. 1985;4:169.
47. Qin G, Gu H, Zhao Y, Ma Z, Shi G, Yang Y, et al. An indole-3-acetic acid carboxyl methyltransferase regulates *Arabidopsis* leaf development. Plant Cell. 2005;17(10):2693–704.
48. Koyama T, Furutani M, Tasaka M, Ohme-Takagi M. TCP transcription factors control the morphology of shoot lateral organs via negative regulation of the expression of boundary-specific genes in *Arabidopsis*. Plant Cell. 2007;19(2):473–84.
49. Van Aken O, Pecenkova T, van de Cotte B, De Rycke R, Eeckhout D, Fromm H, et al. Mitochondrial type-I prohibitins of *Arabidopsis thaliana* are required for supporting proficient meristem development. Plant J. 2007;52(5):850–64.
50. He J, Duan Y, Hua D, Fan G, Wang L, Liu Y, et al. DEXH box RNA helicase-mediated mitochondrial reactive oxygen species production in *Arabidopsis* mediates crosstalk between abscisic acid and auxin signaling. Plant Cell. 2012;24(5):1815–33.
51. Zhang N, Zhang HJ, Zhao B, Sun QQ, Cao YY, Li R, et al. The RNA-seq approach to discriminate gene expression profiles in response to melatonin on cucumber lateral root formation. J Pineal Res. 2014;56(1):39–50.
52. Ivanova A, Law SR, Narsai R, Duncan O, Lee JH, Zhang B, et al. A functional antagonistic relationship between Auxin and mitochondrial retrograde signaling regulates *Alternative Oxidase1a* expression in Arabidopsis. Plant Physiol. 2014;165(3):1233–54.
53. Kerchev PI, De Clercq I, Denecker J, Muhlenbock P, Kumpf R, Nguyen L, et al. Mitochondrial perturbation negatively affects auxin signaling. Mol Plant. 2014;7(7):1138–50.
54. Quint M, Barkawi LS, Fan KT, Cohen JD, Gray WM. Arabidopsis IAR4 modulates auxin response by regulating auxin homeostasis. Plant Physiol. 2009;150(2):748–58.
55. Vanlerberghe GC. Alternative oxidase: a mitochondrial respiratory pathway to maintain metabolic and signaling homeostasis during abiotic and biotic stress in plants. Int J Mol Sci. 2013;14(4):6805–47.
56. Ederli L, Morettini R, Borgogni A, Wasternack C, Miersch O, Reale L, et al. Interaction between nitric oxide and ethylene in the induction of alternative oxidase in ozone-treated tobacco plants. Plant Physiol. 2006;142(2):595–608.
57. Tan DX, Manchester LC, Liu X, Rosales-Corral SA, Acuna-Castroviejo D, Reiter RJ. Mitochondria and chloroplasts as the original sites of melatonin synthesis: a hypothesis related to melatonin's primary function and evolution in eukaryotes. J Pineal Res. 2013;54(2):127–38.
58. Zheng X, Tan DX, Allan AC, Zuo B, Zhao Y, Reiter RJ, et al. Chloroplastic biosynthesis of melatonin and its involvement in protection of plants from salt stress. Sci Rep. 2017;7:41236.
59. Benkova E, Michniewicz M, Sauer M, Teichmann T, Seifertova D, Jurgens G, et al. Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell. 2003;115(5):591–602.
60. Hayashi K, Nakamura S, Fukunaga S, Nishimura T, Jenness MK, Murphy AS, et al. Auxin transport sites are visualized in planta using fluorescent auxin analogs. Proc Natl Acad Sci U S A. 2014;111(31):11557–62.
61. Ng S, Giraud E, Duncan O, Law SR, Wang Y, Xu L, et al. Cyclin-dependent kinase E1 (CDKE1) provides a cellular switch in plants between growth and stress responses. J Biol Chem. 2013;288(5):3449–59.
62. De Clercq I, Vermeirssen V, Van Aken O, Vandepoele K, Murcha MW, Law SR, et al. The membrane-bound NAC transcription factor ANAC013 functions in mitochondrial retrograde regulation of the oxidative stress response in *Arabidopsis*. Plant Cell. 2013;25(9):3472–90.
63. Paponov IA, Paponov M, Teale W, Menges M, Chakrabortee S, Murray JA, et al. Comprehensive transcriptome analysis of auxin responses in *Arabidopsis*. Mol Plant. 2008;1(2):321–37.
64. Lewis DR, Olex AL, Lundy SR, Turkett WH, Fetrow JS, Muday GK. A kinetic analysis of the auxin transcriptome reveals cell wall remodeling proteins that modulate lateral root development in *Arabidopsis*. Plant Cell. 2013;25(9):3329–46.
65. Fury W, Batliwalla F, Gregersen PK, Li W. Overlapping probabilities of top ranking gene lists, hypergeometric distribution, and stringency of gene selection criterion. Conf Proc. 2006;1:5531–4.
66. Walley JW, Kelley DR, Nestorova G, Hirschberg DL, Dehesh K. Arabidopsis deadenylases AtCAF1a and AtCAF1b play overlapping and distinct roles in mediating environmental stress responses. Plant Physiol. 2010;152(2):866–75.
67. Luesse DR, Wilson ME, Haswell ES. RNA sequencing analysis of the *msl2msl3*, *crl*, and *ggps1* mutants indicates that diverse sources of plastid dysfunction do not Alter leaf morphology through a common signaling pathway. Front Plant Sci. 2015;6:1148.
68. Omelyanchuk NA, Wiebe DS, Novikova DD, Levitsky VG, Klimova N, Gorelova V, et al. Auxin regulates functional gene groups in a fold-change-specific manner in *Arabidopsis thaliana* roots. Sci Rep. 2017;7(1):2489.
69. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet. 2000;25(1):25–9.

70. Waese J, Provart NJ. The bio-analytic resource for plant biology. Methods Mol Biol. 2017;1533:119–48.
71. Schmid M, Davison TS, Henz SR, Pape UJ, Demar M, Vingron M, et al. A gene expression map of *Arabidopsis thaliana* development. Nat Genet. 2005;37(5):501–6.
72. Shi H, Zhang S, Lin D, Wei Y, Yan Y, Liu G, et al. Zinc finger of Arabidopsis thaliana 6 is involved in melatonin-mediated auxin signaling through interacting INDETERMINATE DOMAIN15 and INDOLE-3-ACETIC ACID 17. J Pineal Res. 2018;65(2):e12494.
73. Wan J, Zhang P, Wang R, Sun L, Ju Q, Xu J. Comparative physiological responses and transcriptome analysis reveal the roles of melatonin and serotonin in regulating growth and metabolism in *Arabidopsis*. BMC Plant Biol. 2018;18(1):362.
74. Zhang S, Wu J, Yuan D, Zhang D, Huang Z, Xiao L, et al. Perturbation of auxin homeostasis caused by mitochondrial *FtSH4* gene-mediated peroxidase accumulation regulates *Arabidopsis* architecture. Mol Plant. 2014;7(5):856–73.
75. Antoniou C, Chatzimichail G, Xenofontos R, Pavlou JJ, Panagiotou E, Christou A, et al. Melatonin systemically ameliorates drought stress-induced damage in *Medicago sativa* plants by modulating nitro-oxidative homeostasis and proline metabolism. J Pineal Res. 2017;62(4). https://doi.org/10.1111/jpi.12401
76. Millar AH, Wiskich JT, Whelan J, Day DA. Organic acid activation of the alternative oxidase of plant mitochondria. FEBS Lett. 1993;329(3):259–62.
77. Selinski J, Hartmann A, Deckers-Hebestreit G, Day DA, Whelan J, Scheibe R. Alternative oxidase isoforms are differentially activated by Tricarboxylic acid cycle intermediates. Plant Physiol. 2018;176(2):1423–32.
78. Day DA, Whelan J, Millar AH, Siedow JN, Wiskich JT. Regulation of the alternative oxidase in plants and fungi. Funct Plant Biol. 1995;22:497–509.
79. Yu Y, Lv Y, Shi Y, Li T, Chen Y, Zhao D, et al. The Role of Phyto-Melatonin and Related Metabolites in Response to Stress. Molecules. 2018;23(8). https://doi.org/10.3390/molecules23081887
80. Reiter RJ, Rosales-Corral S, Tan DX, Jou MJ, Galano A, Xu B. Melatonin as a mitochondria-targeted antioxidant: one of evolution's best ideas. Cell Mol Life Sci. 2017;74(21):3863–81.
81. Hardeland R. Melatonin in plants - diversity of levels and multiplicity of functions. Front Plant Sci. 2016;7:198.
82. Arnao MB. Phytomelatonin: discovery, content, and role in plants. Adv Bot. 2014;2014:1–11.
83. Wei Y, Hu W, Wang Q, Zeng H, Li X, Yan Y, et al. Identification, transcriptional and functional analysis of heat-shock protein 90s in banana (*Musa acuminata* L.) highlight their novel role in melatonin-mediated plant response to Fusarium wilt. J Pineal Res. 2017;62(1). https://doi.org/10.1111/jpi.12367
84. Liu C, Chen L, Zhao R, Li R, Zhang S, Yu W, et al. Melatonin induces disease resistance to *Botrytis cinerea* in tomato fruit by activating Jasmonic acid signaling pathway. J Agric Food Chem. 2019;67(22):6116–24.
85. Mandal MK, Suren H, Ward B, Boroujerdi A, Kousik C. Differential roles of melatonin in plant-host resistance and pathogen suppression in cucurbits. J Pineal Res. 2018;65(3):e12505.
86. Lee HY, Back K. Mitogen-activated protein kinase pathways are required for melatonin-mediated defense responses in plants. J Pineal Res. 2016;60(3):327–35.
87. Lee HY, Back K. Melatonin is required for H_2O_2 - and NO-mediated defense signaling through MAPKKK3 and OXI1 in *Arabidopsis thaliana*. J Pineal Res. 2017;62(2). https://doi.org/10.1111/jpi.12379
88. Li H, Chang J, Zheng J, Dong Y, Liu Q, Yang X, et al. Local melatonin application induces cold tolerance in distant organs of *Citrullus lanatus* L. via long distance transport. Sci Rep. 2017;7:40858.
89. Yoon YH, Kim M, Park WJ. Foliar Accumulation of Melatonin Applied to the Roots of Maize (*Zea mays*) Seedlings. Biomolecules. 2019;9(1). https://doi.org/10.3390/biom9010026
90. Erland LAE, Yasunaga A, Li ITS, Murch SJ, Saxena PK. Direct visualization of location and uptake of applied melatonin and serotonin in living tissues and their redistribution in plants in response to thermal stress. J Pineal Res. 2019;66(1):e12527.
91. Lee HY, Byeon Y, Tan DX, Reiter RJ, Back K. *Arabidopsis* serotonin *N*-acetyltransferase knockout mutant plants exhibit decreased melatonin and salicylic acid levels resulting in susceptibility to an avirulent pathogen. J Pineal Res. 2015;58(3):291–9.
92. Breen S, Solomon PS, Bedon F, Vincent D. Surveying the potential of secreted antimicrobial peptides to enhance plant disease resistance. Front Plant Sci. 2015;6:900.
93. Dracatos PM, Payne J, Di Pietro A, Anderson MA, Plummer KM. Plant Defensins NaD1 and NaD2 Induce Different Stress Response Pathways in Fungi. Int J Mol Sci. 2016;17(9). https://doi.org/10.3390/ijms17091473
94. Klessig DF, Choi HW, Dempsey DA. Systemic acquired resistance and salicylic acid: past, present, and future. Mol Plant-Microbe Interact. 2018;31(9):871–88.
95. Cui F, Wu S, Sun W, Coaker G, Kunkel B, He P, et al. The *Pseudomonas syringae* type III effector AvrRpt2 promotes pathogen virulence via stimulating *Arabidopsis* auxin/indole acetic acid protein turnover. Plant Physiol. 2013;162(2):1018–29.
96. Wang D, Pajerowska-Mukhtar K, Culler AH, Dong X. Salicylic acid inhibits pathogen growth in plants through repression of the auxin signaling pathway. Curr Biol. 2007;17(20):1784–90.
97. Zhang S, Zheng X, Reiter RJ, Feng S, Wang Y, Liu S, et al. Melatonin attenuates potato late blight by disrupting cell growth, stress tolerance, Fungicide Susceptibility and Homeostasis of Gene Expression in *Phytophthora infestans*. Front Plant Sci. 2017;8:1993.
98. Lay FT, Anderson MA. Defensins--components of the innate immune system in plants. Curr Protein Pept Sci. 2005;6(1):85–101.
99. Rajniak J, Giehl RFH, Chang E, Murgia I, von Wiren N, Sattely ES. Biosynthesis of redox-active metabolites in response to iron deficiency in plants. Nat Chem Biol. 2018;14(5):442–50.
100. Zhou C, Liu Z, Zhu L, Ma Z, Wang J, Zhu J. Exogenous Melatonin Improves Plant Iron Deficiency Tolerance via Increased Accumulation of Polyamine-Mediated Nitric Oxide. Int J Mol Sci. 2016;17(11). https://doi.org/10.3390/ijms17111777
101. Ipcho S, Sundelin T, Erbs G, Kistler HC, Newman MA, Olsson S. Fungal Innate Immunity Induced by Bacterial Microbe-Associated Molecular Patterns (MAMPs). G3 (Bethesda). 2016;6(6):1585–95.
102. Verbon EH, Trapet PL, Stringlis IA, Kruijs S, Bakker P, Pieterse CMJ. Iron and immunity. Annu Rev Phytopathol. 2017;55:355–75.
103. Pre M, Atallah M, Champion A, De Vos M, Pieterse CM, Memelink J. The AP2/ERF domain transcription factor ORA59 integrates jasmonic acid and ethylene signals in plant defense. Plant Physiol. 2008;147(3):1347–57.
104. Knoth C, Eulgem T. The oomycete response gene *LURP1* is required for defense against *Hyaloperonospora parasitica* in *Arabidopsis thaliana*. Plant J. 2008;55(1):53–64.
105. Hagen G, Guilfoyle T. Auxin-responsive gene expression: genes, promoters and regulatory factors. Plant Mol Biol. 2002;49(3–4):373–85.
106. Kant S, Bi YM, Zhu T, Rothstein SJ. *SAUR39*, a small auxin-up RNA gene, acts as a negative regulator of auxin synthesis and transport in rice. Plant Physiol. 2009;151(2):691–701.
107. Majda M, Robert S. The Role of Auxin in Cell Wall Expansion. Int J Mol Sci. 2018;19(4). https://doi.org/10.3390/ijms19040951
108. Rawat R, Schwartz J, Jones MA, Sairanen I, Cheng Y, Andersson CR, et al. REVEILLE1, a Myb-like transcription factor, integrates the circadian clock and auxin pathways. Proc Natl Acad Sci U S A. 2009;106(39):16883–8.
109. Zazimalova E, Murphy AS, Yang H, Hoyerova K, Hosek P. Auxin transporters--why so many? Cold Spring Harb Perspect Biol. 2010;2(3):a001552.
110. Wen D, Gong B, Sun S, Liu S, Wang X, Wei M, et al. Promoting Roles of Melatonin in Adventitious Root Development of *Solanum lycopersicum* L by Regulating Auxin and Nitric Oxide Signaling. Front Plant Sci. 2016;7:718.
111. Davies PJ. Plant hormones. Dodrecht: Springer; 2010. p. 1–15.
112. Bilgin DD, Zavala JA, Zhu J, Clough SJ, Ort DR, DeLucia EH. Biotic stress globally downregulates photosynthesis genes. Plant Cell Environ. 2010;33(10):1597–613.
113. Pajerowska-Mukhtar KM, Wang W, Tada Y, Oka N, Tucker CL, Fonseca JP, et al. The HSF-like transcription factor TBF1 is a major molecular switch for plant growth-to-defense transition. Curr Biol. 2012;22(2):103–12.
114. Xu G, Yuan M, Ai C, Liu L, Zhuang E, Karapetyan S, et al. uORF-mediated translation allows engineered plant disease resistance without fitness costs. Nature. 2017;545(7655):491–4.
115. Liu X, Afrin T, Pajerowska-Mukhtar KM. Arabidopsis GCN2 kinase contributes to ABA homeostasis and stomatal immunity. Commun Biol. 2019;2:302.
116. Mutwil M, Klie S, Tohge T, Giorgi FM, Wilkins O, Campbell MM, et al. PlaNet: combined sequence and expression comparisons across plant networks derived from seven species. Plant Cell. 2011;23(3):895–910.
117. Bray NL, Pimentel H, Melsted P, Pachter L. Near-optimal probabilistic RNA-seq quantification. Nat Biotechnol. 2016;34(5):525–7.
118. Cheng CY, Krishnakumar V, Chan AP, Thibaud-Nissen F, Schobel S, Town CD. Araport11: a complete reannotation of the *Arabidopsis thaliana* reference genome. Plant J. 2017;89(4):789–804.

Publisher's Note

2.1 Supplementary Information

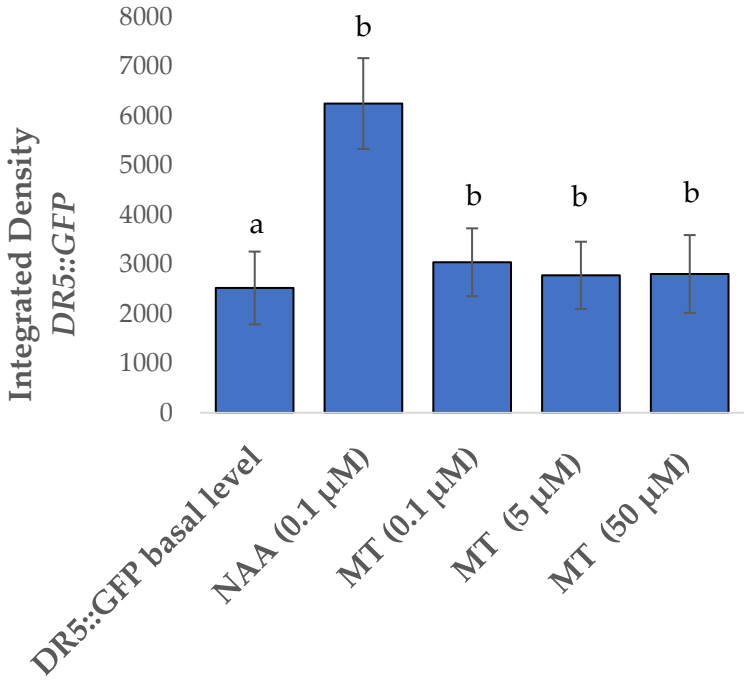


Figure S1. **Effect of melatonin on expression of auxin-responsive marker line *DR5::GFP* in *Arabidopsis thaliana* primary root.** GFP quantification by Integrated Density in ImageJ. Statistical differences analyzed between treatment by One-way ANOVA and Tukey post-hoc test with *p*-value <0.05. Different letters denote statistical significance. Statistics was conducted on absolute values. 10 seedlings per treatment, 1 plate per treatment. Bars represent mean integrated density of treated seedlings (n=8-10). Error bars represent standard deviation. Experiment repeated thrice on independent days with similar results.

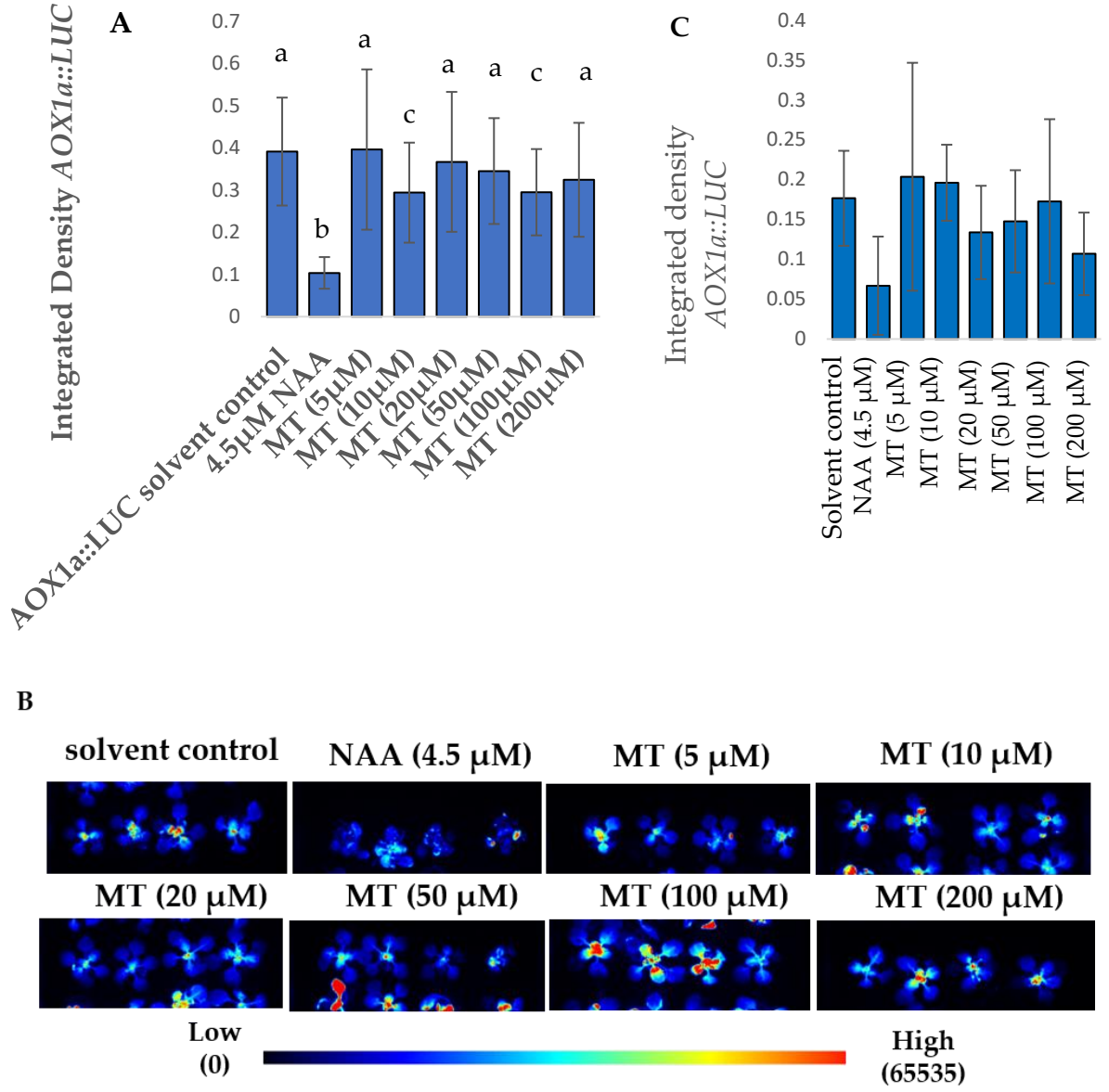


Figure S2. Effect of melatonin on expression of auxin-responsive gene *AOX1a::LUC* in rosette leaves of *Arabidopsis thaliana* (A) Quantification of bioluminescence of *AOX1a::LUC* +/- Antimycin A treatment by Integrated density method (ImageJ). (B) Bioluminescence images of *AOX::LUC* (wild-type Col-0) grown on +/- MT or NAA containing media for 3 days and visualized for *LUC* activity under normal growth conditions in ChemiDoc (BioRad). No statistically significant differences observed (C) Quantification of bioluminescence of *AOX1a::LUC* under normal growth conditions by Integrated density method (ImageJ). Data are expressed as mean integrated density (sum of pixels) of treated seedlings (n=12-16). Error bars represent standard deviation. Different alphabets denote statistical significance (One-way ANOVA and Tukey post-hoc), $p < 0.05$. Experiments were repeated thrice on independent days with similar results.

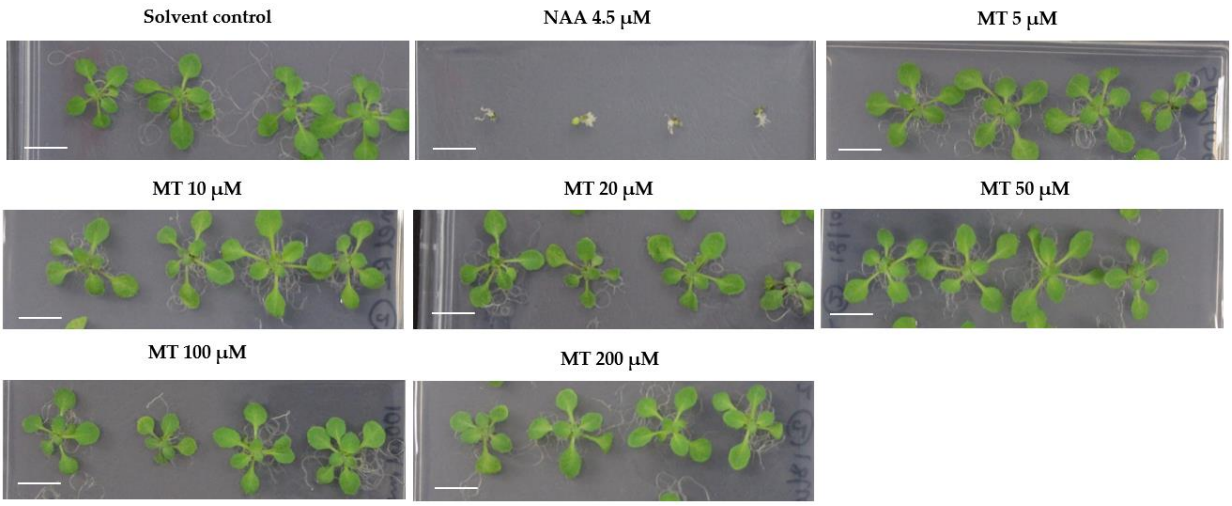


Figure S3. Differential seed development response of wild type AOX::LUC (Col-0) toward melatonin or auxin.

Differential seed developmental responses of 14-day old *AOX1a::LUC* plants directly germinated on Gamborg's B5 media supplemented with either melatonin or NAA. Shown are representative images. Scale bar = 1cm. Experiment was repeated thrice on independent days with similar results.

Table S1. Overlap analysis of differentially expressed genes (DEGs) related to auxin-responsive GO terms

N/A refers to transcript levels of genes not altered by the corresponding treatment

Gene ID	Our Data	Omelyanchuk et al., 2017 [68] 1 µM IAA-treated roots (6h)
AT1G29460 (SAUR65)	N/A	N/A
AT5G17300 (RVE1)	N/A	N/A
AT3G48360 (BT2)	down	up
AT5G63160 (BT1)	down	down
AT1G52830 (IAA6)	down	N/A
AT2G17500 (PILS5)	up	N/A
AT2G34650 (PIID)	up	up
AT5G12330 (LRP1)	up	N/A
AT1G56650 (PAP1)	up	N/A
AT5G13930 (TT4)	up	N/A
AT1G27740 (RSL4)	up	N/A
AT2G23170 (GH3.3)	up	up

Table S1. ***Contd.***

Gene ID	**Our Data**	**Omelyanchuk et al., 2017 [68] 1 µM IAA-treated roots (6h)**
AT2G14960 (GH3.1)	up	up
AT4G12550 (AIR1)	up	N/A
AT5G16530 (PIN5)	up	N/A
AT3G14370 (WAG2)	up	N/A
AT4G37390 (GH3.4)	up	up
AT1G52830 (IAA6)	down	N/A
AT4G02520 (GSTF2)	up	up
AT3G62680 (PRP3)	up	down
AT2G47000 (ABCB4)	up	up
AT5G07990 (TT7)	up	N/A
Overlap: Approx. 40% match		

Table S2. Overlap analysis of DEGs with previous transcriptome data sets on melatonin in *Arabidopsis.*

N/A refers to transcript levels of genes not altered by the corresponding treatment

Gene ID	**Our Data**	**Weeda et al., 2014 [37]**		**Wan et al., 2018 [73]**
		100 pM MT-treated whole seedlings 16h	1 mM MT-treated whole seedlings 16h	10 µM-MT treated whole seedlings 2 days
AT3G59930	up	yes	N/A	up
AT5G33355	up	yes	N/A	N/A
AT1G34047	up	N/A	N/A	N/A
AT2G26010 (PDF1.3)	up	N/A	N/A	N/A
AT2G26020 (PDF1.2b)	up	N/A	yes	N/A
AT2G47950	up	yes	N/A	N/A
AT1G06160(AP2/ERF59)	up	N/A	N/A	N/A
AT3G51910 (HSFA7A)	up	N/A	N/A	N/A

Table S2 contd.

Gene ID	Our Data	Weeda et al., 2014 [37]		Wan et al., 2018 [73]
		100 pM MT-treated whole seedlings 16h	1 mM MT-treated whole seedlings 16h	10 µM-MT treated whole seedlings 2 days
AT5G07100 (WRKY26)	up	N/A	N/A	N/A
AT5G17300 (RVE1)	down	N/A	N/A	N/A
ATCG00490 (RBCL)	down	N/A	N/A	N/A
ATCG00270 (PSBD)	down	N/A	N/A	N/A
ATCG00340 (PSAB)	down	N/A	N/A	N/A
ATCG00280 (PSBC)	down	N/A	N/A	N/A
ATCG00350 (PSAA)	down	N/A	N/A	N/A
ATCG00020 (PSBA)	down	N/A	N/A	N/A

Table S2 contd.

Gene ID	Our Data	Weeda et al., 2014 [37]		Wan et al., 2018 [73]
		100 pM MT-treated whole seedlings 16h	1 mM MT-treated whole seedlings 16h	10 µM-MT treated whole seedlings 2 days
ATCG00330 (RPS14)	down	N/A	N/A	down
AT4G31940 (CYP82C)	up	N/A	N/A	up
AT3G13310 (DJC66)	up	N/A	N/A	N/A
AT1G75040 (PR5)	up	N/A	N/A	N/A
AT5G27420 (CNI1)	up	N/A	N/A	N/A
AT2G15310 (ARFB1A)	up	N/A	N/A	N/A
AT1G10140 (UCP031279)	up	N/A	N/A	N/A
AT1G78450	up	N/A	N/A	N/A

Table S2 contd.

Gene ID	Our Data	Weeda et al., 2014 [37]		Wan et al., 2018 [73]
		100 pM MT-treated whole seedlings 16h	1 mM MT-treated whole seedlings 16h	10 µM-MT treated whole seedlings 2 days
AT2G40095	up	N/A	N/A	N/A
AT1G29460 (SAUR65)	down	N/A	N/A	N/A
AT1G21240 (WAK3)	up	N/A	yes	N/A
AT4G18250	up	N/A	N/A	N/A
AT2G16720	up	N/A	yes	N/A
AT5G13170 (SAG29)	down	N/A	N/A	N/A
AT1G15520 (ABCG40)	up	N/A	N/A	N/A
AT1G14880 (PCR1)	up	N/A	yes	N/A
AT2G44130 (KMD3)	up	N/A	yes	N/A

Table S2 contd.

Gene ID	Our Data	Weeda et al., 2014 [37]		Wan et al., 2018 [73]
		100 pM MT-treated whole seedlings 16h	1 mM MT-treated whole seedlings 16h	10 µM-MT treated whole seedlings 2 days
AT3G50480 (HR4)	up	N/A	yes	N/A
AT3G22231 (PCC1)	up	N/A	yes	N/A
AT1G32960 (ATSBT3.3)	up	N/A	yes	down
AT2G14560 (LURP1)	up	N/A	yes	N/A
AT3G48080	up	N/A	N/A	N/A
AT2G26400	up	N/A	yes	N/A
AT5G25250 (FLOT1)	up	N/A	yes	N/A
AT1G10340	up	N/A	yes	down
AT4G02520 (GSTF2)	up	N/A	N/A	N/A

Table S2 contd.

Gene ID	**Our Data**	**Weeda et al., 2014 [37]**		**Wan et al., 2018 [73]**
		100 pM MT-treated whole seedlings 16h	1 mM MT-treated whole seedlings 16h	10 µM-MT treated whole seedlings 2 days
AT2G44480 (BGLU17)	up	N/A	N/A	N/A
AT5G55450 (ATLTP4.4)	up	N/A	N/A	N/A
AT5G10770	up	N/A	N/A	down
AT5G53830 (MVQ3)	up	N/A	N/A	N/A
AT4G04840 (MSRB6)	down	N/A	N/A	N/A
Overlap: Approx.			**31% match**	**10% match**

Table S3. Summary statistics of RNA-Seq data

	Total read counts (#)	Total pseudo-aligned (#)	Average read length (bp)	Q30 bases ratio (%)
Contrep1	23,874,897	17,267,556	75	93
Contrep2	23,772,799	17,562,147	75	93
Contrep3	25,553,637	18,955,141	75	93
MT5rep1	22,499,526	16,920,223	75	93
MT5rep2	22,565,852	16,933,173	75	93
MT5rep3	24,218,870	18,370,942	75	93
MT100rep1	29,105,294	21,607,241	75	93
MT100rep2	29,858,496	21,859,754	75	93
MT100rep3	24,357,119	18,875,010	75	93
NAA rep1	23,752,930	18,051,162	75	93
NAA rep2	21,771,965	16,510,212	75	93
NAA rep3	23,698,912	17,727,290	75	93

Table S4: DEGs uniquely regulated by MT (100 µM) in comparison with hormones-responsive transcriptome (microarray) database

Gene ID	Annotation	Log2 fold change						
		Our data	*Arabidopsis* eFP Browser					
		MT	ABA	SA	MeJA	BL	GA	CK
Defensins (5)								
AT3G59930	*Defensin-like (DEFL) family protein*	4.92	-0.02	0.04	-0.23	-0.16	0.27	-0.34
AT5G33355	*Defensin-like (DEFL) family protein*	4.92	-0.02	0.04	-0.23	-0.16	0.27	-0.34
AT1G34047	*Defensin-like (DEFL) family protein*	4.88	N/A	N/A	N/A	N/A	N/A	N/A
AT2G26010	*Plant defensin PDF1.3*	4.62	N/A	N/A	N/A	N/A	N/A	N/A
AT2G26020	*Plant defensin PDF1.2b*	4.33	-0.54	-1.28	-0.15	-0.84	0.22	-0.32
Transcription factors (5)								
AT2G47950	*Myelin-transcription factor like protein*	4.11	0.67	-0.47	2.25	0.32	0.75	0.19
AT1G06160	*Ethylene responsive factor AP2/ERF59*	2.47	-5.81	-0.2	0.4	-0.57	-0.25	-1.18
AT3G51910	*Heat shock transcription factor HSFA7A*	1.52	-0.48	-0.11	-0.27	0.3	0.52	-0.98
AT5G07100	*WRKY DNA binding protein WRKY26*	1.23	-1.34	-0.29	-0.15	-0.66	0.05	-1.89
AT5G17300	*Myb-like transcription factor RVE1*	-1.42	0.79	-0.62	-0.02	0.31	-0.05	-0.48
Photosynthesis (7)								
ATCG00330	*Chloroplast ribosomal protein RPS14*	-1.55	0.26	0.06	-0.54	-0.3	0.36	0.0
ATCG00020	*Photosystem II reaction center A PSBA*	-1.49	0.02	0.35	-0.09	-0.34	-0.04	-0.01
ATCG00350	*Photosystem I, PsaA/PsaB*	-1.47	-0.11	-0.09	0.01	-0.1	-0.3	-0.12
ATCG00280	*Photosystem II reaction center C, PSBC*	-1.43	-0.01	0.33	0.02	0.0	-0.15	-0.19
ATCG00340	*Photosystem I, PsaA/PsaB*	-1.41	-0.44	0.2	-0.11	-0.71	-0.98	0.03
ATCG00270	*Photosystem II reaction center D PSBD*	-1.40	0.01	-0.15	-0.04	0.01	-0.13	-0.35
ATCG00490	*Ribulose-biphosphate carboxylases RBCL*	-1.25	-0.25	-0.15	-0.27	-0.09	0.02	-0.26

Table S4: Contd.

Gene ID	Annotation	Log2 fold change						
		Our data	*Arabidopsis* eFP Browser					
		MT	ABA	SA	MeJA	BR	GA-3	CK
Post-translational protein modification (3)								
AT3G13310	*Chaperone DnaJ-domain superfamily DJC66*	3.80	-0.25	0.38	-0.27	-0.09	-0.17	-1.05
AT2G15310	*ADP-ribosylation factor ARFB1A*	2.02	0.89	2.36	0.42	-0.27	0.27	-0.59
AT5G10770	*Eukaryotic aspartyl protease family protein*	1.26	0.46	-2.07	-1.83	0.0	0.23	-0.44
Response to iron ion and indole glucosinolate metabolic process (1)								
AT4G31940	*Cytochrome P450 CYP82C4*	4.46	-0.01	0.46	-3.17	0.04	-0.42	-3.13
Systemic acquired resistance (1)								
AT1G75040	*Pathogenesis-related gene 5, PR5*	3.69	0.18	-1.81	0.15	0.37	0.72	-0.76
Response to abscisic acid (1)								
AT5G27420	*Carbon/nitrogen insensitive ubiquitin ligase CNI1*	2.22	0.2	1.17	-0.81	0.48	0.48	-1.63
Auxin response, polar transport and activated-signaling (1)								
AT1G29460	*SAUR-like auxin responsive SAUR65*	-1.26	-4.96	-1.21	-0.72	1.4	0.5	0.2
Miscellaneous (4)								
AT1G10140	*Uncharacterized conserved protein*	1.50	-0.14	-0.89	0.76	0.41	0.31	-0.36
AT1G78450	*SOUL-heme binding protein*	1.43	-0.1	-1.33	-0.79	0.03	0.08	-1.36
AT2G40095	*Alpha/beta hydrolase related protein*	1.36	N/A	N/A	N/A	N/A	N/A	N/A
AT2G07706	*Hypothetical protein*	-1.53	N/A	N/A	N/A	N/A	N/A	N/A

N/A= The requested primary gene / probeset ID could not be found in Affymetrix ATH1 datasource in *Arabidopsis* eFP browser. Treatments in eFP browser: ABA=Abscisic acid (10 µM); SA=salicylic acid (10 µM); MeJA=methyl jasmonate (10 µM); BR=brassinolide (10 nM); GA-3=gibberellic acid (1 µM); CK=cytokinin (20 µM). 7-day old wild-type (Col-0) plants were grown in liquid MS media under continuous light conditions at 23 °C. The duration of treatments in the media was 3 hours. RNA was isolated from whole seedlings and hybridised to the ATH1 gene chip. An exception to growth conditions are plants treated with CK which were 21-day old wild-type (Col-0) plants grown on agar. The light conditions have not been specified for CK treated plants.

Table S5 DEGs commonly regulated by MT (100 µM) and auxin (NAA; 4.5 µM) in comparison with expression profiles in hormones-responsive transcriptome (microarray) database

Gene ID	Annotation	Log2 fold change							
		Our data		*Arabidopsis* eFP Browser					
		MT	NAA	ABA	SA	MeJA	BL	GA	CK
Kinases (3)									
AT1G21240	*Wall-associated kinase 3, WAK3*	3.55	3.91	0.42	0.24	0.13	2.42	2.42	0.45
AT4G18250	*Receptor serine/threonine kinase-like*	3.43	3.07	-0.08	1.88	0.64	1.2	1.05	-1.29
AT1G21250	*Wall-associated kinase 1, WAK1*	1.55	3.18	-0.42	4.3	-0.56	0.35	0.32	-0.81
Transcription factors (1)									
AT2G16720	*Myb domain protein 7, MYB7*	1.81	1.36	1.14	0.04	0.31	0.08	-0.06	0.32
Senescence (1)									
AT5G13170	*Senescence-associated gene, SAG12*	-1.46	2.70	7.31	-0.92	-3.05	0.05	0.05	-1.4
Transporter (1)									
AT1G15520	*ABC transporter family, ABCG40*	3.01	3.00	-0.01	0.99	0.71	0.13	0.56	0.5
Oxidation-reduction process (1)									
AT4G04840	*Methionine sulfoxide reductase, MSRB6*	-1.36	3.05	-1.35	-1.31	2.09	-0.28	-0.3	0.18
Lipid metabolic process (3)									
AT3G48080	*Alpha-beta hydrolases ABH*	2.68	3.16	-0.46	2.27	-0.22	-0.02	0.08	-0.88
AT2G26400	*Acireductone dioxygenase, ARD3*	2.62	2.62	-0.55	6.76	-0.28	0.11	0.13	-1.58
AT3G22231	*Pathogen circadian controlled PCC1*	2.95	4.98	-0.73	4.35	-0.97	-0.16	0.52	-1.09
Plant type hypersensitive response (1)									
AT3G50480	*Homolog of RPW8, HR4*	3.03	1.70	-0.37	1.73	-0.43	0.19	0.46	-0.16

Table S5 contd.

Gene ID	Annotation	Log2 fold change							
		Our data		*Arabidopsis* eFP Browser					
		MT	NAA	ABA	SA	MeJA	BL	GA	CK
Induced systemic resistance (1)									
AT1G32960	*Subtilase family protein, ATSBT3.3*	2.90	2.21	2.65	1.6	2.76	2.55	1.91	0.1
Response to other organisms (2)									
AT2G14560	*Late upregulated in response to downy mildew LURP1*	2.90	5.13	-2.09	5.35	-1.55	0.51	0.81	-2.38
AT5G25250	*Flotillin-like protein 1, FLOT1*	2.61	2.18	3.01	0.74	2.24	5.13	3.83	-0.82
Regulation of phenylpropanoid metabolic process (1)									
AT2G44130	*Galactose/oxidase kelch repeat protein*	3.40	3.06	0.33	-0.79	0.47	0.46	0.05	-1.28
Response to abiotic stresses (2)									
AT1G14880	*Plant cadmium resistance1, PCR1*	6.55	4.33	-0.21	1.9	0.17	-0.57	-0.11	-1.42
AT4G02520	*Glutathione-S transferase GSTF2*	1.82	1.80	-0.28	0.52	-0.07	0.07	0.28	0.09
Miscellaneous (4)									
AT1G10340	*Ankyrin repeat family protein*	2.37	1.7	-0.53	1.85	-1.21	0.57	0.25	-1.47
AT2G44480	*Beta glucosidase 17, BGLU17*	1.81	1.84	0.28	-2.99	-0.6	-2.33	0.1	-0.68
AT5G55450	*Bifunctional inhibitor/lipid transfer, ATLTP4.4*	1.47	3.01	-0.51	2.82	-0.7	0.06	0.04	-0.5
AT5G53830	*VQ-motif containing protein, MVQ3*	1.25	1.41	1.38	-0.78	-0.02	1.01	0.13	-0.34

N/A=The requested primary gene / probeset ID could not be found in Affymetrix ATH1 datasource in *Arabidopsis* eFP browser. Treatments in eFP browser: ABA=Abscisic acid (10 µM); SA=salicylic acid (10 µM); MeJA=methyl jasmonate (10 µM); BR=brassinolide (10 nM); GA-3=gibberellic acid (1 µM); CK=cytokinin (20 µM). 7-day old wild-type (Col-0) plants were grown in liquid MS media under continuous light conditions at 23 °C. The duration of treatments in the media was 3 hours. RNA was isolated from whole seedlings and hybridised to the ATH1 gene chip. An exception to growth conditions are plants treated with CK which were 21-day old wild-type (Col-0) plants grown on agar. The light conditions have not been specified for CK treated plants.

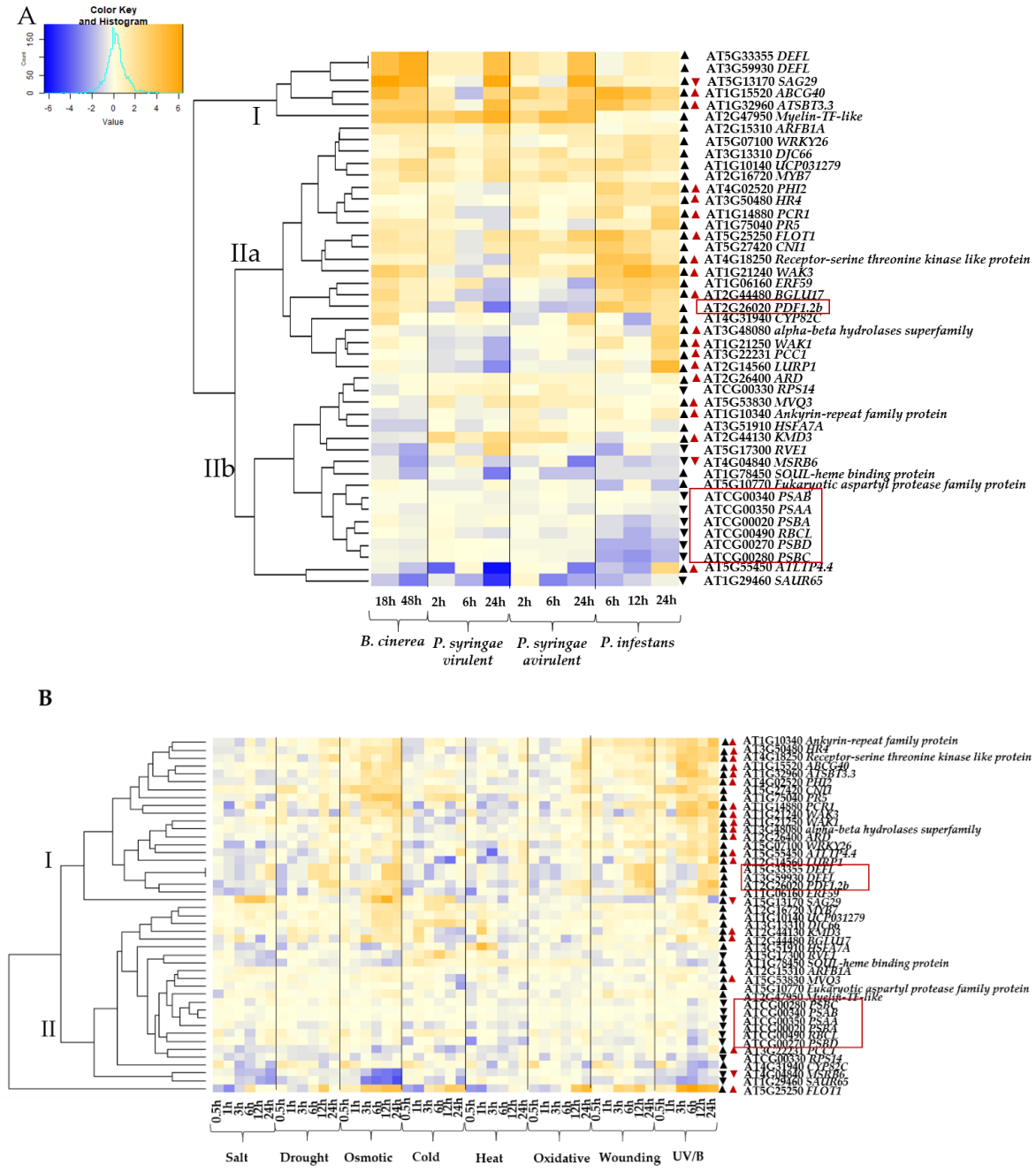
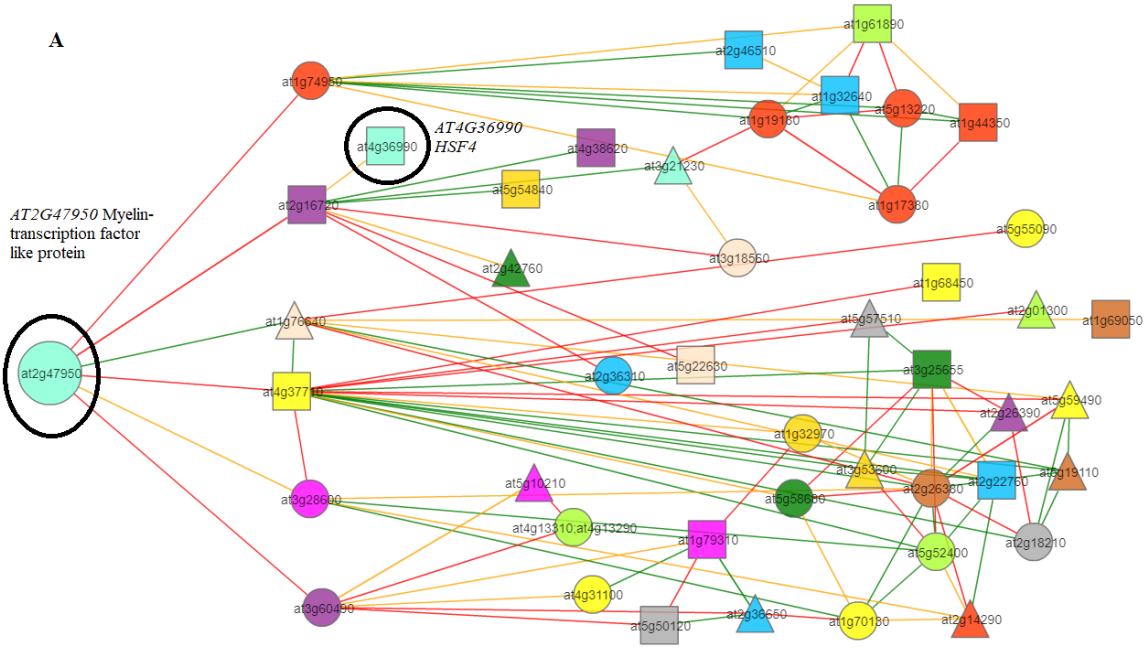


Figure S4. Microarray-based expression pattern of unique or commonly regulated DEGs by MT under stresses. (A) expression patterns of DEGs in 4-week old *Arabidopsis* leaves in different biotic conditions and (B) expression patterns of DEGs in 18-day old *Arabidopsis* shoot in different abiotic conditions. Arrows indicate trend of regulation (up or down) in our RNA-Seq data in response to melatonin (100 μM) (black arrows) and/or NAA (red arrows). Clusters are numbered as I and II.

The heat map represents hierarchical clustering of log2 transformed fold change of signal values as compared to control. Prior to clustering, the values were standardized by z-scoring method in 'scale' function in R (version R-3.3.2). The colour key and histogram (same for A and B) represent the scale for relative expression with trace levels. Blue colour represents low expression levels, light represent medium expression and orange represents highest expression level. Different clusters are denoted by I and II. Boxed in red are the DEGs genes forming part of each cluster and are further discussed.

A



B

Gene ID	Description
AT1G74950 (TIFY10B)	TIFY domain/Divergent CCT motif family protein
AT2G16720 (MYB7)	Encodes a member of MYB3R- and R2R3- type MYB- encoding genes
AT4G36990 (HSF4)	Heat shock transcription factor /TL1 binding transcription factor
AT1G76640 (CML39)	Calcium-binding EF-hand family protein
AT4G37710 (VQ29)	VQ motif-containing protein
AT3G28600	P-loop containing nucleoside triphosphate hydrolases superfamily protein
AT3G60490 (ERF35)	encodes a member of the DREB subfamily A-4 of ERF/AP2 transcription factor family. The protein contains one AP2 domain. There are 17 members in this subfamily including TINY.

Figure S5: Co-expression network of the gene *AT2G47950* (myelin transcription factor-like protein) induced by melatonin (100 µM). Circled are *AT2G47950* and co-expressed gene

AT4G36990 (HSF4) (b). Genes co-expressed with *AT2G47950* in the first rank. Data are sourced from publicly available software PlaNet (http://aranet.mpimp-golm.mpg.de/ [114].

Chapter 3: Melatonin treatment reveals differences between the *serotonin-N-acetyltransferase1* mutant and wild-type and induces conserved and ecotype-dependent responses in *Arabidopsis thaliana*

3.1 Abstract

Since the discovery of melatonin in plants, numerous studies have observed growth regulating effects under optimum and stress conditions. Among melatonin biosynthetic genes identified in plants, serotonin *N*-acetyltransferase (*SNAT1*) encoding SNAT1 protein plays a key role in diverse melatonin-mediated processes such as tolerance to abiotic stresses and pathogen infection. Melatonin acts as an antioxidant and affects gene expression, possibly regulating important biological processes that are conserved for survival and/or unique to aid in adaptation of distinct local habitat environments of plant species. Melatonin-induced gene expression differences due to geographic isolation of plant populations is currently unknown. This is a two-fold study where the phenotypic and transcriptomic effects of *Arabidopsis thaliana* root applied melatonin were analysed in; (1) a T-DNA insertional mutant for a key melatonin biosynthetic gene *AtSNAT1* (*AT1G32070*); and (2) in 29 selected *Arabidopsis thaliana* ecotypes (representing diverse geographical locations). Comparison of *AtSNAT1* versus wild type (Columbia-0, Col-0) in the absence and presence of applied melatonin identified 111 and 374 differentially expressed genes associated to melatonin-mediated functions in plant growth and abiotic and biotic stress defence. A large phenotypic response to melatonin, based on projected rosette area, was observed in the ecotypes ranging from a moderate to high response. Three ecotypes, Bur-0, Col-0, and Hn-0, with respectively moderate and high melatonin responsiveness, were further selected for whole transcriptome analysis in response to exogenous melatonin. The expression levels of a set of 118 core-conserved genes were identified as being regulated in the melatonin-treated ecotypes compared to their respective controls. Ecotype-dependent transcriptional regulation in Bur-0 (101 DEGs), Col-0 (289 DEGs) and Hn-0 (237 DEGs) indicated the role of melatonin in adaptation to distinct environments. Overall, these findings demonstrate that melatonin is essential for many conserved aspects of plant physiology and there exists flexibility in transcriptional regulation among the tested genotypes in the 'wiring' of the biological processes underlying melatonin signalling.

Keywords

Arabidopsis, melatonin, *AtSNAT1*, ecotype, variation, transcriptome

3.2 Introduction

Melatonin (MT) is a metabolite discovered in plants in 1995 (Dubbels et al., 1995, Hattori et al., 1995). Its role as a plant growth regulator under normal and stressful conditions has become widely accepted and makes it a potential candidate for a new plant hormone (Hernández et al., 2015, Wei et al., 2018, Arnao and Hernández-Ruiz, 2019). The endogenous levels of melatonin have been shown to increase under a variety of environmental stressors such as salt, drought, cold and pathogenic infection in a variety of plants, including *Arabidopsis* (Arnao and Hernández-Ruiz, 2013, Bajwa et al., 2014, Ahmad et al., 2019). However, melatonin content can also vary within and between species, from 20 µg/g to up to a 200 µg/g between tissues (Hardeland, 2016). This points to the presence of natural variation in endogenous melatonin levels in plants. Different growth conditions and environments (stimuli) impact the endogenous melatonin levels within plant species (Arnao and Hernández-Ruiz, 2013). For example, plant species (*Glycyrrhiza uralensis*) adapted to high natural light or UV intensities exhibit higher melatonin levels as compared to the species from distinct habitats (Simopoulos et al., 2005). The high levels of melatonin found in species of *G. uralensis* plants have been hypothesised to play a role in stress protection against high light levels (Simopoulos et al., 2005). The hypothesis, that melatonin-rich plants are able to better withstand environmental challenges than plants producing less melatonin, has been investigated in few studies (Okazaki et al., 2010, Park et al., 2013). Melatonin content was found to be higher in the rice (*Oryza sativa* L.) cultivar cv. Zhandao 88 which is a resistant variety to rice stripe virus infection, as compared to the susceptible variety Nipponbare, in response to the infection (Lu et al., 2019). Furthermore, melatonin content was induced more (20.79%) in watermelon drought-resistant genotype M20 than the sensitive genotype Y34 (Li et al., 2019b).

There are six key genes, tryptophan decarboxylase (*TDC*), tryptamine 5-hydroxylase (*T5H*), serotonin *N*-acetyltransferase (*SNAT*), *N*-acetylserotonin-*O*-methyltransferase (*ASMT*), caffeic-acid-*O*-methyltransferase (*COMT*), and histone deacetylase (*HDAC*), involved in the biosynthesis of melatonin in plants, including rice (*Oryza sativa*) and *Arabidopsis* (Back et al., 2016, Lee et al., 2018). Much research has been focussed on functional characterisation of the penultimate key genes encoding serotonin *N*-acetyltransferase (SNAT) and *N*-acetylserotonin-*O*-methyltransferase (ASMT) in *Arabidopsis* and rice (Kang et al., 2011, Lee et al., 2014, Back et al., 2016). In *Arabidopsis* (ecotype Columbia, Col-0), there are two *SNAT* isogenes, *AtSNAT1 (AT1G32070)* and *AtSNAT2 (AT1G26220)* that have been characterised (Lee et al., 2015, Lee et al., 2019).

They share low amino acid identity (27%) and their recombinant proteins have been shown to exhibit SNAT activity based on in vitro enzyme kinetics (Lee et al., 2014, Lee et al., 2019). *AtSNAT1* and *AtSNAT2* have distinct expression patterns. In Columbia ecotype (Col-0), *AtSNAT1* has high expression in mature leaves and *AtSNAT2* has high expression levels in flowers under control conditions (Lee et al., 2015, Lee et al., 2019). Both AtSNAT1 and AtSNAT2 enzymes have shown to be localised in chloroplasts (Lee et al., 2015, Lee et al., 2019). Overexpression and T-DNA insertional mutant analysis (SALK_020577) of *AtSNAT1* in Col-0 background, revealed roles ranging from melatonin synthesis, disease resistance to the bacterium, *Pseudomonas syringae* DC3000, anthocyanin metabolism, abiotic stress tolerance, and photosynthesis-related processes (Lee et al., 2015, Zhang et al., 2016, Koskela et al., 2018, Lee and Back, 2018). Variation in the levels of melatonin biosynthetic gene expression have been associated with differences in melatonin levels in plants, and also exists under environmental stimuli (Hardeland, 2016). The increase in melatonin levels (up to 6.6-fold compared to control) upon cadmium chloride ($CdCl_2$; 0.2 mM) exposure in rice leaves has been associated with enhanced expression levels of *TDC*, *T5H* and *ASMT* and decreased expression levels of *SNAT1* (Byeon et al., 2015). The genotype and melatonin interactions have been recently reported for a few crop species (Fu et al., 2017, Hu et al., 2017, Li et al., 2019b, Lu et al., 2019, Huang et al., 2020). The upregulated expression levels of *TDC*, *T5H*, *SNAT* and *ASMT* under drought stress were shown to be relatively higher in the leaves of the drought-sensitive apple (*Malus*) species *M. hupehensis*, than the drought-tolerant species *M. prunifolia* resulting in drought tolerance in the sensitive genotype (Li et al., 2015).

In this study the effect of melatonin on the phenotype and transcriptome of *Arabidopsis* rosette leaves was investigated in the absence and presence of melatonin applied exogenously through roots in the growth medium. First, the impact of a melatonin biosynthetic gene *(AtSNAT1)* T-DNA insertion mutation was analysed to characterize the role of AtSNAT1 in plant growth, development and gene expression. Secondly, 29 geographically diverse *Arabidopsis* ecotypes were screened for their phenotypic response to melatonin to identify the most extreme melatonin responsive ecotypes and then to further analyse their transcriptomes. Overall, two distinct hypotheses were tested: 1) AtSNAT1 determines growth patterns in *Arabidopsis* in presence and absence of applied melatonin; 2) *Arabidopsis* ecotypes have different transcriptomes in response melatonin. Altogether, the study addresses the effect of melatonin across a range of genotypes.

3.3 Results

3.3.1 Phenotypic response to melatonin in *atsnat1* and wild-type (Col-0)

A range of melatonin concentrations (5, 20, 50, 200 and 400 µM) was assessed in T-DNA insertion mutant line *atsnat1* (SALK_020577, background Col-0 ecotype) and Col-0 to quantify differences in projected rosette area as a parameter for the melatonin phenotypic response (MPR). Seeds were germinated on agar containing melatonin and the seedling assessed after 12 days. The concentrations were chosen based on literature search where 5-50 µM has been reported to enhance overall seedling biomass and higher concentrations have shown to inhibit rosette growth in *Arabidopsis* (Col-0) to delay onset of endoreduplication (Bajwa et al., 2014, Hernández et al., 2015, Wang et al., 2017). The rosette tissue was studied (as projected rosette area) to analyse the systemic effects of melatonin application through roots as reported in Chapter 2 (Zia et al., 2019). In the current study, it was observed that under control conditions (no melatonin), *atsnat1* showed significantly reduced projected rosette area as compared to Col-0 ($p<0.05$) (Figure 3.1). This finding is in line with a study which demonstrated that *atsnat1* showed smaller leaf sizes as compared to the WT (Lee et al., 2015). However, melatonin treatments from 5 – 200 µM did not result in a significant rosette growth difference, compared to no-melatonin control for each genotype. In *atsnat1* mutant, melatonin treatment (20 µM) resulted in statistically significantly higher growth compared to other melatonin concentrations but was still not significantly different to the no-melatonin control. The highest concentration of melatonin (400 µM) led to a significant response in both genotypes with reduced rosette growth compared to their respective controls. The pairwise statistical comparison of fold changes of treatment over control between genotypes (Col-0 versus *atsnat1*) revealed no significant differences in the degree of reduced rosette growth (relative ratios) in response to melatonin (400 µM) compared to the no-melatonin controls between Col-0 (0.22 ± 0.09) and *atsnat1* (0.21 ± 0.04). This indicated that both Col-0 and *atsnat1* responded similarly to melatonin at higher concentration. Notably, all melatonin concentrations did not affect germination in both WT and *atsnat1*. The melatonin concentration (400 µM) was selected for further experiments as it was the only concentration effective in inducing a statistically significant phenotypic response in Col-0 and *atsnat1*.

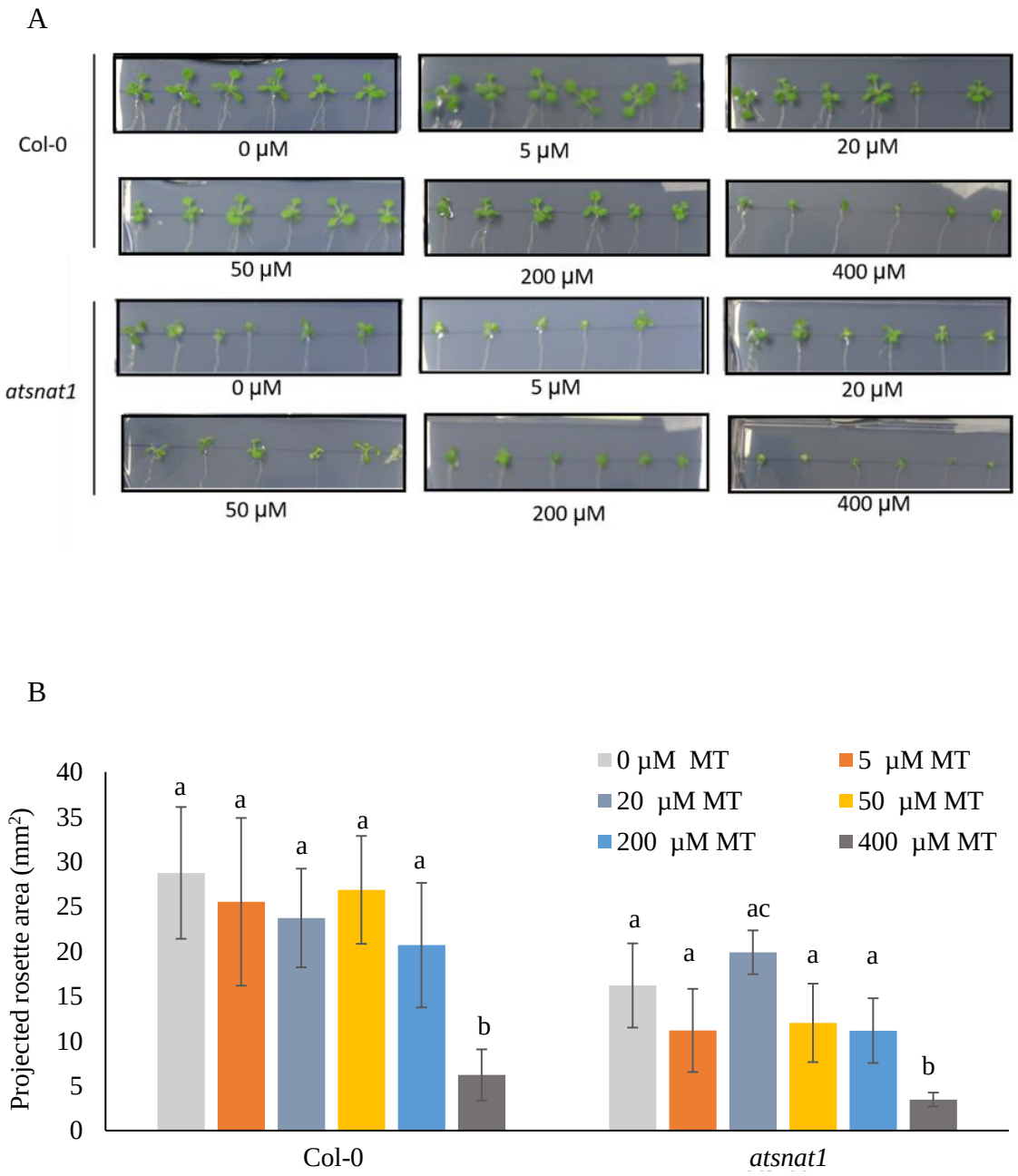


Figure 3.1: Phenotypic response of Col-0 and *atsnat1* to melatonin (MT) concentrations.

Sterilised seeds were directly germinated on +/- melatonin-supplemented ½ MS (Murashige and Skoog) medium for 12 days. All treatments including control contain 0.2% ethanol (EtOH 0.2% v/v) as a solvent for melatonin. Bars represent mean projected rosette area (mm^2). n=12 seedlings. Error bars represent standard deviation. ± in the text refers to standard deviation. Pairwise comparisons of values in response to melatonin treatments for each genotype were compared to its untreated control separately in a one-way ANOVA followed by Games Howell post-hoc test. The pairwise analysis of fold change (treatment over control) between genotypes for 400 µM MT was determined by a student's t-test. Different letters denote statistical significance among each genotype.

3.3.2 Transcriptome differences between *atsnat1* and Col-0 without melatonin treatment

The global transcript profiles of Col-0 and *atsnat1* were compared by RNA-sequencing using rosette leaves from seven-day old plants grown for three days on media with no melatonin. The data were analysed using a threshold criteria of cut-off at false discovery rate (FDR) adjusted probability p-value < 0.05 and a $\log_2$ fold change ≥ 1.2. No further arbitrary thresholds to limit the low expressed genes were employed in order to avoid any data bias and to be able to identify the low expressed genes with potential biological significance (Raithel et al., 2016, Rahmati Ishka et al., 2018). In the absence of melatonin 111 genes displayed significantly different expression (based on the threshold criteria) between *atsnat1* and Col-0 (Table 3.1a). Among these differentially expressed genes, 73 were downregulated (denoted as minus sign (-)) and 38 upregulated in the *atsnat1* mutant as compared to Col-0. These included large $\log_2$ fold differences ranging from 11.01 to -10. The $\log_2$ fold change values are indicated in brackets for genes mentioned next. The genes with large $\log_2$ fold differences in upregulated expression included: *AT2G01422* (*other RNA*) (11.01); *AT1G53480* (*Mto1 responding down, MRD1*) (9.77); *AT1G72290* (*Kunitz-protease inhibitor 1, KTI2*) (8.98); *AT1G08037* (*long noncoding RNA, lncRNA*) (7.43); *AT1G64795* (encoding a hypothetical protein) (7.25); and *AT1G26390* (encoding a FAD-binding Berberine family protein, *FOX2*) (6.86). The genes with large $\log_2$ fold differences in downregulated expression were: *AT3G08785 (antisense RNA*) (10); *AT3G01505 (antisense long noncoding RNA* (-9.98); *AT5G51190 (Ethylene response factor 105, ERF105*) (-8.35); *AT2G07475 (antisense long noncoding RNA*) (-8.22); (*AT3G17520* (encoding a late embryogenesis abundant protein, *LEA*) (-7.06); *AT4G28755* (encoding an hypothetical protein) (-7.03). Moreover, among the top 10 significantly differentially expressed genes (FDR adjusted p-value < 0.0001) and large differences in expression levels (based on raw read counts) in *atsnat1* compared to Col-0 comprised: 1) AT1G07887 (*antisense long non-coding RNA, lncRNA*) (7.39); 2) *AT1G32070* (*serotonin N-acetyltransferase, AtSNAT1*) (- 2.70); 3) *AT3G05640 (E-growth regulating1, EGR1*) (-1.49); 4) *AT5G11330* (- 1.42); 5) *AT2G42540* (cold-regulated 15, *COR15A*) (-2.15); 6) *AT4G28755* (*Responsive to dessication 29A, RD29A*) (-1.73); 7) *AT4G28755* (gene encoding an hypothetical protein) (-7.03); 8) *AT2G42530* (*cold-regulated 15B, COR15B*) (-1.98); 9) *AT1G67105* (other_RNA) (-2.24); and 10) *AT1G07737* (*antisense_lncRNA*) (-1.46). Notably, *AT1G32070 (serotonin N-acetyl transferase, AtSNAT1)* has a significantly

lower expression (-2.70) in *atsnat1* as compared to Col-0 further indicating the robustness of the data and complementing the qRT-PCR analysis (Figure S3.1).

Table 3.1 Transcriptomic pairwise comparisons of *atsnat1* and Col-0 rosette leaf transcriptomes under varying exposure of roots to melatonin (MT).

	Comparisons	Total number of significantly differentially expressed genes* (% of 24,621 quantifiable genes)	Number of differentially expressed genes with abundance changes (% of total changes)	
			Increased	**Decreased**
a)	*atsnat1* (0 µM MT) vs Col-0 (0 µM MT)	111 (0.45%)	38 (34%)	73 (66%)
b)	Col-0 (400 µM MT) vs Col-0 (0 µM MT)	482 (2%)	204 (42%)	278 (58%)
c)	*atsnat1* (400 µM MT) vs *atsnat1* (0 µM MT)	630 (3%)	347 (55%)	283 (45%)
d)	*atsnat1* (400 µM MT) vs Col-0 (400 µM MT)	374 (1.5%)	210 (56%)	164 (43%)

* Genes classified as statistically significantly different according to the threshold criteria of FDR adjusted p value < 0.05 and a $\log_2$ fold change ≥ and ≤ 1.2. Gene expression differences in **a)** *atsnat1* compared to Col-0 under no-MT control conditions (0 µM MT **b)** Col-0 treated with MT (400 µM MT) compared to no-MT control conditions (0 µM MT) **c)** *atsnat1* treated with MT (400 µM MT) compared to no-MT control conditions (0 µM MT) **d)** *atsnat1* treated with MT (400 µM MT) compared to Col-0 treated with MT (400 µM MT).

3.3.3 Transcriptome differences between *atsnat1* and Col-0 with melatonin treatment

The transcriptome of rosette leaves in *atsnat1* was significantly more impacted by melatonin (400 µM) by a 1.3-fold increase in the total number of DEGs compared to total DEGs in Col-0 (Table 3.1 b, c). In contrast to the relatively smaller transcriptome differences (i.e. 111) between *atsnat1*and Col-0 under no-melatonin control conditions, there were 3.4 times more DEGs (i.e. 374 DEGs) in response to 400 µM melatonin (Figure 3.2, Table 3.1d). Among the total DEGs in *atsnat1*, 61% of the upregulated and 58% of the downregulated DEGs were unique to *atsnat1* (Figure 3.2).

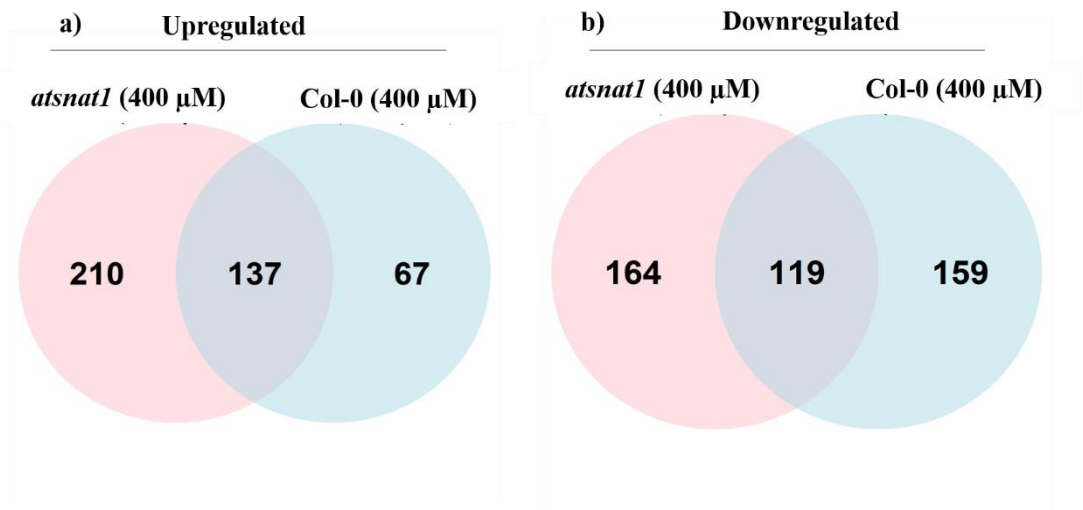


Figure 3.2: Venn diagrams showing number of significant melatonin responsive-differentially expressed genes (DEGs) that are unique to *atsnat1* or Col-0 and/or shared between *atsnat1* and Col-0 as compared to their respective no-melatonin controls. Genes classified as differentially expressed according to the threshold criteria FDR adjusted p- value < 0.05 and a $\log_2$ fold change $\geq$ 1.2.

3.3.4 Differentially expressed transcription factors in *atsnat1* compared to Col-0 in response to melatonin

Dramatic differences in the number of transcription factors (TFs) were observed between *atsnat1* and Col-0 in response to melatonin. A total of 23 TFs were uniquely expressed in *atsnat1* upon melatonin treatment as compared to Col-0. Twelve of the TFs were upregulated in expression and 11 downregulated, all of which largely comprised the TF families of NAC and ERF, bHLH and WRKYs (Table 3.2). Moreover, melatonin treatment regulated TFs in the mutant with functions that are known to be associated with melatonin including flowering (*AT2G39250 Schnarchzapfen*, *SNZ*); (*AT5G62430*.1, Cycling *Dof factor 1, CDF1*), root growth (*AT3G11260, Wuschel related homeobox* 5, *WOX5*), biotic stress in the form of pathogens (*AT2G25000 WRKY DNA-BINDING PROTEIN 60, WRKY60*) and abiotic stress tolerance related to high light, cold stress, (*AT5G59820, ZAT12*), oxygen (*AT3G10040, Hypoxia response attenuator 1, HRA*) and iron deficiency responses (*AT2G47520, Ethylene response factor 71, ERF071*) (Table 3.2).

Table 3.2: Transcription factors uniquely expressed in *atsnat1* in response to melatonin (400 µM) compared to no-melatonin control

Gene ID	Gene name/Description	Log2 fold change	p-value
NAM (NO APICAL MERISTEM, PETUNIA) ATAF 1-2 (ARABIDOPSIS THALIANA ACTIVATING FACTOR) AND CUC2 (CUP-SHAPED COTYLEDON) (NAC)			
AT3G44350	*ANAC061, NAC DOMAIN CONTAINING PROTEIN 61*	2.85	2.53E-02
AT1G01010	*NAC001, NAC DOMAIN CONTAINING PROTEIN 1*	2.11	1.17E-13
AT5G18270.1	*ANAC087, ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 87*	1.25	6.26E-03
ETHYLENE RESPONSIVE FACTOR (ERF)			
AT2G47520	*ERF071, ETHYLENE RESPONSE FACTOR 71*, encodes a member of the ERF (ethylene response factor) subfamily B-2 of ERF/AP2 transcription factor family. It plays a role in hypoxia-induced root slanting.	2.21	3.12E-03
AT5G13330	*ERF113*, encodes a member of the ERF (ethylene response factor) subfamily B-4 of ERF/AP2 transcription factor family.	1.82	2.63E-06
AT1G06160	*ERF094, ETHYLENE RESPONSIVE FACTOR 59*	1.56	1.20E-10
AT1G03800	*ERF10, ERF DOMAIN PROTEIN 10, encodes a member of the ERF (ethylene response factor) subfamily B-1 of ERF/AP2 transcription factor family (ATERF-10)*	-1.38	1.07E-02
BASIC-HELIX-LOOP-HELIX (BHLH)			
AT5G04150.1	*BHLH101*, encodes a member of the basic helix-loop-helix transcription factor family protein. It likely regulates genes involved in the distribution of iron within the plant.	1.49	6.13E-03
AT5G41315	*GL3, GLABRA 3*, encodes a basic helix loop helix domain protein that interacts with GL1 in trichome development. GL3 interacts with JAZ and DELLA proteins to regulate trichome initiation.	-2.53	1.35E-02
WRKY			
AT5G22570	*WRKY38, WRKY DNA-BINDING PROTEIN 38*, member of WRKY Transcription Factor; Group III WRKY	-4.25	3.78E-02
AT5G43290	*WRKY49, WRKY DNA-BINDING PROTEIN 49*, member of WRKY Transcription Factor; Group II-c WRKY	-1.38	4.63E-03
AT2G25000	*WRKY60, WRKY DNA-BINDING PROTEIN 60, pathogen-induced transcription factor*.	-1.35	2.93E-02
(MYELOBLASTOSIS) MYB and MYB-RELATED			
AT5G16600.1	*MYB43, MYB DOMAIN PROTEIN 43*, encodes a putative transcription factor (MYB43).	-2.45	1.54E-03
AT5G37260	*RVE2, REVEILLE 2*, encodes a MYB family transcription factor Circadian 1 (CIR1). It is involved in circadian regulation in *Arabidopsis*.	1.65	0.00E+00
AT5G06800	It encodes a myb-like HTH transcriptional regulator family protein	-1.33	1.87E-02

Table 3.2: contd.

Gene ID	Gene name/Description	Log2 fold change	p-value
Miscellaneous			
AT3G11260	*WOX5, WUSCHEL RELATED HOMEOBOX 5*, required for quiescent center (QC) function and columella stem cell maintenance in the root meristem	2.79	2.56E-04
AT2G38250	*GT-3B,* TRI-HELIX transcription factor, encodes a homeodomain-like superfamily protein	2.34	2.52E-08
AT5G59820	*ZAT12,* encodes a zinc finger protein involved in high light and cold acclimation.	2.19	0.00E+00
AT4G34590	*BZIP11, BASIC LEUCINE-ZIPPER 11,* encodes a basic domain leucine zipper (bZip) transcription factor bZIP11, directly regulates gene expression of ASN1 and ProDH2, which are enzyme-coding genes involved in amino acid metabolism.	1.52	5.44E-12
AT5G62430.1	*CDF1, CYCLING DOF FACTOR 1,* Dof-type zinc finger domain-containing protein. It represses expression of Constans (CO), a circadian regulator of flowering time.	-1.58	1.77E-04
AT2G39250	*SNZ, SCHNARCHZAPFEN,* encodes an AP2 domain transcription factor that can repress flowering.	-1.43	4.28E-05
AT1G26960	*ATHB-23, HOMEOBOX PROTEIN 23,* encodes a homeodomain leucine zipper class I (HD-Zip I) protein. It participates in the gene regulatory network controlling root branching by mediating the regulation of LAX3 by ARF7/19.	-1.34	6.63E-04
AT3G10040	*HRA1, HYPOXIA RESPONSE ATTENUATOR1, a low oxygen-inducible transcription factor*	-1.24	7.55E-07

3.3.5 Natural variation in phenotypic response of ecotypes to melatonin in *Arabidopsis*

The presence and extent of phenotypic natural variation in response to melatonin was assessed in *Arabidopsis.* A total of 29 ecotypes were selected for screening based on their geographical diversity. The geographical and climatological information of the 29 ecotypes tested is detailed in Table S3.1. A strongly observable melatonin-associated phenotype of *Arabidopsis* plants in vitro is reduced rosette growth observed at higher concentrations (200 µM and above) (Bajwa et al., 2014, Hernández et al., 2015, Wang et al., 2017). Quantification of projected rosette area resultant from roots exposed to a higher concentration of melatonin (400 µM) was to ensure a clear phenotypic response to melatonin to efficiently screen the large set of ecotypes.

The bioassay revealed significant differences in terms of reduced projected rosette area (mm^2) among ecotypes in their response to melatonin (400 µM). All 29 ecotypes had significantly reduced projected rosette area with respect to absolute values in response to melatonin as compared to their no-melatonin controls (Figure S3.2). Based on the two-way analysis of variance (ANOVA, F-value 3.106) significant (p=0.037*). This revealed the extensive presence of natural variation in response to melatonin. Upon further pairwise post-hoc analysis of the relative projected rosette area (i.e. ratio of response to melatonin relative to untreated control, with normalisation to Col-0 as a reference ecotype on the same plate), ecotypes with varying degrees of responses to melatonin were observed which helped in identifying the ecotypes with moderate responsiveness to the most responsive (Figure 3.3). Two classes of ecotypes could be identified, one with moderate response to melatonin in terms of rosette growth and the second set with strong response to melatonin resulting in significantly less rosette growth. It was observed that Bur-0, Kondara and Lp2-2 had moderate response to melatonin in terms of projected rosette area and were statistically significantly different to Nfa-10, Gd-1, WS-2, Bl-1, Wl-0, Wa-1, Rrs-7, Bor-4, Nfa-8, Hn-0 and Ler-1 which were most responsive to melatonin with reduced rosette growth as indicated by p-values in Table S3.2. The ecotypes Rrs-7, Nfa-8, Hn-0 and Ler-1 were among the most responsive ecotypes that were significantly different to Col-0, implying that these ecotypes had significantly reduced rosette growth than Col-0 and were more responsive to melatonin than Col-0. One ecotype from each class of responsiveness, that different significantly in their response to melatonin were selected, i.e. Bur-0 (moderate response) and Hn-0 (high response), along with Col-0 as a reference ecotype (moderate response) for transcriptomic analysis. Along with statistical analysis, literature search was conducted before choosing the ecotypes, Bur-0, Col-0 and Hn-0 for further

experiments. The literature search results revealed that there are recombinant inbred lines (RIL) populations generated from Bur-0 crossed with Col-0 as useful comparative genetic resources. These have been extensively exploited in previous studies for quantitative trait loci (QTL) mapping to study diverse traits such as club root resistance, shoot growth, cold tolerance, cadmium tolerance, flowering time, stem lignin content and cell wall digestibility, seed size, and expression trait variation (Jubault et al., 2008, Simon et al., 2008, Brachi et al., 2010, Gery et al., 2011, Herridge et al., 2011, Chavigneau et al., 2012, Cubillos et al., 2012). The basis of selecting Hn-0 was statistically significant difference with the reference ecotype Col-0. A stronger probability value (p-value=0.001) was shown by Hn-0 than other high responsive ecotypes such as Ler-1 (p=0.019) (Table S3.2).

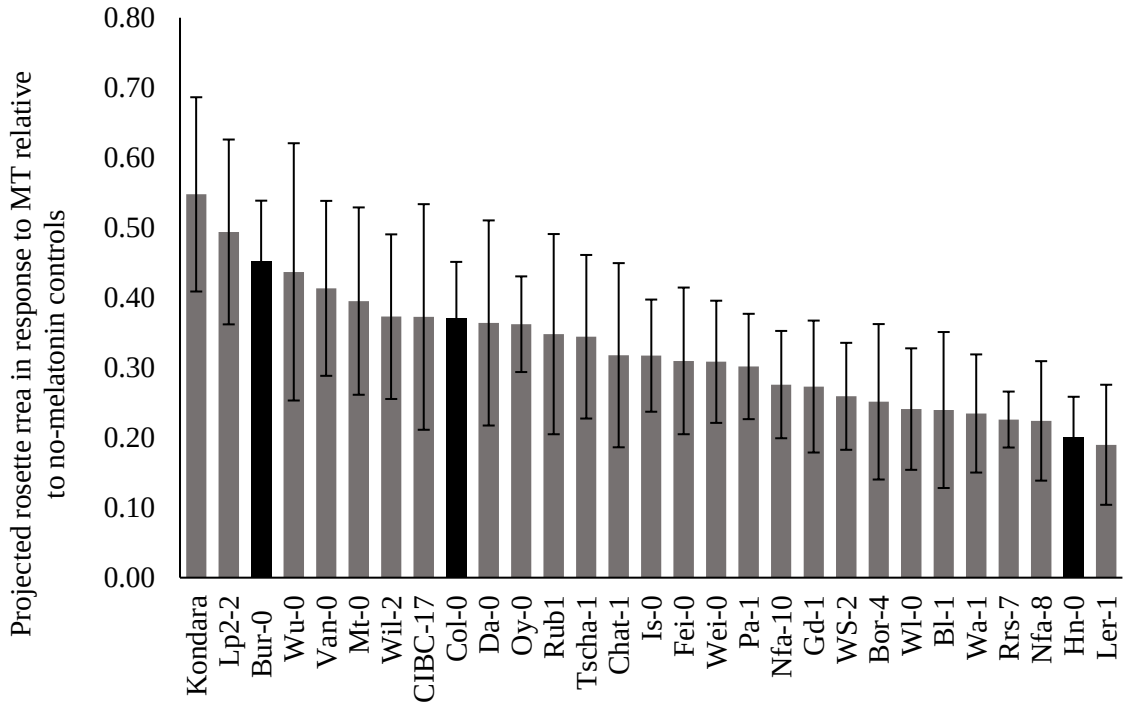


Figure 3.3: Natural variation in phenotypic response of *Arabidopsis* ecotypes to melatonin (400 µM). Phenotypic responses to melatonin in 29 ecotypes were determined for relative projected rosette area in 12-day old-seedlings (n=8-12 seedlings) directly germinated on +/- MT-supplemented growth ½ MS agar media. Each ecotype was grown with Col-0 in the same Petri dish as a reference ecotype for normalisation. Relative projected rosette area was calculated as = Ratio of ecotype x (400 µM)/Col-0 same plate (400 µM) divided by Ratio of ecotype x (0 µM)/Col-0 same plate (0 µM). Bars indicate mean of relative projected rosette area. Error bars represent standard deviations. For simplicity, the p-values of all combinations of pairwise analyses are indicated in Table S3.2. Ecotypes selected for subsequent transcriptome analysis by RNA-Sequencing are highlighted in black. Experiments repeated twice with similar results. P-values of pairwise analyses are shown in table S3.2. Data of absolute projected rosette area (mm^2) are shown in (Figure S3.2).

3.3.6 Total number of differentially expressed genes (DEGs) in response to melatonin in Bur-0, Col-0 and Hn-0

The total rosette leaf transcriptome of the selected ecotypes, Bur-0, Col-0 and Hn-0 were analysed in response to melatonin (400 µM) to identify the underlying molecular mechanisms that are likely shaping their phenotypic response to melatonin. Transcriptional responses to melatonin relative to no-melatonin controls among ecotypes revealed abundance changes in a range of transcripts (Figure 3.4). In terms of the total DEGs numbers, the data matched the trend observed in the phenotypic analysis. Hn-0, being the most responsive ecotype to melatonin, had the highest number of total DEGs of 501, with marginal difference in total numbers with Col-0 (492 DEGs) and Bur-0 being the ecotype with less responsiveness to melatonin showed least number of total changes corresponding to 324. The principal component analysis (PCA) compared the data from each ecotype with all the control samples and melatonin-treated samples for all the total DEGs, irrespective of the log2 fold changes (Figure S3.3). The analysis indicated that the gene expression patterns of Hn-0, at control and melatonin treatments, were most different to Col-0 and Bur-0.

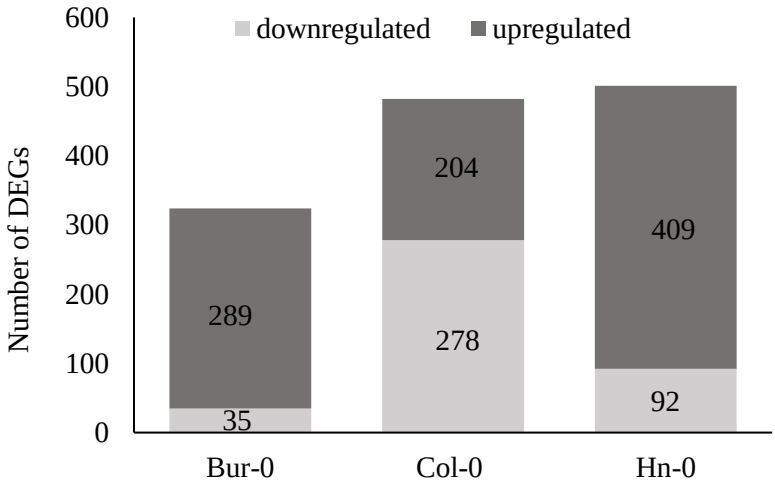


Figure 3.4: Total number of differentially expressed genes (DEGs) in rosette tissue of *Arabidopsis* ecotypes (Bur-0, Col-0 and Hn-0) in response to three days of root exposure to melatonin (400 µM) relative to no-melatonin controls. Genes called as differentially expressed using a threshold criteria of cut-off at false discovery rate (FDR) adjusted probability p-value < 0.05 and a $\log_2$ fold change $\geq$ 1.2.

3.3.7 Common and specific differentially expressed genes (DEGs) in response to melatonin in ecotypes, Bur-0, Col-0 and Hn-0

A total of 118 DEGs were common between all three ecotypes, Bur-0, Col-0 and Hn-0 among which large proportion was upregulated in expression (91%) compared to downregulated DEGs (9.3%) (Figure 3.5). The commonalities were also present between each pair of ecotypes. For example, Bur-0 had a total number of 223 common DEGs (including between either Col-0, Hn-0 and both) out of which 91% were upregulated and 9% downregulated. Col-0 showed a total number of 193 common DEGs (including between either Bur-0, Hn-0 and both) out of which 80% were upregulated and 20% downregulated. Lastly, Hn-0 exhibited a total number of 264 common DEGs (including between either Bur-0, Col-0 and both) out of which 84% were upregulated and 16% downregulated. Ecotype dependent changes were also observed in the number of DEGs for the three ecotypes in response to melatonin compared to their respective no-melatonin controls (Figure 3.5). In total, 289 DEGs were unique to Col-0, out of which only 17% (i.e. 50 DEGs) had upregulated expression and 83% had downregulated expression (i.e. 239). A total of 101 DEGs were unique to Bur-0 out of which 86% (i.e. 87 DEGs) had upregulated expression and only 11% (i.e. 14 DEGs) had downregulated expression. In case of Hn-0, a total of 237 DEGs were uniquely expressed, out of which 78% (i.e. 186 DEGs) were upregulated in expression compared to 22% (i.e. 51 DEGs) with downregulated expression. In general, there were more common transcripts among the ecotypes than unique ones. The notable exception was Col-0 which had a higher number (i.e. 239) of unique downregulated DEGs than commonly downregulated DEGs (i.e. 39).

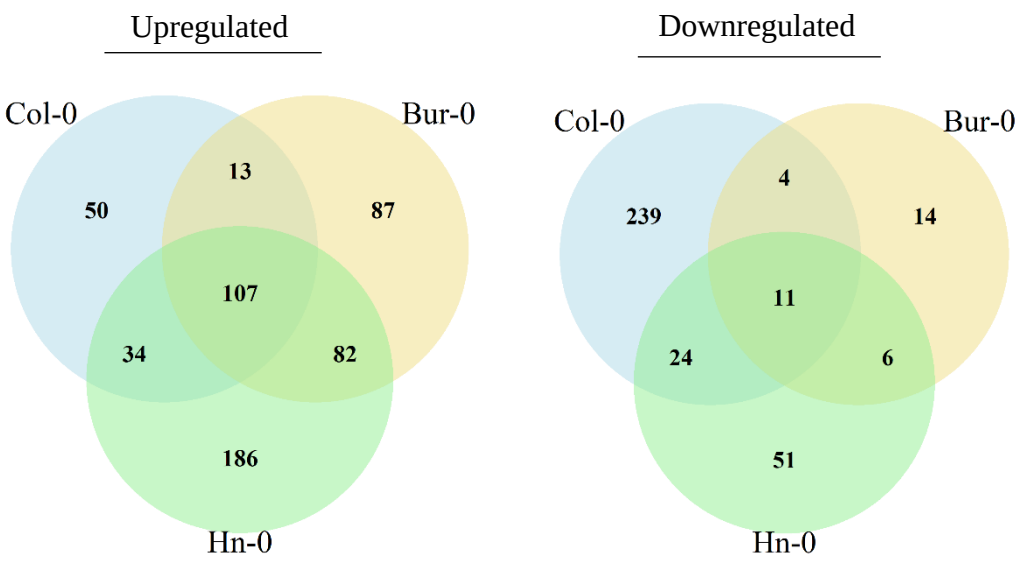


Figure 3.5: Triple-Venn diagrams showing unique and common DEGs among *Arabidopsis* ecotypes Bur-0, Col-0 and Hn-0 in response to melatonin (400 µM). Up- and downregulated expression resulting from rosette tissue grown for three days on melatonin are shown on the left and right, respectively. DEGs in response to melatonin were normalised to the no-melatonin controls for each ecotype.

3.3.8 Gene Ontology (GO) enriched terms and transcription factors (TFs) in commonly expressed genes in response to melatonin in Bur-0, Col-0 and Hn-0

A GO enrichment analysis was performed in the list of common DEGs (including both upregulated and downregulated) in response to melatonin between the three ecotypes (Table 3.3). These included a total of four GO parent terms that were commonly enriched between Bur-0, Col-0, and Hn-0. These were largely related to phenylpropanoid metabolic process, responses to decreased oxygen levels (hypoxia) and responses to biotic stress defence. Melatonin treatment also provoked a similar transcriptional response among ecotypes to that of organic signalling compounds, karrikins. The GO analysis reveals that phenylpropanoid metabolic process, response to karrikins, cellular response to hypoxia and defence response to other organisms are common responses elicited by melatonin irrespective of the type of ecotype. However, to avoid any bias in data interpretation, the GO enrichment analysis was also extended to each ecotype's specific transcriptome in response to melatonin (Table 3.5). While no common GO terms between all three ecotypes

were identified through this approach, it led to a thorough teasing apart of more exact and related common GO enriched terms between individual pairwise comparisons of ecotypes. For example, Bur-0 shared the related GO terms “response to water deprivation” and “response to dessication” with Col-0 and shared the exact GO term “response to abscisic acid (ABA) with Hn-0. The GO terms “response to insect” and “regulation of defence response” were shared between Col-0 and Hn-0. The related terms of “response to reactive oxygen species” and “response to oxidative stress” were also common between Col-0 and Hn-0.

The commonly up and downregulated DEGs across the ecotypes consisted of 10 TFs with varied functions (Table 3.4). The common and similar pattern of gene expression of these TFs among ecotypes indicates the downstream genes regulated by these TFs govern the common melatonin response. Furthermore, an overlap analysis of the previously identified uniquely DEGs (28) in Col-0 in response to melatonin at relatively lower concentration (100 µM) (Chapter 2) with the current data revealed 46% overlap (Table S2). Interestingly, the 46% comprised genes that were common with not only Col-0 but also other ecotypes, Bur-0 and Hn-0 with a similar expression pattern.

Table 3.3: Significant Gene Ontology (GO) parent terms enriched for common DEGs in response to melatonin (400 µM) among Bur-0, Col-0 and Hn-0 compared to their respective no-melatonin controls.

GO biological process parent terms	# genes	Fold enrichment	p-value
Shared			
phenylpropanoid metabolic process (GO:0009698)	6	13	2.59E-02
response to karrikin (GO:0080167)	6	11.68	4.64E-02
cellular response to hypoxia (GO:0071456)	10	10.65	1.48E-04
defense response to other organisms (GO:0098542)	17	5.55	3.82E-05

Table 3.4: Transcription factors commonly up and downregulated in expression across ecotypes, Bur-0, Col-0 and Hn-0 in response to melatonin (400 µM) relative to their respective no-melatonin controls.

Gene ID	**Gene name/Description**	**Log2 fold change**					
		Bur-0	**p-value**	**Col-0**	**p-value**	**Hn-0**	**p-value**
AT5G07100	*WRKY26, WRKY DINA-BINDING PROTEIN 26*	1.90	0.00E+00	1.60	2.47E-12	1.25	9.12E-08
AT2G16720.1	*MYB7, MYB DOMAIN PROTEIN 7*	3.77	0.00E+00	2.45	1.95E-06	3.61	0.00E+00
AT3G46090	*ZAT7*, C2H2 and C2HC zinc fingers superfamily protein	4.46	0.00E+00	2.42	0.00E+00	7.71	0.00E+00
AT4G00870	*BHLH14*, interacts with JAZ proteins, and functions redundantly with bHLH3, bHLH13 and bHLH17 to negatively regulate jasmonate responses.	2.28	8.60E-04	2.80	8.54E-03	2.32	1.12E-02
AT1G35515	*MYB8*, high response to osmotic stress	3.64	9.78E-04	3.72	1.93E-08	3.93	1.63E-02
AT3G51910.1	*HSFA7A, HEAT SHOCK TRANSCRIPTION FACTOR A7A*	1.54	7.31E-12	1.60	7.92E-08	2.12	0.00E+00
AT5G59570	*BOA, BROTHER OF LUX ARRHYTHMO*, a component of the circadian clock. It binds to specific sites on CCA1 promoter leading to CCA1 activation	1.50	1.30E-12	1.71	0.00E+00	1.27	3.29E-10
AT4G36990	*HSFB1, CLASS B HEAT SHOCK FACTOR B1*	2.59	0.00E+00	1.76	6.74E-08	3.18	2.75E-12
AT4G38620.1	*MYB4*, encodes a R2R3 MYB protein which is involved in the response to UV-B.	2.80	0.00E+00	2.61	0.00E+00	3.47	0.00E+00
AT5G39860	*PRE1, PACLOBUTRAZOL RESISTANCE1, BASIC HELIX-LOOP-HELIX PROTEIN 136* integrates multiple signalling pathways to regulate cell elongation and plant development.	-2.00	3.54E-06	-1.88	3.23E-04	-1.72	1.26E-04

3.3.9 Gene Ontology (GO) enriched terms and transcription factors (TFs) in uniquely expressed genes in response to melatonin in Bur-0, Col-0 and Hn-0

Gene ontology (GO) enrichment analysis was conducted on the DEGs unique to Bur-0, Col-0 or Hn-0 in order to determine the classes of genes uniquely responding to melatonin in each ecotype (Table 3.5). Enrichment in transcripts related to GO terms categories exhibited by individual ecotypes was observed, e.g. Col-0 exhibited enrichment in transcripts related to GO parent term categories which exhibited fold enrichment relative to the genome ranging from 3.42 to 27.43-fold. These included terms such as: "glucosinolate biosynthetic process (GO:0019761)"; "response to jasmonic acid (GO:0009753)"; "regulation of jasmonic acid mediated signalling pathway (GO:2000022)". The GO terms related to melatonin precursors were enriched 12 to 17-fold. These were "tryptophan metabolic process (GO:0006568)" and "indole-containing compound biosynthetic process (GO:0042435)". Overall, 57% of total GO enriched terms in Col-0 were stress-related (Table 3.5).

The relatively less responsiveness to melatonin in Bur-0 was further revealed by only two enriched GO parent terms. These were "response to water deprivation (GO:0009414)" and "response to abscisic acid (GO:0009737)". In contrast, Hn-0 revealed diverse enriched GO parent terms in response to melatonin. These showed signatures of abiotic and biotic stress responses (about 74% of enriched GO terms were related to stresses) such as oxidative stress, light stress and salicylic acid-activated biotic defence processes, such as systemic acquired resistance (SAR). The ability to synthesize secondary metabolites was also provoked by melatonin in Hn-0 as seen by the enrichment of GO term "secondary metabolite biosynthetic process (GO:0044550)". These responses provide further insight into the highest responsiveness of Hn-0 to melatonin. Notably, as mentioned in section 3.3.8, there were some overlapping GO terms observed in unique gene sets. These have been flagged by colour text and not considered a part of uniquely enriched GO terms (Table 3.5).

In order to determine whether the differences in response to melatonin across ecotypes was contributed by differential expression of transcription factors, a focused comparison of melatonin-dependent transcript changes for transcription factors was conducted (Table 3.6). There were a total of 23 transcription factors in the DEGs unique to Col-0 in response to melatonin. These consisted of three TFs with upregulated expression which were *AT3G12820 MYB10,* member of the R2R3 factor gene family, *AT1G78600 LZF1, LIGHT-REGULATED ZINC FINGER PROTEIN 1*, and *AT5G51790 bHLH, BASIC HELIX-LOOP HELIX*. Among the 20 TFs with downregulated expression included TFs belonging to

classes of MYB (MYELOBLASTOSIS), ERF (ETHYLENE RESPONSE FACTORS), NAC (NAM, ATAF, and CUC), BHLH (BASIC HELIX-LOOP-HELIX) and HSF (HEAT-SHOCK FACTORS). The expression of fewer TFs (five) was regulated in Bur-0 which provides an indication of the fewer number of downstream transcriptome differences as observed by total DEGs in response to melatonin. Three of the TFs were related to drought and ABA responses which were *AT2G46680 ATHB-7, ARABIDOPSIS THALIANA HOMEO-BOX 7, RD26, RESPONSIVE TO DESSICATION 27* and *DREB1B, C-REPEAT-DRE BINDING FACTOR 1*. Response to melatonin in Bur-0 induced expression of the TFs which might result in downstream responses of drought and ABA-related genes. This is evident by the enrichment of these responses in terms of GO parent terms in Bur-0 (Table 3.5). Hn-0 exhibited a range of TFs in response to melatonin which included TF families such as MYB, WRKY and BHLH. The TFs related to biotic stress defence, such as *WRKY70* and *WRKY18,* were upregulated in expression. This indicates that melatonin response in Hn-0 primarily elicits biotic stress defence-related changes.

Table 3.5: Significant Gene Ontology (GO) enriched parent terms for total DEGs (up and downregulated) uniquely to ecotypes Bur-0, Col-0 and Hn-0 in response to melatonin (400 µM) compared to their no-melatonin controls.

GO biological process parent terms	# genes	Fold enrichment	p-value
Bur-0 (2)			
response to water deprivation (GO:0009414)*	11	9.86	5.83E-05
response to abscisic acid (GO:0009737)*	11	6.39	3.90E-03
Col-0 (14)			
response to desiccation (GO:0009269)*	5	27.43	7.54E-03
glucosinolate biosynthetic process (GO:0019761)	10	26.73	8.27E-08
regulation of jasmonic acid mediated signaling pathway (GO:2000022)	10	26.06	1.03E-07
jasmonic acid biosynthetic process (GO:0009695)	6	24.06	1.41E-03
response to insect (GO:0009625)*	6	20.18	3.48E-03
tryptophan metabolic process (GO:0006568)	5	17.97	4.57E-02
response to wounding (GO:0009611)	29	14.6	2.85E-20
indole-containing compound biosynthetic process (GO:0042435)	6	12.76	3.76E-02
response to jasmonic acid (GO:0009753)	26	12.49	2.23E-16
regulation of defense response (GO:0031347)*	14	5.63	1.12E-03
response to oxidative stress (GO:0006979)*	16	4.25	5.87E-03
response to salt stress (GO:0009651)	19	4.22	7.16E-04
response to drug (GO:0042493)	18	3.75	7.68E-03
defense response (GO:0006952)	33	3.42	3.91E-06
Hn-0 (19)			
photosynthesis, light harvesting in photosystem I (GO:0009768)	5	26.73	7.61E-03
response to insect (GO:0009625)*	6	23.8	1.34E-03
induced systemic resistance (GO:0009682)	5	20.49	2.40E-02
response to far red light (GO:0010218)	6	14.46	1.83E-02
response to red light (GO:0010114)	7	12.12	9.07E-03
systemic acquired resistance (GO:0009627)	6	12.09	4.71E-02
secondary metabolite biosynthetic process (GO:0044550)	10	8.36	1.75E-03
cellular response to hypoxia (GO:0071456)	14	7.36	4.52E-05
response to reactive oxygen species (GO:0000302)*	9	7.33	1.78E-02
response to salicylic acid (GO:0009751)	12	7.16	6.41E-04
regulation of response to biotic stimulus (GO:0002831)	10	6.68	1.19E-02
response to wounding (GO:0009611)	11	6.53	4.83E-03
regulation of response to external stimulus (GO:0032101)	10	6.5	1.49E-02
response to nutrient levels (GO:0031667)	12	6.39	2.04E-03
regulation of defense response (GO:0031347)*	11	5.22	3.71E-02
response to fungus (GO:0009620)	13	4.98	1.00E-02
response to abscisic acid (GO:0009737)*	16	3.75	2.50E-02
regulation of biological quality (GO:0065008)	25	2.67	2.95E-02
cell communication (GO:0007154)	32	2.52	4.97E-03

*****exact and related common GO terms are presented in colour text

Table 3.6: Transcription factors (TF) differentially expressed in response to melatonin (MT) (400 μM) relative to no-melatonin controls in Bur-0, Col-0, and Hn-0

Gene ID	Gene name/Description	Log2 fold change	p-value
Bur-0 specific (5)			
AT2G46680	*ATHB-7, ARABIDOPSIS THALIANA HOMEO-BOX 7*, transcriptionally regulated in an ABA-dependent manner	1.33	4.88E-02
AT1G32640.1	*MYC2, JAI1, JASMONATE INSENSITIVE-1*,	1.29	1.49E-02
AT4G27410	*RD26, RESPONSIVE TO DESSICATION 27, NAC domain containing-protein 72*	1.22	4.15E-06
AT4G25490	*DREB1B, C-REPEAT-DRE BINDING FACTOR 1*, involved in response to low temperature and abscisic acid.	-2.98	1.20E-02
AT1G16490.1	*MYB58, MYB DOMAIN PROTEIN 58*, Member of the R2R3 factor gene family.	-2.40	6.99E-03
Col-0 specific (23)			
AT3G12820	*MYB10*, member of the R2R3 factor gene family.	1.71	2.74E-02
AT1G78600	*LZF1, light-regulated zinc finger protein 1*	1.58	0.00E+00
AT5G51790	*bHLH, basic helix-loop helix*	1.39	2.96E-11
AT2G41240	*BHLH100, BASIC HELIX-LOOP-HELIX PROTEIN 100*, functions as a key regulator of iron-deficiency responses	-3.54	6.50E-03
AT1G52890	*NAC019, NAC DOMAIN CONTAINING PROTEIN 19*, encodes a NAC transcription factor whose expression is induced by drought, high salt, and abscisic acid.	-3.34	5.96E-07
AT1G56650	*MYB75*, encodes a putative MYB domain containing transcription factor involved in anthocyanin metabolism and radical scavenging.	-3.22	8.59E-04
AT2G20880	*ERF053, ERF DOMAIN 53*, encodes ERF53, a drought-induced transcription factor.	-3.17	3.43E-04
AT5G43840.1	*HSFA6A, HEAT-SHOCK TRANSCRIPTION FACTOR A61*	-3.08	7.78E-03
AT4G34410	*ERF109, ETHYLENE RESPONSIVE FACTOR 109*	-2.78	2.85E-02
AT2G22200	*ERF056*, encodes a member of the DREB subfamily A-6 of ERF/AP2 transcription factor family.	-2.52	1.41E-04
AT1G18710	*AtMYB47*, member of the R2R3 factor gene family	-2.28	3.71E-04
AT5G07700.1	*MYB76, MYB-DOMAIN PROTEIN 76*, encodes a putative transcription factor (MYB76).	-2.19	1.14E-04
AT4G34530	*BHLH63*, encodes a transcription factor CIB1 (cryptochrome-interacting basic-helix-loop-helix).	-2.08	8.54E-03

Table 3.6: contd.

Gene ID	Gene name/Description	Log2 fold change	p-value
Col-0 specific			
AT4G28140	*ERF054, ETHYLENE RESPONSIVE FACTOR 54,* encodes a member of the DREB subfamily A-6 of ERF/AP2 transcription factor family. regulated by heat shock	-1.97	1.61E-02
AT5G47220	*ERF2, ETHYLENE-RESPONSE FACTOR-2*	-1.70	7.45E-04
AT5G64750	*ABR1, ABA REPRESSOR 1,* is a member of the ERF (ethylene response factor) subfamily B-4 of ERF/AP2 transcription factor family.	-1.66	8.28E-03
AT2G22770	*NAI1*, regulates the development of ER bodies. also involved in response to the endophytic fungus *Piriformospora indica.*	-1.58	2.60E-08
AT2G46270.1	*GBF3, G-BOX BINDING FACTOR 3,* encodes a bZIP G-box binding protein whose expression is induced by ABA	-1.56	1.12E-07
AT5G60890.1	*MYB34,* Myb-like transcription factor that modulates expression of ASA1, a key point of control in the tryptophan pathway	-1.41	5.30E-04
AT1G69490	*NAC029, NAC DOMAIN CONTAINING PROTEIN 9*	-1.41	1.62E-03
AT3G15500	*NAC055, NAC DOMAIN CONTAINING PROTEIN 55,*	-1.34	4.75E-04
AT1G59640	*BPE, BIG PETAL,* A basic helix-loop-helix encoding gene (BIGPETAL, BPE) involved in the control of petal size.	-1.32	1.23E-02
AT1G74430.1	*MYB95, MYB DOMAIN CONTAINING PROTEIN 95*	-1.29	8.12E-03
Hn-0 specific (21)			
AT4G39250.1	*RL1, RADIALIS-LIKE SANT/MYB 2,*	1.70	2.87E-02
AT1G27730	*ZAT10, SALT TOLERANCE ZINC FINGER*	1.96	7.13E-05
AT3G56970	*ORG2, OBP3-RESPONSIVE GENE 2, BASIC HELIX-LOOP-HELIX 38*	4.64	6.28E-12
AT5G50915	*BHLH137, BASIC HELIX-LOOP-HELIX 137,*	2.22	8.90E-05
AT3G04030	*MYR2,* encodes homeo-domain like superfamily protein	1.46	7.68E-05
AT3G56400	*WRKY70,* Member of WRKY Transcription Factor; Group III. Function as activator of SA-dependent defence genes and a repressor of JA-regulated genes.	1.68	4.02E-02
AT1G01010	*NAC001, NAC-DOMAIN CONTAINING PROTEIN 1,*	1.90	3.30E-04

Table 3.6: contd.

Gene ID	Gene name/Description	Log2 fold change	p-value
Hn-0 specific			
AT2G41240.1	*BHLH100, BASIC HELIX-LOOP-HELIX 100,* functions as a key regulator of iron-deficiency responses	5.98	0.00E+00
AT5G11260.1	*HY5, BZIP TRANSCRIPTION FACTOR HY5*	1.43	2.96E-02
AT4G22070	*WRKY31, WRKY DNA-BINDING PROTEIN 31,*	5.53	1.69E-02
AT2G46400	*WRKY46,* encodes a WRKY transcription factor that contributes to the feed-forward inhibition of osmotic/salt stress-dependent LR inhibition via regulation of ABA signalling and auxin homeostasis.	3.06	7.65E-03
AT1G21910	*ERF012,* encodes a member of the DREB subfamily A-5 of ERF/AP2 transcription factor family	1.21	2.73E-03
AT3G56980	*ORG3, OBP3-RESPONSIVE GENE 3,* encodes a member of the basic helix-loop-helix transcription factor protein.	4.32	3.90E-08
AT1G18570.1	*MYB51, MYB DOMAIN PROTEIN 51,* encodes a member of the R2R3-MYB transcription family. Involved in indole glucosinolate biosynthesis	1.37	7.67E-05
AT4G31800	*WRKY18, WRKY DNA-BINDING PROTEIN 18,* pathogen-induced transcription factor.	1.62	7.54E-12
AT5G59820	*ZAT12,* encodes a zinc finger protein involved in high light and cold acclimation.	2.53	1.92E-05
AT5G16600.1	*MYB43, MYB DOMAIN PROTEIN 43,* encodes a putative transcription factor	-2.32	2.10E-02
AT3G21330	*BHLH87, BASIC HELIX-LOOP-HELIX 87,*	-2.82	3.19E-02
AT1G22640.1	*MYB3, MYB DOMAIN PROTEIN 3,* MYB-type transcription factor (MYB3) that represses phenylpropanoid biosynthesis gene expression	-1.32	1.62E-03
AT5G62430.1	*CDF1, CYCLING DOF FACTOR 1,* encodes Dof-type zinc finger domain-containing protein, represses expression of Constans (CO), a circadian regulator of flowering time.	-2.66	1.32E-04
AT1G22490	*BHLH94, BASIC HELIX-LOOP-HELIX 4*	-1.49	1.18E-04

3.4 Discussion

3.4.1 Parallel comparison of wild-type (Col-0) and melatonin biosynthetic gene mutant (*atsnat1*) reveals differences in phenotype and transcriptome

A parallel comparison of Col-0 and the *AtSNAT1* knock out mutant, referred to as *atsnat1* revealed growth differences, where *atsnat1*showed significantly reduced projected rosette area as compared to Col-0 under no-melatonin control conditions. This indicates that a functional *SNAT1* gene and the ability to synthesize melatonin is likely to be important for rosette growth. Melatonin inhibits rosette growth at higher concentrations by reducing cell proliferation and endoreduplication processes. Although, the growth inhibition is to put the plant on a 'stand-by' mode as melatonin treatment increased the expression of histone and ribosomal-related genes (Hernández et al., 2015, Wang et al., 2017). The fact that *atsnat1* and Col-0 were equally responsive to high melatonin concentration (400 µM), in terms of reduced projected rosette area, indicates that melatonin is taken up by both genotypes that caused them both to respond. AtSNAT1 encoded by *AtSNAT1* gene potentially does not play a key role for the melatonin-mediated leaf development that occurs by targeting the endoreduplication processes as this process was not disrupted in the mutant.

For transcriptome analysis in this study, the duration for melatonin treatment was shortened from 12 days to 3 days to avoid any secondary growth and developmental effects arising from a longer durations and effectively capture the true melatonin response in the form of early transcriptional events upon melatonin treatment. Similarly, previous three studies assessing the effect of melatonin on transcriptome in *Arabidopsis* (Col-0) have short durations ranging from 16 hours to 3 days (Weeda et al., 2014, Wan et al., 2018, Zia et al., 2019). The approach of shortening the treatment duration to avoid any potential secondary effects has also been previously used to assess transcriptome response to auxin in *Arabidopsis* ecotypes (Delker et al., 2010).

The differences observed in the transcriptome between the *atsnat1* and Col-0 under no-melatonin control conditions are potentially essential in regulating melatonin functions. The presence of non-protein coding transcripts were abundant among the 111 genes different between *atsnat1* and Col-0. Non-coding RNAs such as microRNAs (miRNA) and long non-coding RNAs (lncRNA) are considered important regulators of gene expression in *Arabidopsis* (Guttman and Rinn, 2012, Ding et al., 2019) and computational analysis has recently revealed novel miRNA-mRNA interactions are predicted to play a role in melatonin biosynthesis in *Hypericum* species (Petijova et al., 2020).

AtSNAT1 plays an essential role in melatonin-mediated tolerance to abiotic stress such as high light and iron deficiency in *Arabidopsis* (Lee and Back, 2018, Wan et al., 2018). In

line with these findings, melatonin treatment of *atsnat1* regulated the expression levels of TFs such as those encoded by *AT5G59820* (*zinc finger protein, ZAT12)* and *AT5G04150.1* (*basic helix-loop-helix, bHLH101)* which are involved in acclimation to high light and distribution of iron in the plant, respectively (Davletova et al., 2005, Sivitz et al., 2012). Induction of TF encoded by *AT4G34590* (*BZIP11*) related to amino acid metabolism indicates that melatonin treatment likely induced downstream genes involving secondary metabolic processes in the mutant. Melatonin treatments also affected the expression levels of circadian-regulated TFs, *AT5G37260* (*Reveille 2, RVE2*) and *AT5G62430.1* (*Cycling DOF factor, CDF1*). Both these TFs play a role in repressing photoperiodic flowering (Zhang et al., 2007, Goralogia et al., 2017). It has been recently shown that AtSNAT2 (encoded by the isogene of *AtSNAT1*) plays a role in melatonin-mediated delayed flowering in *Arabidopsis* which is not dependent on the circadian-regulated pathways but on the Gibberellic acid (GA)-responsive pathways (Lee et al., 2019). It is likely that AtSNAT1 plays a role in melatonin-mediated flowering which, unlike AtSNAT2, is dependent on the circadian-regulated pathways governed by TFs (*RVE2*) and (*CDF1*). This is possible as gene isoforms in *Arabidopsis* have been shown to have distinct or specific roles (Kijima et al., 2018). The expression of *AtSNAT2* was not statistically significantly different in response to melatonin compared to no-melatonin controls in Col-0 and *atsnat1* mutant based on the RNA-Seq data (data not shown).

3.4.2 Natural variation is present in phenotypic response to root-applied melatonin

The study of natural variation in *Arabidopsis* has previously targeted those phenotypic traits that have proposed roles in stress adaptation, such as flowering time and seed dormancy (Clerkx et al., 2004, Koornneef et al., 2004, Rosas et al., 2014). However, there are integrated signalling pathways such as those of auxin that play an essential role in coordinating a multitude of developmental and environmental stimuli. Plant hormones and signalling molecules like auxin and salicylic acid (SA) have previously been used as a stimuli to serve as a model for range of responses to identify natural variation in *Arabidopsis* (Van Leeuwen et al., 2007, Delker et al., 2010). The presence of extensive natural variation in phenotypic responses to auxin (IAA, NAA and 2,4-D) in terms of root growth inhibition and hypocotyl elongation have been demonstrated in *Arabidopsis* (Delker et al., 2010). Melatonin as well has been shown to coordinate multiple growth and developmental and environmental stimuli (Bajwa et al., 2014, Shi et al., 2015b, Shi et al., 2016, Chen et al., 2020)

This study demonstrated that variation in projected rosette area growth was evident in response to melatonin (400 µM) as compared to the no-melatonin control. This observation also served as the first indication that the tested ecotypes were able to use melatonin albeit with varying degrees from the growth medium that triggered changes in their rosette phenotypes. This has been previously shown with ecotype Col-0 in Chapter 2 of this thesis (Zia et al., 2019). Two distinct clusters were identified, moderate responsive and highly responsive. Among the moderate responsive cluster is ecotype Bur-0 which has previously been shown to be tolerant to abiotic stresses such as salt (100-500 mM NaCl) and cadmium (1.5 – 2 µM $CdCl_2$) (Katori et al., 2010, Fischer et al., 2017). Bur-0 has relatively low Cd accumulation in terms of Cd content (µg/g DW) compared to Col-0, which would explain its tolerance to cadmium (Fischer et al., 2017). The moderate responsiveness of Bur-0 could be firstly due to ecotype-specific gene expression differences in response to melatonin and/or high endogenous melatonin content in Bur-0 such that external treatment did not potentially have a major effect in uptake or accumulation of melatonin. The opposite trend would be expected for highly melatonin-responsive ecotypes such as Hn-0. While the first possibility was tested further in this study by gene expression analyses by RNA-sequencing, the second possibility of endogenous melatonin levels variation could not be tested due to time and budget constraints. However, this needs to be further tested in terms of melatonin quantification.

3.4.3 Melatonin leads to certain common responses between ecotypes

A set (total 118) of genes commonly responded to melatonin across Col-0, Bur-0 and Hn-0. The overall nature of the common transcriptomic responses was revealed by the GO terms enriched in certain processes such as “cellular response to hypoxia”, “response to karrikin”, “defence response to other organisms” and “phenylpropanoid metabolic process”. Melatonin has been shown to induce the expression of phenylpropanoid pathway-related genes to improve reactive oxygen species (ROS) scavenging capacity and counteract pathogenic infection (Zhang et al., 2016, Li et al., 2019a). A direct role of melatonin in benefiting plants under a limited oxygen supply has not been shown to date. However, there is a close link between hypoxia and oxidative stress resulting in excessive ROS production in *Arabidopsis* (Pucciariello et al., 2012). Karrikins are small compounds containing a butanolide ring and are produced by wildfires (Flematti et al., 2004). They are structurally similar to plant hormones, strigolactones, and have many biological properties such as enhancing seed germination in both fire and non-fire prone environments and conferring tolerance to a range of abiotic stresses in plants (Li et al., 2017b). These are

achieved by interacting with other phytohormones such as strigolactones (Meng et al., 2016). While it is shown that melatonin signalling is antagonistic to strigolactones to mediate delay in flowering, the interaction of signalling pathways between melatonin and karrikins has not been studied. (Zhang et al., 2019). A parallel transcriptome comparison of melatonin with karrikin treatments in plants would help identifying any overlapping transcriptome responses. However, manual inspection of the GO analysis on gene lists unique to each ecotype indicated that there were some common terms between ecotypes not previously identified by the GO analysis conducted on common gene lists. This shows that the traditional GO analysis might not be sufficiently sensitive and a supplementary manual inspection is necessary as a quality check for robust biological interpretation as highlighted by (Choi et al., 2019). The common GO terms between Bur-0 and Col-0 as well as Bur-0 and Hn-0 indicate the potential role of melatonin alone or melatonin and ABA signalling in drought adaptation in both the ecotypes. The positive effect of melatonin application in alleviating drought stress has been shown in a variety of plant species and genotypes (Li et al., 2015, Sadak and Bakry, 2020). The enrichment of defence responses to biotic (insect) and oxidative stress by melatonin indicate the stress mitigating role of melatonin in both Col-0 and Hn-0 ecotypes. It has been reported in numerous studies that melatonin confers stress (abiotic and biotic) tolerance in plants by alleviating the resulting oxidative damage due to stresses (Park et al., 2013, Li et al., 2017a, Cao et al., 2018). Moreover, while the role of melatonin in enhancing plant immunity against a wide range of pathogens (bacteria, fungi and oomycetes) has been shown, a similar effect of melatonin in plant-insect interactions is yet to be explored (Shi et al., 2015a, Li et al., 2018, Mandal et al., 2018).

3.4.4 Ecotype-specific differences in transcriptional response to melatonin indicate the potential role of melatonin in local habitat adaptation

The ecotype-specific transcript changes in melatonin response suggested ecotype-dependent effects of melatonin. Relative fewer total number (324) of DEGs were regulated in the moderately responsive ecotype, Bur-0. On the other hand, the most responsive ecotype, Hn-0 exhibited an increased number of total DEGs (501) in melatonin response. These observations of differences in transcriptome responses among contrasting ecotypes are in accordance with previous studies where salicylic acid treatment resulted in an increased number of DEGs in the hyper-SA-responsive ecotype, Mt-0 as compared to the hypo-responsive ecotype, Cvi-0 (Van Leeuwen et al., 2007). The tryptophan synthesis related biological process terms “tryptophan metabolic process (GO:0006568)” and

"indole-containing compound biosynthetic process (GO:0042435)" were apparent in Col-0. This indicates that melatonin treatment potentially resulted in enhanced synthesis of melatonin (or other indolic compounds) in Col-0. This would need further analysis of endogenous melatonin content quantification. Similar to this is the case with the highly-responsive ecotype, Hn-0, which showed enrichment in GO term "secondary metabolite biosynthetic process (GO:0044550)".

In contrast, the comparatively low responsiveness of Bur-0 can be explained by enrichment of two specific biological processes that are related to water and responses to ABA. No enrichment in secondary metabolic processes in Bur-0 potentially indicates that this process is not activated by melatonin treatment. While the GO terms related to water stress response were also enriched in Col-0, these terms are the only responses enriched in Bur-0 indicating the specific response in Bur-0. The enrichment of hormone, ABA and water deficit response-related transcripts in Bur-0 might reflect the suite of melatonin-triggered changes in this particular ecotype related to water stress adaptation. This is not surprising considering the unique climatic environment of Bur-0 (Burren, Ireland) (Webb and Scannell, 1983). Bur-0 is an ecotype that has evolved and adapted to a cool and damp climate (Huang et al., 2014). It originates from Burren in Ireland which is a region of karstified limestone and unique botanical features such as unusual distribution of plant life ranging from Mediterranean to Arctic-alpine species growing together in the area (Webb, 1961, Webb and Scannell, 1983, Mcnamara and Hennessy, 2010). The ability of soils for water conservation in karst areas is extremely poor (Mcnamara and Hennessy, 2010). This means that plants growing in these regions such as Burren must have unique adaptation mechanisms. The link of water availability and plants in Burren has been previously studied in drought tolerant local plants *Teucrium scorodonia* L., *Mycelis muralis* L. Dumort and *Corrylus avellane L.* (Osborne et al., 2003). It was shown that water limitation due to poor water conservation ability in a karst landscape of Burren is a stressful factor for the native plants (Osborne et al., 2003). Interestingly, melatonin treatment uniquely enriched GO terms of genes related to water stress and water stress-induced ABA hormone in Bur-0 indicating the possibility of the specific role of melatonin in water stress adaptation in Bur-0. This is probable as melatonin treatment (100 µM) has shown to confer drought tolerance in apple species (*Malus prunifolia* and *Malus hupehensis*) by enhancing water conservation in leaves by regulating stomatal closure and reducing endogenous ABA contents (Li et al., 2015). It was also shown recently that melatonin-regulated stomatal closure is dependent on the plant melatonin receptor PMTR1 in *Arabidopsis* (Col-0) (Wei et al., 2018). However, the expression levels of *PMTR1* (*AT3G05010*) based on the RNA-

Seq data in this study were not statistically significant in response to melatonin among the ecotypes (data not shown). Transcription factors such as NACs, DREBs and R2R3 MYBs play crucial role in drought stress adaptation in *Arabidopsis* (Fujita et al., 2004, Re et al., 2014, Butt et al., 2017, Kudo et al., 2017). In the current study melatonin treatment led to specific differential expression of five (*ATHB-7, MYC2, RD26, DREB1B* and *MYB58*) drought-related transcription factors in Bur-0. This further strengthens the role of melatonin in water stress adaptation specific to Bur-0 compared to Col-0 and Hn-0. Moreover, Bur-0 has revealed to be tolerant to a wide range of stress factors such as salinity and heavy metals such as cadmium and zinc and is often compared with Col-0 which is relatively sensitive to these stress factors (Katori et al., 2010, Fischer et al., 2017). The role of melatonin in conferring tolerance to heavy metals and salinity in a range of plant species including *Arabidopsis* (Col-0) has been shown (Chen et al., 2017, Wang et al., 2019). It is probable that melatonin has a role in tolerance of Bur-0 to the mentioned stress factors.

Hn-0 has been previously included in the set of ecotypes studied for response to plant-growth promoting beneficial rhizobacterium (*Pseudomonas simiae* WCS417r) where it was shown to be responsive to WCS417r with significantly enhanced lateral root numbers (45-fold) as compared to the control (Wintermans et al., 2016). Hn-0 has also been studied for its response to salinity but with no definitive category of response in terms of resistance or susceptibility (Julkowska et al., 2016). However, the local habitat of Hn-0 (Hennetalsperre, Germany) receives relative less average precipitation yearly (570 mm) compared to Bur-0 (732.5 mm) and Col-0 (1192 mm) (Table S3.1). Response to melatonin triggered a wide range of growth and stress-responses in Hn-0 related to photomorphogenesis, abiotic (light, low oxygen levels, nutrient levels) and biotic stresses as shown by the enrichment of the related GO biological processes and the TFs that were specifically differentially expressed in Hn-0. This included TFs affecting growth-related processes such as organ elongation and flowering under stresses such as *AT4G39250.1* (*Radialis-like SANT/MYB, RL1), AT5G50915* (*basic helix-loop-helix 137, bHLH137), AT3G04030* (*MYB-related protein 2, MYR2)* (Josse et al., 2011, Zhao et al., 2011, Yang et al., 2018). This indicates that Hn-0 potentially requires melatonin for growth-related processes and simultaneous adaptation to a wide range of stress factors as opposed to Bur-0 which exhibited only water-stress related responses to melatonin.

3.4.5 Implications of studying ecotypes in *Arabidopsis*

The effects of melatonin in *Arabidopsis* have been primarily studied in Col-0 as a common reference ecotype (Bajwa et al., 2014, Hernández et al., 2015, Wang et al., 2017, Wan et

al., 2018). By including geographically diverse ecotypes, the complete set of melatonin-responsive genes within the *Arabidopsis* species can be revealed and the robustness of a gene core set strengthened. The ecotype-specific effects of melatonin in this study strongly demonstrate the importance of including more than one ecotype in studying melatonin. This has also been previously pointed out in studying the response to the defence signalling molecule, salicylic acid (SA) in *Arabidopsis* ecotypes where significant variation and ecotype-specific effects were observed. For example, an entire gene network was found to be significantly decreased in response to SA only in Col-0, but not in other tested ecotypes which might have implications in the species-dependent role of SA in plant-pathogen interactions (Van Leeuwen et al., 2007).

3.5 Conclusion and Next Steps

This study has provided insights into the role of melatonin biosynthetic gene *AtSNAT1* in diverse melatonin-mediated effects by phenotypic and transcriptomic analyses. In addition, core-conserved transcriptional effects of melatonin between ecotypes (Bur-0, Col-0 and Hn-0) are detailed. The identified ecotype-specific effects of melatonin highlight a possible role for melatonin in unique local habitat adaptation. Overall, the findings of the transcriptome analysis in the form of upregulated and downregulated gene expression in melatonin response has provided tools for further generation of hypotheses.

To gain further genetic insights and as an immediate next step, the analysis of this study will be extended to identify the potential alteration by melatonin of alternative splicing in transcriptomes of ecotypes, Bur-0, Col-0, and Hn-0 and *atsnat1* mutant. The results will help to investigate the transcript variants underlying melatonin-responsive genotypic variation. Future research directions could include utilising the available and well-researched recombinant inbred lines (RILs) of Bur-0 and Col-0 (Jubault et al., 2008). These could be used to map responses to melatonin to identify the quantitative trait loci (QTL) and candidate genes underlying variation between the two ecotypes. The inclusion of a large number of ecotypes in the screening set can also enable a genome-wide association analyses (GWAS) to identify candidate genes underlying response to melatonin. To gain functional insights, the findings of the current GO analysis can be further investigated to see whether, for example, melatonin alters abiotic (water stress, hypoxia, oxidative) and biotic stress (microbes and insects) responses in the ecotypes (Bur-0, Col-0 and Hn-0) and if they are altered in *atsnat1* mutant. Furthermore, T-DNA mutants of the identified transcription factors of interest could also be screened for melatonin response under the above-mentioned stressors. Finally, the expression levels of all the

genes involved in melatonin synthesis and endogenous melatonin concentration in ecotypes and *atsnat1* mutant could be tested to provide the potential link between observed genotypic differences and endogenous melatonin synthesis and production.

Taken together, the knowledge of the genetic and functional basis of variation across distinct genotypes in melatonin response in *Arabidopsis* can lay a foundation to transfer the information to crops of agricultural interest.

3.6 Materials and Methods

3.6.1 Plant Materials and growth conditions

29 *Arabidopsis* ecotypes were selected for this study based on their wide geographical distribution and a priori literature search revealing their responses to variety of abiotic and biotic stresses (Table S3.1). Seeds of all the ecotypes and *atsnat1* (*AT1G32070*) mutant (SALK_020577) were obtained from *Arabidopsis* Biological Resource Center (ABRC) https://abrc.osu.edu/ and bulked up, with care taken to avoid cross pollination, to propagate enough seed material for the study. Seeds were directly sown on soil (standard potting mix:vermiculite:perlite, 5:1:1) in glasshouse with growth conditions of 23°C, 16h/8h light-dark cycle with 120 µmol/m^2/s^2 light intensity. Late-flowering accessions were grown for 2 weeks until a fully developed rosette was formed and then subjected to vernalisation treatment for 4 weeks at 4°C, after which they were returned to normal growth conditions once bolting was visible, as per the instructions specified by ABRC. Seeds were surface-sterilized by vapour-phase method of chlorine gas for 3 hours and then stratified by immersing in 0.1% agarose-filled Eppendorf tubes, stored at 4 °C for a week to synchronize germination. For phenotypic analysis, sterilised seeds were directly sown equi-distantly on +/- melatonin-supplemented 1/2 MS medium (square plates 100 X 100mm, LabServ) and placed vertically at an angle of approximately 65° in controlled environment room at 23 °C, 16h/8h light-dark cycle and 56% humidity with 120 µmol/m^2/s^2 light intensity (as above). For transcriptome analysis, the sterilised seeds were directly sown on ½ MS medium for seven days and then transferred onto +/- MT-supplemented medium for a further three days also in controlled environment room with above mentioned growth conditions. The plates were randomised to avoid any positional effects.

3.6.2 Melatonin treatment

Melatonin (Sigma, M5250) was dissolved in 99.9% absolute ethanol (v/v) as a 200 mM (0.05 g/mL) initial stock as per the manufacturer's instructions and then further diluted to stock concentrations with 99.9% ethanol, which were eventually diluted in the growth medium to give the desired final concentrations. All treatments contained the same concentration of ethanol (final concentration 0.2%) to limit any potential effect arising from this solvent. Freshly prepared melatonin solutions were filter sterilized and added into cooled autoclaved medium (55 °C set on a water bath) and an equal volume of media (50 mL) poured into each plate. All *Arabidopsis* plants in this study were grown on half-strength Murashige and Skoog (MS) media (Sigma, M5524) containing sucrose (0.25%), 1% agar (Sigma, A7921) and pH 5.7 unless otherwise indicated. Plates were transferred to the controlled environment room and placed vertically (65° angle) for efficient root growth along the surface of agar for 12 days.

3.6.3 Projected rosette area quantification

Twenty-nine ecotypes were screened for projected rosette area among treatments with and without melatonin, with four plates per treatment and three seedlings of each ecotype alongside three seedlings of Col-0 as a reference ecotype per plate. There were total 12 seedlings for each treatment. The projected rosette area was determined by a well-established high-throughput and non-invasive method in *Arabidopsis* using chlorophyll fluorometer (Technologica, CF imager www.technologica.uk) (Barbagallo et al., 2003, Jansen et al., 2009). Briefly, the pictures of rosette area were captured from top-view after picture segmentation and background removal by the in-built black-and white charge-coupled device (CCD) camera (Dolphin F-145B, Allied Vision Technologies GmbH, Stadtroda, Germany) in the CF imager. For normalisation and precise estimation, plates were positioned in the same focal plane (185 mm from the camera as per the manual instructions). Each pixel of the image corresponded to an area of 0.02 mm^2 at the recommended settings. All the settings were set at default as indicated in the instruction manual (Technologica, CF imager www.technologica.uk).

3.6.4 RNA-extraction

Rosettes from ten day old *Arabidopsis* seedlings of Col-0, Bur-0 and Hn-0 and *atsnat1* mutant (SALK_020577) were separated from the roots by careful cutting through scissors and immediately flash-frozen followed by homogenization in liquid nitrogen. Five individual rosettes were pooled per replicate (three plates per treatment) with three

independent biological replicates for each treatment. The Spectrum™ Plant Total RNA kit (Sigma, NSW, Australia) was used to extract total RNA which was subjected to DNaseI removal before elution by using the On-column DNase I digestion kit (Sigma, NSW, Australia) following manufacturer's guidelines. The RNA quality and quantity were checked by the Spectrophotometric analysis using Nanodrop™ ND-1000 (Analytical Technologies, Australia).

3.6.5 RNA-Sequencing

The RNA samples were provided to La Trobe University Genomics Platform for cDNA library preparation. The TruSeq stranded mRNA library kit (Illumina, catalogue no. RS-122-9004) was used following manufacturer's protocol to construct 24 RNA libraries which comprised three biological replicates for each treatment of each genotype, i.e. 0 (control) and 400 µM melatonin. The NextSeq500 system (Illumina) was used to sequence the constructed libraries. The run type was NextSeq500/500 high output v2 kit (75 cycles) or 75 bp single-end reads.

3.6.6 Bioinformatic analysis of RNA-Sequencing data

RNA-Seq data were analysed by the La Trobe University Genomics Platform. The quality control, trimming and mapping of reads and differential expression analysis were conducted in CLC Genomics Workbench package 20.0 (www.qiagenbioinformatics.com). All the sequenced libraries had Phred quality scores in a range of 30-36 indicating 99.9% base call accuracy. A Phred quality score of 20 or above indicates high quality sequencing (Ewing et al., 1998, Van Veen et al., 2016). Therefore, reads of all the samples were mapped to the well-annotated *Arabidopsis* Col-0 genome (TAIR) from EnsemblPlants (https://plants.ensembl.org/Arabidopsis_thaliana/Info/Index). The transcript abundance was determined as transcripts per million (TPM). Before comparing samples, a per-sample library size normalization was conducted based on TMM (trimmed mean of M values) to adjust for sequencing depth differences between the samples (Robinson and Oshlack, 2010). For differential gene expression analysis, the sleuth program was used which utilizes a Wald test to determine differential gene expression (Bray et al., 2016). Genes with a false discovery rate (FDR)-adjusted p-values of < 0.05 and $\log_2$ fold change of at least 1.2 were classified as differentially expressed as per study in Chapter 2.

3.6.7 Data visualisation

Venn diagrams were generated by 'VennDiagram' and 'gplots' packages in RStudio (version 3.3.2) by using 'draw.pairwise.venn' 'draw.triple.venn' functions, respectively. in RStudio version 3.3.2.

3.6.8 Gene ontology (GO) term enrichment analysis and Transcription factor (TF) identification

GO term enrichment for biological processes of DEGs sets (both upregulated and downregulated together) were obtained from www.geneontology.com (Ashburner et al., 2000). Fisher's exact test was used to identify statistically significant GO terms enriched against all the annotated genes in the reference genome background of *Arabidopsis* (Col-0). Multiple testing of the analysis was corrected by Bonferroni correction at $p < 0.05$. Transcription factors in the lists of up- and downregulated DEGs were obtained from the Plant Transcription Factor Database (Plant TFDB) www.planttfdb.cbi.pku.edu.cn. (Jin et al., 2017).

3.6.9 qRT-PCR analysis

The *atsnat1* mutant was PCR-screened for homozygosity and sequenced to confirm the T-DNA insertion site (data not shown) which was in the 7th intron as also reported by (Koskela et al., 2018). qRT-PCR was conducted to compare the *AtSNAT1* gene expression in wild-type Col-0 to *atsnat1* mutant. The *AtSNAT1* (*AT1G32070*) gene and the reference gene *ACTIN2 (AT3G18780*) sequences for all the tested ecotypes were downloaded from the 1001 genomes collection www.1001genomes.org. The choice of reference genes was done based on literature search on melatonin effect on gene expression in *Arabidopsis* (Bajwa et al., 2014, Wang et al., 2017, Ai and Zhu, 2018, Koskela et al., 2018, Wan et al., 2018, Wei et al., 2018). The sequences were aligned to the reference sequence (Col-0) in alignment editor software, BioEdit version 7.2 to create a consensus sequence. The primers were designed in regions conserved between all the ecotypes to have a single pair of primers of each gene for all the ecotypes. The single pair of primers designed on a consensus sequence also allowed to avoid any potential differences in primer efficiency between the ecotypes. Care was taken to design primers that were predicted to have 100% specificity to the *ATSNAT1* gene only which was further confirmed by PCR. The oligonucleotides were designed by using primer3 (version 4.1.0) and were synthesized by Bioneer Pacific (Kew East, VIC, 3102, Australia). The primer sequences and other

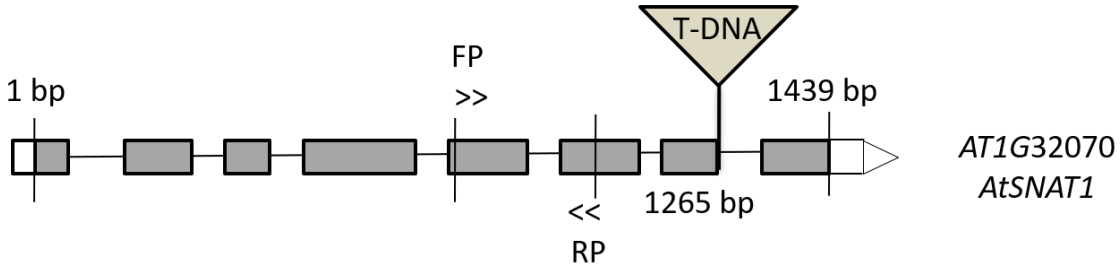
information of *AtSNAT1* were: Forward primer (5`-3`): GATGGCCAAGGAGACCGTTA, Tm=59.46°C; reverse primer (5`-3`): TCGCACGTGCCATACCAATA, Tm=59.82°C; product size = 165 bp and for *AtACTIN2* were: Forward primer (5`-3`): CATGCCATCCTCCGTCTTGA, Tm=59.82°C; reverse primer (5`-3`): GGTTTCCATCTCCTGCTCGT, Tm=59.75°C; product size=171 bp. The same set of primers were also used to assess *SNAT1* expression in *atsnat1*mutant. The cDNA was synthesised from RNA (1 µg) by using Tetro cDNA synthesis kit and Oligodt primers (Bioline, Catalogue Number, BIO-65043) as per manufacturer's recommendations. Standard curves were generated to calculate PCR primer amplification efficiencies which lay above 90% for both primer pairs. Briefly, synthesised cDNA was serially diluted at a dilution factor of 1:4 for up to five dilution points. 2 µL of cDNA was used for total qPCR reaction. SYBR Green chemistry (Luna Universal qPCR master mix, M3003, NEB) was used for qPCR and conducted on QuantStudio^{TM} 5 Real-Time PCR System, 384-well, Applied Biosystems (Catalog #A28140). The cycling parameters were Initial Denaturation, 95°C, 20 seconds; Denaturation, 95°C, 20 seconds; Extension, 60°C, 30 seconds, (40 cycles); Melt Curve, 95°C for 1 second, 60°C for 20 seconds, 95°C for 1 second.

3.6.10 Statistical analyses

Welch ANOVA and Games-Howell post-hoc test with p-value<0.05 was utilized in this study unless otherwise stated (Drake et al., 2016, Tsugama et al., 2019). The statistical analyses for phenotypic data were conducted on SPSS (IBM SPSS Statistics Version 25). Statistical analyses for RNA-Seq data were conducted on CLC Genomics Workbench (Schaarschmidt et al., 2020). A generalized linear model (GLM) based on negative binomial distribution was used for differential gene expression analysis (Robinson et al., 2010). The *p*-values were corrected for multiple testing with false discovery rate (FDR) statistics. The Wald test was applied to calculate the p-values and FDR adjusted p-values for comparison of all group pairs (Chen et al., 2011).

3.7 Supplementary Information

A



B

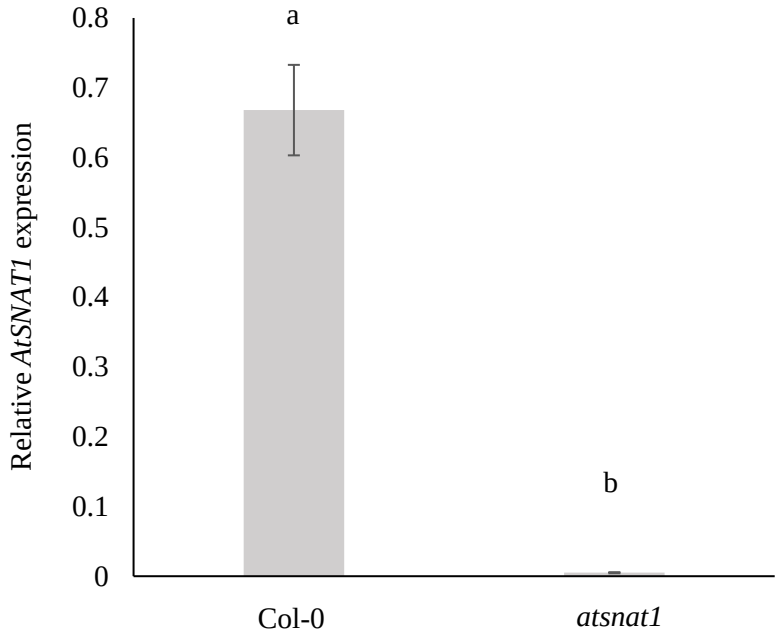


Figure S3.1: Melatonin biosynthetic gene *AtSNAT1* expression in rosette leaves of Col-0 and T-DNA insertion mutant, *atsnat1*, under no-melatonin control. All treatments including control contain ethanol (EtOH 0.2% v/v) as a solvent for melatonin. Seeds were germinated on un-supplemented ½ MS media plates for seven days and transferred to no-melatonin control plates for another 3 days. (A) Schematic representation of *ATSNAT1* gene structure (not to scale) showing T-DNA insertion site and *ATSNAT1* qPCR primers (FP=forward primer; RP=reverse primer) annealing site. (B) Bars represent the mean *AtSNAT1* expression quantified by qRT-PCR relative to the reference gene *ACTIN2* expression. Error bars denote standard deviation. Three biological replicates per treatment were assessed and with pooled five rosettes comprised each biological replicate. Pairwise analyses were conducted by a Student's t-test. Different letters denote statistical significance at p-value <0.05

Table S3.1: Geographic and climatological information of 29 *Arabidopsis thaliana* ecotypes tested in the study.

Ecotype	ABRC stock number	Origin	Latitude (°N)	Longitude (°E)	Altitude (metres above sea level)	Average temperature (°C yearly)	Average precipitation (mm yearly)	Habitat of plant collection location (available from TAIR)
Bl-1	CS76450	Bologna, Italy	44.5	11.33	54	14	774	Botanic garden
Bor-4	CS76454	Borky, Czech Republic	49.4	16.2	488	8.3	234.3	N/A
Bur-0*	**CS76734**	**Burren, Ireland**	**54.1**	**-6.2**	**31**	**11**	**732.5**	**Wall by roadside**
Chat-1	CS76463	Chateaudun, France	48.07	1.33	127	10.8	632	Country road
CIBC-17	CS76770	Ascot, United Kingdom	51.4	-0.67	68	13.8	60	Flower bed infront of CAB International Institute of Biological Control
Col-0*	**CS70000**	**Columbia, USA**	**38.3**	**-92.3**	**89**	**17.2**	**1192**	**N/A**
Da-0	CS76791	Darmstadt, Germany	49.87	8.65	144	9.5	653	N/A
Fei-0	CS76412	St. Maria d. Feira Village, Portugal	40.92	-8.54	140	14.3	1148	Roadside
Gd-1	CS76491	Gudow, Germany	53.5	10.5	25	8.4	700	roadside
Hn-0*	**CS76513**	**Hennetalsperre, Germany**	**51.34**	**8.28**	**272**	**12.5**	**570**	**N/A**
Is-0	CS76517	Isenburg, Germany	50.5	7.5	130	9.9	655	N/A
Kondara	CS76532	Khurmatov, Tadjikistan	38.48	68.49	3000	24.3	1289	Southern slope of Gisserky mountain ridge
Ler-1	CS77021	Landsberg, Germany	47.98	10.87	630	8.1	941	N/A
Lp2-2	CS76546	Lipovec, Czech Republic	49.38	16.81	550	11.75	711.2	N/A
Mt-0	CS77112	Martuba, Libya	32.34	22.46	100-200	15-16	130	N/A
Nfa-8	CS22598	Ascot, UK	51.40	-0.6	68	13.8	60	N/A
Nfa-10	CS77126	Ascot, UK	51.40	-0.6	68	13.8	60	N/A
Oy-0	CS77156	Oystese, Norway	60.38	6.19	4	7.2	2190	N/A
Pa-1	CS76570	Palermo, Italy	38.07	13.22	14	18.4	605	N/A
Rrs-7	CS76593	Indiana, USA	41.56	-86.42	230	-5 – 29.4	939.8	N/A
Rub1	CS76594	Rubezhnoe, Ukraine	49	38.28	74	8.4	490	near lake

Table S3.1 contd.

Ecotype	**ABRC stock number**	**Origin**	**Latitude (°N)**	**Longitude (°E)**	**Altitude (metres above sea level)**	**Average temperature (°C yearly)**	**Average precipitation (yearly)**	Habitat of plant collection location
Tscha-1	CS76616	Tsagguns, Austria	47.07	9.9	685	8.4	1071	camping site
Van-0	CS76623	Vancouver, Canada	49.3	-123	34	9.9	1283	field border
Wa-1	CS76626	Warsaw, Poland	42.3	21	78–116	7.7	501	N/A
Wl-0	CS76630	Wildbad, Germany	47.92	10.81	425	8.7	876	N/A
WS-2	CS78920	Wassilewskija, Russia	52.3	30	N/A	N/A	N/A	sandy rye field
Wu-0	CS78858	Wurzburg, Germany	49.78	9.93	117	9.5	603	sandy soil

*Highlighted in bold font are the ecotypes selected for RNA-Sequencing analysis; N/A means the information is not available in TAIR.

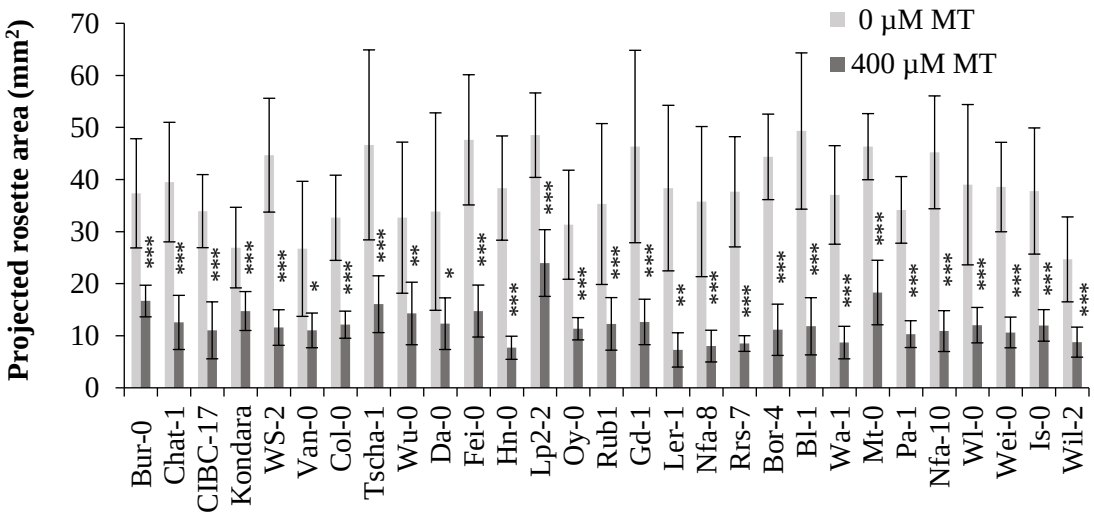


Figure S3.2: Phenotypic response (projected rosette area) of 29 *Arabidopsis* ecotypes to exogenous melatonin (400 µM) compared to respective no-melatonin controls. Statistical differences analyzed between treatment and no-melatonin controls of each individual ecotype. All treatments including control contain 0.2% ethanol (EtOH 0.2% v/v) as a solvent for melatonin Significant differences marked by asterisks are assessed by two-way ANOVA to identify ecotype effect ($p<0.0001$***), treatment effect ($p<0.0001$***) and interaction effect of treatment * ecotype ($p=0.037$*; F-value=3.1), To identify simple main effects, one-way ANOVA and Games-Howell post hoc with *p*-value <0.05 was used. Statistically significant differences are denoted by asterisk where p-value <0.05 (*), <0.01 (**) and <0.0001 (***). Bars represent mean rosette area of treated seedlings (n=8-12). All statistical analyses were conducted on SPSS (IBM SPSS Statistics Version 25).

Table S3.2: Pairwise comparisons of relative projected rosette area among 29 ecotypes of *Arabidopsis*.

Table shows p-values of relative projected rosette areas for each pairwise comparison. Significant differences ($p<0.05$) are highlighted in red. The p-values <0.001 are indicated as zero.

	Bur-0	Kondara	Lp2-2	Wu-0	Van-0	Pa-1	Wil-2	CIBC-17	Col-0	Da-0	Oy-0	Rub1	Tscha-1	Chat-1	Is-0	Fei-0	Wei-0	Nfa-10	Gd-1	WS-2	Bl-1	Wl-0	Wa-1	Mt-0	Rrs-7	Bor-4	Nfa-8	Hn-0	Ler-1
Bur-0		0.925	1	1	1	0.012	0.859	0.995	0.681	0.966	0.392	0.879	0.604	0.722	0.101	0.142	0.034	0.002	0.01	0	0.05	0.001	0	1	0	0.007	0	0	0
Kondara	0.925		1	0.995	0.894	0.015	0.223	0.555	0.175	0.388	0.117	0.278	0.131	0.235	0.034	0.034	0.021	0.006	0.007	0.003	0.009	0.002	0.001	0.63	0.001	0.004	0.001	0.001	0
Lp2-2	1	1		1	0.999	0.053	0.696	0.941	0.586	0.848	0.425	0.713	0.468	0.559	0.134	0.142	0.082	0.018	0.025	0.008	0.032	0.005	0.003	0.979	0.002	0.014	0.002	0.001	0.001
Wu-0	1	0.995	1		1	0.842	1	1	1	1	1	1	0.998	0.993	0.947	0.939	0.9	0.628	0.656	0.469	0.5	0.359	0.302	1	0.203	0.503	0.24	0.12	0.119
Van-0	1	0.894	0.999	1		0.874	1	1	1	1	1	1	1	0.998	0.969	0.964	0.929	0.664	0.699	0.502	0.552	0.393	0.333	1	0.234	0.547	0.269	0.147	0.142
Pa-1	0.012	0.015	0.053	0.842	0.874		0.954	0.999	0.898	1	0.951	1	1	1	1	1	1	1	1	1	0.998	0.984	0.941	0.917	0.464	1	0.83	0.187	0.415
Wil-2	0.859	0.223	0.696	1	1	0.954		1	1	1	1	1	1	1	0.999	0.998	0.991	0.622	0.729	0.307	0.579	0.204	0.114	1	0.012	0.507	0.065	0.003	0.025
CIBC-17	0.995	0.555	0.941	1	1	0.999	1		1	1	1	1	1	1	1	1	1	0.967	0.975	0.871	0.884	0.75	0.659	1	0.435	0.9	0.547	0.252	0.281
Col-0	0.681	0.175	0.586	1	1	0.898	1	1		1	1	1	1	1	0.996	0.995	0.975	0.476	0.621	0.195	0.519	0.137	0.069	1	0.006	0.413	0.038	0.001	0.019
Da-0	0.966	0.388	0.848	1	1	1	1	1	1		1	1	1	1	1	1	1	0.973	0.981	0.875	0.894	0.747	0.646	1	0.392	0.91	0.524	0.211	0.252
Oy-0	0.392	0.117	0.425	1	1	0.951	1	1	1	1		1	1	1	0.999	0.999	0.993	0.564	0.711	0.238	0.59	0.171	0.085	1	0.005	0.493	0.048	0.001	0.025
Rub1	0.879	0.278	0.713	1	1	1	1	1	1	1	1		1	1	1	1	1	0.997	0.998	0.97	0.969	0.902	0.835	1	0.602	0.979	0.732	0.372	0.418
Tscha-1	0.604	0.131	0.468	0.998	1	1	1	1	1	1	1	1		1	1	1	1	0.991	0.994	0.911	0.932	0.778	0.655	1	0.313	0.947	0.508	0.141	0.218
Chat-1	0.722	0.235	0.559	0.993	0.998	1	1	1	1	1	1	1	1		1	1	1	1	1	1	1	0.998	0.993	1	0.951	1	0.978	0.827	0.841
Is-0	0.101	0.034	0.134	0.947	0.969	1	0.999	1	0.996	1	0.999	1	1	1		1	1	1	1	0.989	0.989	0.93	0.841	0.991	0.406	0.995	0.695	0.183	0.326
Fei-0	0.142	0.034	0.142	0.939	0.964	1	0.998	1	0.995	1	0.999	1	1	1	1		1	1	1	1	0.999	0.99	0.967	0.989	0.729	1	0.902	0.423	0.55
Wei-0	0.034	0.021	0.082	0.9	0.929	1	0.991	1	0.975	1	0.993	1	1	1	1	1		1	1	0.998	0.997	0.973	0.917	0.967	0.463	0.999	0.793	0.192	0.387
Nfa-10	0.002	0.006	0.018	0.628	0.664	1	0.622	0.967	0.476	0.973	0.564	0.997	0.991	1	1	1	1		1	1	1	1	1	0.646	0.951	1	0.997	0.652	0.813
Gd-1	0.01	0.007	0.025	0.656	0.699	1	0.729	0.975	0.621	0.981	0.711	0.998	0.994	1	1	1	1	1		1	1	1	1	0.707	0.995	1	1	0.874	0.924
WS-2	0	0.003	0.008	0.469	0.502	1	0.307	0.871	0.195	0.875	0.238	0.97	0.911	1	0.989	1	0.998	1	1		1	1	1	0.414	0.999	1	1	0.912	0.957
Bl-1	0.05	0.009	0.032	0.5	0.552	0.998	0.579	0.884	0.519	0.894	0.59	0.969	0.932	1	0.989	0.999	0.997	1	1	1		1	1	0.546	1	1	1	1	1
Wl-0	0.001	0.002	0.005	0.359	0.393	0.984	0.204	0.75	0.137	0.747	0.171	0.902	0.778	0.998	0.93	0.99	0.973	1	1	1	1		1	0.289	1	1	1	1	1
Wa-1	0	0.001	0.003	0.302	0.333	0.941	0.114	0.659	0.069	0.646	0.085	0.835	0.655	0.993	0.841	0.967	0.917	1	1	1	1	1		0.212	1	1	1	1	1
Mt-0	1	0.63	0.979	1	1	0.917	1	1	1	1	1	1	1	1	0.991	0.989	0.967	0.646	0.707	0.414	0.546	0.289	0.212		0.093	0.514	0.147	0.04	0.056
Rrs-7	0	0.001	0.002	0.203	0.234	0.464	0.012	0.435	0.006	0.392	0.005	0.602	0.313	0.951	0.406	0.729	0.463	0.951	0.995	0.999	1	1	1	0.093		1	1	1	1
Bor-4	0.007	0.004	0.014	0.503	0.547	1	0.507	0.9	0.413	0.91	0.493	0.979	0.947	1	0.995	1	0.999	1	1	1	1	1	1	0.514	1		1	0.999	0.999
Nfa-8	0	0.001	0.002	0.24	0.269	0.83	0.065	0.547	0.038	0.524	0.048	0.732	0.508	0.978	0.695	0.902	0.793	0.997	1	1	1	1	1	0.147	1	1		1	1
Hn-0	0	0.001	0.001	0.12	0.147	0.187	0.003	0.252	0.001	0.211	0.001	0.372	0.141	0.827	0.183	0.423	0.192	0.652	0.874	0.912	1	1	1	0.04	1	0.999	1		1
Ler-1	0	0	0.001	0.119	0.142	0.415	0.025	0.281	0.019	0.252	0.025	0.418	0.218	0.841	0.326	0.55	0.387	0.813	0.924	0.957	1	1	1	0.056	1	0.999	1	1	

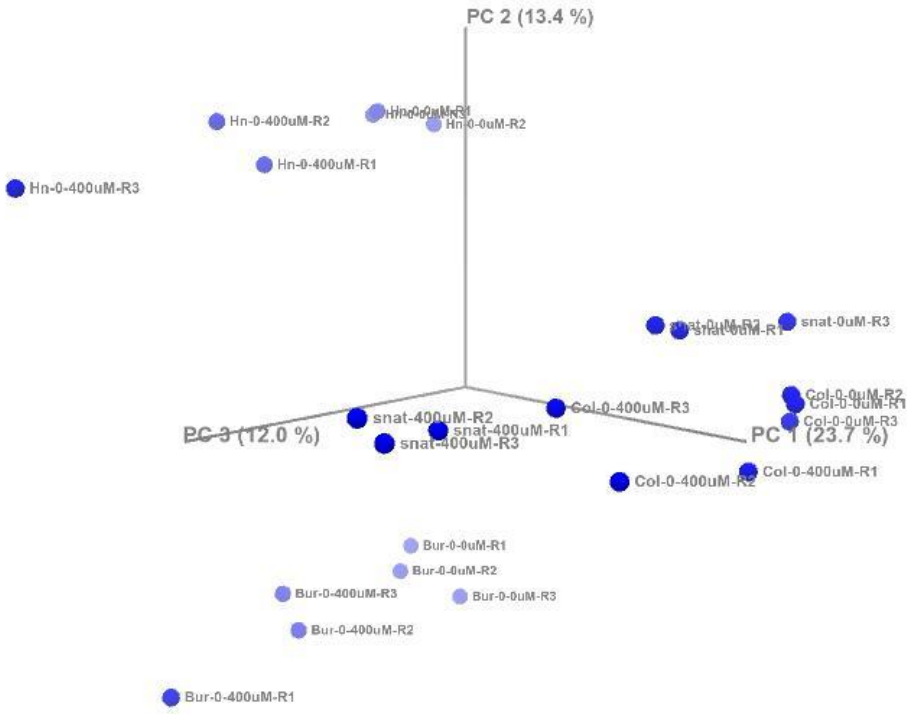


Figure S3.3: Principal component analysis (PCA) of the total DEGs in ecotypes Bur-0, Col-0, and Hn-0 and mutant *atsnat1*. PCA plot of the expression values of the three biological replicates for Col-0, Bur-0, Hn-0 and *atsnat1* under each treatment, i.e. melatonin (400 µM) or their respective controls (0 µM).

Table S3.3: Summary statistics of RNA-Seq data

	Total read counts (#)	**Total reads mapped (#)**	**Reads mapped (%)**
Bur-0-0µM-R2	18,138,369	17,218,912	94.93087278
Bur-0-0µM-R3	16,298,297	15,345,517	94.15411316
Bur-0-0µM-R4	16,073,557	15,087,932	93.86803431
Bur-0-400µM-R1	19,807,632	18,911,663	95.47664759
Bur-0-400µM-R2	19,013,586	18,079,823	95.08896954
Bur-0-400µM-R3	18,060,563	16,899,889	93.57343401
Col-0-0µM-R1	19,693,041	18,791,973	95.42443445
Col-0-0µM-R2	18,389,067	17,556,399	95.47193993
Col-0-0µM-R3	14,733,349	14,019,095	95.15212733
Col-0-400µM-R1	18,484,603	17,662,604	95.55306111
Col-0-400µM-R2	23,185,858	22,123,224	95.41688731
Col-0-400µM-R4	24,693,727	23,188,970	93.9063188
Hn-0-0µM-R1	16,128,278	15,163,164	94.01601336
Hn-0-0µM-R2	17,952,528	16,606,166	92.50043225
Hn-0-0µM-R3	15,676,411	14,605,837	93.17079656
Hn-0-400µM-R1	19,437,878	18,089,886	93.06512779
Hn-0-400µM-R2	16,608,092	15,017,733	90.42419201
Hn-0-400µM-R3	23,932,521	22,494,880	93.99293957
snat-0µM-R1	23,629,452	22,146,067	93.72230469
snat-0µM-R2	25,750,198	24,507,896	95.17556331
snat-0µM-R4	18,602,870	17,616,925	94.70003822
snat-400µM-R2	22,289,924	20,990,967	94.1724476
snat-400µM-R3	20,645,474	19,490,623	94.4062752
snat-400µM-R4	23,737,113	22,351,478	94.16257992

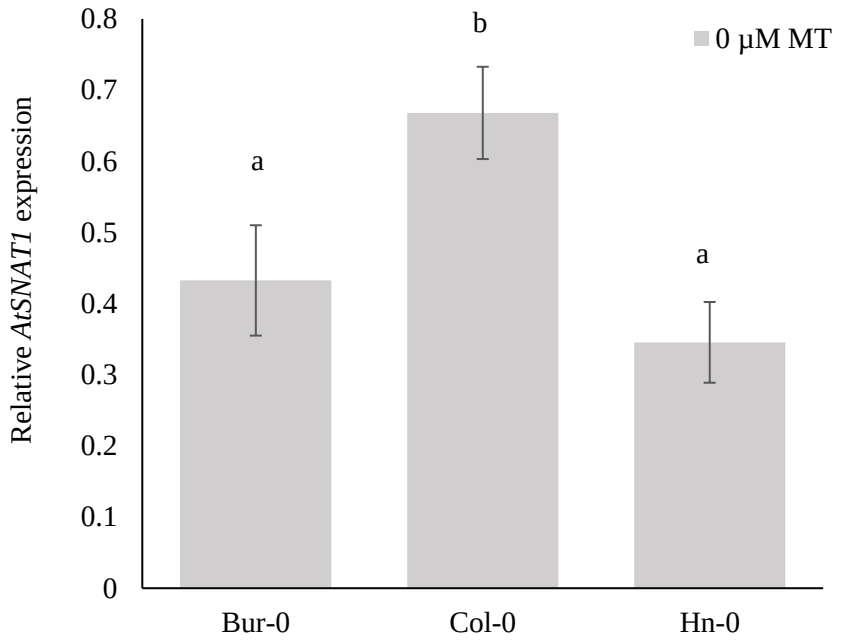


Figure S3.4: Expression levels of *AtSNAT1* in ecotypes Bur-0, Col-0 and Hn-0 under no-melatonin control conditions. Seeds were germinated on un-supplemented ½ MS media plates for seven days and transferred to no-melatonin control plates for another 3 days. All treatments including control contain 0.2% ethanol (EtOH 0.2% v/v). as a solvent for melatonin. Bars represent the mean *AtSNAT1* expression quantified by qRT-PCR relative to the reference gene *ACTIN2* expression. Error bars denote standard deviation. Four biological replicates per treatment were assessed and with pooled five rosettes comprised each biological replicate. Pairwise analyses were conducted by a one-way ANOVA followed by post-hoc Tukey test. Different letters denote statistical significance at p-value <0.05

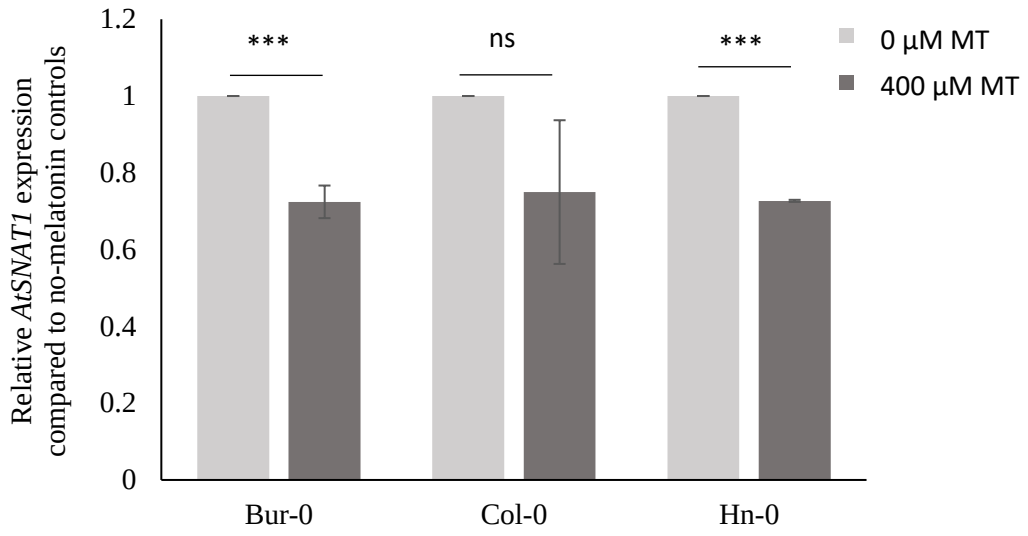


Figure S3.5: Effect of melatonin on *AtSNAT1* expression levels in ecotypes Bur-0, Col-0 and Hn-0 in response to melatonin (400 µM) relative to no-melatonin controls. Seeds were germinated on un-supplemented ½ MS media plates for seven days and transferred to no-melatonin control plates for another 3 days. All treatments including control contain 0.2% ethanol (EtOH 0.2% v/v) as a solvent for melatonin. Bars represent the mean *AtSNAT1* expression quantified by qRT-PCR relative to the no-melatonin control expression which was set at 1. All the expression values were first normalised to expression of reference gene *ACTIN2*. Error bars denote standard deviation. Four biological replicates per treatment were assessed and with pooled five rosettes comprised each biological replicate. Pairwise analyses were conducted by a one-way ANOVA followed by post-hoc Tukey test, ns = no statistical significance. Statistically significant differences are denoted by asterisk where p-value <0 <0.0001 (***).

Table S3.4: Overlap analysis of current study with previous transcriptomic data in response to melatonin (Chapter 2) (Zia et al., 2019)

Gene ID	Gene name/Description	Log_2 fold change (compared to no-melatonin control in same ecotype)			
		Chapter 2 (Zia et al., 2019)	This study		
		Col-0 (100 µM)	Col-0 (400 µM)	Bur-0 (400 µM)	Hn-0 (400 µM)
AT3G59930	*DEFL*	4.92	8.84	4.80	8.57
AT2G26010	*PDF 1.3*	4.62	5.42	3.76	4.96
AT2G26020	*PDF1.2B*	4.33	5.42	13.84	5.33
AT2G47950	*Myelin-TF-like*	4.11	5.46	5.79	5.44
AT3G51910	*HSFA7A*	1.52	1.6	2.91	2.12
AT5G07100	*WRKY26*	1.59	1.23	1.90	1.25
AT3G13310	*Chaperone DnaJ-domain superfamily DJC66*	3.8	4.14	4.11	4.01
AT2G15310	*ADP-ribosylation factor ARFB1A*	2.02	3.26	2.67	3.62
AT5G10770	*Eukaryotic aspartyl protease family protein*	1.26	2.05	2.04	2.19
AT1G75040	*Pathogenesis-related gene 5, PR5*	3.69	3.75	3.41	4.51
AT5G27420	*Carbon/nitrogen insensitive ubiquitin ligase CNI1/ATL31*	2.22	2.42	2.21	3.88
AT1G10140	*Uncharacterized conserved protein*	1.5	2.1	3.34	2.04
AT1G78450	*SOUL-heme binding protein*	1.43	2.45	1.11	2.84
AT5G33355	*Defensin-like (DEFL) family protein*	4.92	N/A	5.93 ^{ns}	6.97 ^{ns}
AT1G34047	*Defensin-like (DEFL) family protein*	4.88	N/A	0.26 ^{ns}	4.23 ^{ns}
AT1G06160	*Ethylene responsive factor AP2/ERF59*	2.47			
AT5G17300	*Myb-like transcription factor RVE1*	-1.42	0.31 ^{ns}	0.44 ^{ns}	0.35 ^{ns}
ATCG00330	*Chloroplast ribosomal protein RPS14*	-1.55	-0.42 ^{ns}	0.09 ^{ns}	-0.22 ^{ns}
ATCG00020	*Photosystem II reaction center A PSBA*	-1.49	0.76 ^{ns}	0.28 ^{ns}	0.30 ^{ns}
ATCG00350	*Photosystem I, PsaA/PsaB*	-1.47	-0.17 ^{ns}	0.08 ^{ns}	-0.52 ^{ns}

Table S3.4: contd.

Gene ID	Gene name/Description	Log$_2$ fold change (compared to no-melatonin control in same ecotype)			
		Chapter 2 (Zia et al., 2019)	This study		
		Col-0 (100 µM) Chapter 2	Col-0 (400 µM)	Bur-0 (400 µM)	Hn-0 (400 µM)
ATCG00280	*Photosystem II reaction center C, PSBC*	-1.43	0.32 ^{ns}	0.34 ^{ns}	0.10 ^{ns}
ATCG00340	*Photosystem I, PsaA/PsaB*	-1.41	-0.31 ^{ns}	0.07 ^{ns}	-0.48 ^{ns}
ATCG00270	*Photosystem II reaction center D PSBD*	-1.40	0.28 ^{ns}	0.36 ^{ns}	0.08 ^{ns}
ATCG00490	*Ribulose-biphosphate carboxylases RBCL*	-1.25	0.58 ^{ns}	0.21 ^{ns}	0.34 ^{ns}
AT4G31940	*Cytochrome P450 CYP82C4*	4.46	N/A	N/A	N/A
AT1G29460	*SAUR-like auxin responsive SAUR65*	-1.26	-0.76 ^{ns}	-0.93 ^{ns}	-1.31 ^{ns}
AT2G40095	*Alpha/beta hydrolase related protein*	1.36	0.84 ^{ns}	0.90 ^{ns}	1.85 ^{ns}
AT2G07706	*Hypothetical protein*	-1.53	1.99 ^{ns}	-0.75 ^{ns}	-2.91 ^{ns}

Highlighted in grey are the statistically significant genes according to the FDR-adjusted p-values; ns = not statistically significantly different; N/A = values not available as transcripts not detected in control and treated samples

3.8 References

Ahmad, S., Kamran, M., Ding, R., Meng, X., Wang, H., Ahmad, I., et al. 2019. Exogenous melatonin confers drought stress by promoting plant growth, photosynthetic capacity and antioxidant defense system of maize seedlings. *PeerJ,* 7**,** e7793

Ai, Y. & Zhu, Z. 2018. Melatonin Antagonizes Jasmonate-Triggered Anthocyanin Biosynthesis in *Arabidopsis thaliana*. *J Agric Food Chem,* 66**,** 5392-5400

Arnao, M. B. & Hernández-Ruiz, J. 2013. Growth conditions determine different melatonin levels in *Lupinus albus* L. *J Pineal Res,* 55**,** 149-55

Arnao, M. B. & Hernández-Ruiz, J. 2019. Melatonin: A New Plant Hormone and/or a Plant Master Regulator? *Trends Plant Sci,* 24**,** 38-48

Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., et al. 2000. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet,* 25**,** 25-9

Back, K., Tan, D. X. & Reiter, R. J. 2016. Melatonin biosynthesis in plants: multiple pathways catalyze tryptophan to melatonin in the cytoplasm or chloroplasts. *J Pineal Res,* 61**,** 426-437

Bajwa, V. S., Shukla, M. R., Sherif, S. M., Murch, S. J. & Saxena, P. K. 2014. Role of melatonin in alleviating cold stress in *Arabidopsis thaliana*. *J Pineal Res,* 56**,** 238-45

Barbagallo, R. P., Oxborough, K., Pallett, K. E. & Baker, N. R. 2003. Rapid, noninvasive screening for perturbations of metabolism and plant growth using chlorophyll fluorescence imaging. *Plant Physiol,* 132**,** 485-93

Brachi, B., Faure, N., Horton, M., Flahauw, E., Vazquez, A., Nordborg, M., et al. 2010. Linkage and association mapping of *Arabidopsis thaliana* flowering time in nature. *PLoS Genet,* 6**,** e1000940

Bray, N. L., Pimentel, H., Melsted, P. & Pachter, L. 2016. Near-optimal probabilistic RNA-seq quantification. *Nat Biotechnol,* 34**,** 525-7

Butt, H. I., Yang, Z., Gong, Q., Chen, E., Wang, X., Zhao, G., et al. 2017. *GaMYB85*, an R2R3 MYB gene, in transgenic *Arabidopsis* plays an important role in drought tolerance. *BMC Plant Biol,* 17**,** 142

Byeon, Y., Lee, H. Y., Hwang, O. J., Lee, H. J., Lee, K. & Back, K. 2015. Coordinated regulation of melatonin synthesis and degradation genes in rice leaves in response to cadmium treatment. *J Pineal Res,* 58**,** 470-8

Cao, S., Shao, J., Shi, L., Xu, L., Shen, Z., Chen, W., et al. 2018. Melatonin increases chilling tolerance in postharvest peach fruit by alleviating oxidative damage. *Sci Rep,* 8**,** 806

Chavigneau, H., Goué, N., Delaunay, S., Courtial, A., Jouanin, L., Reymond, M., et al. 2012. QTL for floral stem lignin content and degradability in three recombinant inbred line (RIL) progenies of *Arabidopsis thaliana* and search for candidate genes involved in cell wall biosynthesis and degradability. *Open J Genet,* 02**,** 7-30

Chen, L., Liu, L., Lu, B., Ma, T., Jiang, D., Li, J., et al. 2020. Exogenous melatonin promotes seed germination and osmotic regulation under salt stress in cotton (*Gossypium hirsutum* L.). *PLoS One,* 15**,** e0228241

Chen, Z., Liu, J., Ng, H. K., Nadarajah, S., Kaufman, H. L., Yang, J. Y., et al. 2011. Statistical methods on detecting differentially expressed genes for RNA-seq data. *BMC Syst Biol,* 5 S1

Chen, Z., Xie, Y., Gu, Q., Zhao, G., Zhang, Y., Cui, W., et al. 2017. The AtrbohF-dependent regulation of ROS signaling is required for melatonin-induced salinity tolerance in *Arabidopsis*. *Free Radic Biol Med,* 108**,** 465-477

Choi, W. G., Barker, R. J., Kim, S. H., Swanson, S. J. & Gilroy, S. 2019. Variation in the transcriptome of different ecotypes of *Arabidopsis thaliana* reveals signatures of oxidative stress in plant responses to spaceflight. *Am J Bot,* 106**,** 123-136

Clerkx, E. J., El-Lithy, M. E., Vierling, E., Ruys, G. J., Blankestijn-De Vries, H., Groot, S. P., et al. 2004. Analysis of natural allelic variation of *Arabidopsis* seed germination and seed longevity traits between the accessions Landsberg erecta and Shakdara, using a new recombinant inbred line population. *Plant Physiol,* 135**,** 432-43

Cubillos, F. A., Yansouni, J., Khalili, H., Balzergue, S., Elftieh, S., Martin-Magniette, M. L., et al. 2012. Expression variation in connected recombinant populations of *Arabidopsis thaliana* highlights distinct transcriptome architectures. *BMC Genom,* 13**,** 117

Davletova, S., Schlauch, K., Coutu, J. & Mittler, R. 2005. The zinc-finger protein Zat12 plays a central role in reactive oxygen and abiotic stress signaling in *Arabidopsis*. *Plant Physiol,* 139**,** 847-56

Delker, C., Poschl, Y., Raschke, A., Ullrich, K., Ettingshausen, S., Hauptmann, V., et al. 2010. Natural variation of transcriptional auxin response networks in *Arabidopsis thaliana*. *Plant Cell,* 22**,** 2184-200

Ding, Z., Wu, C., Tie, W., Yan, Y., He, G. & Hu, W. 2019. Strand-specific RNA-seq based identification and functional prediction of lncRNAs in response to melatonin and simulated drought stresses in cassava. *Plant Physiol Biochem,* 140**,** 96-104

Drake, T., Keating, M., Summers, R., Yochikawa, A., Pitman, T. & Dodd, A. N. 2016. The Cultivation of *Arabidopsis* for Experimental Research Using Commercially Available Peat-Based and Peat-Free Growing Media. *PLoS One,* 11**,** e0153625

Dubbels, R., Reiter, R. J., Klenke, E., Goebel, A., Schnakenberg, E., Ehlers, C., et al. 1995. Melatonin in edible plants identified by radioimmunoassay and by high performance liquid chromatography-mass spectrometry. *J Pineal Res,* 18**,** 28-31

Ewing, B., Hillier, L., Wendl, M. C. & Green, P. 1998. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res,* 8**,** 175-85

Fischer, S., Spielau, T. & Clemens, S. 2017. Natural variation in *Arabidopsis thaliana* Cd responses and the detection of quantitative trait loci affecting Cd tolerance. *Sci Rep,* 7**,** 3693

Flematti, G. R., Ghisalberti, E. L., Dixon, K. W. & Trengove, R. D. 2004. A compound from smoke that promotes seed germination. *Science,* 305**,** 977

Fu, J., Wu, Y., Miao, Y., Xu, Y., Zhao, E., Wang, J., et al. 2017. Improved cold tolerance in *Elymus nutans* by exogenous application of melatonin may involve ABA-dependent and ABA-independent pathways. *Sci Rep,* 7**,** 39865

Fujita, M., Fujita, Y., Maruyama, K., Seki, M., Hiratsu, K., Ohme-Takagi, M., et al. 2004. A dehydration-induced NAC protein, RD26, is involved in a novel ABA-dependent stress-signaling pathway. *Plant J,* 39**,** 863-76

Gery, C., Zuther, E., Schulz, E., Legoupi, J., Chauveau, A., Mckhann, H., et al. 2011. Natural variation in the freezing tolerance of *Arabidopsis thaliana*: Effects of RNAi-induced CBF depletion and QTL localisation vary among accessions. *Plant Sci,* 180**,** 12-23

Goralogia, G. S., Liu, T. K., Zhao, L., Panipinto, P. M., Groover, E. D., Bains, Y. S., et al. 2017. CYCLING DOF FACTOR 1 represses transcription through the TOPLESS co-repressor to control photoperiodic flowering in *Arabidopsis*. *Plant J,* 92**,** 244-262

Guttman, M. & Rinn, J. L. 2012. Modular regulatory principles of large non-coding RNAs. *Nature,* 482**,** 339-46

Hardeland, R. 2016. Melatonin in Plants - Diversity of Levels and Multiplicity of Functions. *Front Plant Sci,* 7**,** 198

Hattori, A., Migitaka, H., Iigo, M., Itoh, M., Yamamoto, K., Ohtani-Kaneko, R., et al. 1995. Identification of melatonin in plants and its effects on plasma melatonin levels and binding to melatonin receptors in vertebrates. *Biochem Mol Biol Int,* 35**,** 627-34

Hernández, I. G., Gomez, F. J., Cerutti, S., Arana, M. V. & Silva, M. F. 2015. Melatonin in *Arabidopsis thaliana* acts as plant growth regulator at low concentrations and preserves seed viability at high concentrations. *Plant Physiol Biochem,* 94**,** 191-6

Herridge, R. P., Day, R. C., Baldwin, S., Macknight, R. C. & Macknight, R. C. 2011. Rapid analysis of seed size in *Arabidopsis* for mutant and QTL discovery. *Plant Methods* [Online], 7.

Hu, W., Yang, H., Tie, W., Yan, Y., Ding, Z., Liu, Y., et al. 2017. Natural Variation in Banana Varieties Highlights the Role of Melatonin in Postharvest Ripening and Quality. *J Agric Food Chem,* 65**,** 9987-9994

Huang, Z., Footitt, S. & Finch-Savage, W. E. 2014. The effect of temperature on reproduction in the summer and winter annual *Arabidopsis thaliana* ecotypes Bur and Cvi. *Ann Bot,* 113**,** 921-9

Huang, Z., Xie, W., Wang, M., Liu, X., Ashraf, U., Qin, D., et al. 2020. Response of rice genotypes with differential nitrate reductase-dependent NO synthesis to melatonin under ZnO nanoparticles' (NPs) stress. *Chemosphere,* 250

Jansen, M., Gilmer, F., Biskup, B., Nagel, K. A., Rascher, U., Fischbach, A., et al. 2009. Simultaneous phenotyping of leaf growth and chlorophyll fluorescence via GROWSCREEN FLUORO allows detection of stress tolerance in *Arabidopsis thaliana* and other rosette plants. *Funct Plant Biol***,** 902-914

Jin, J., Tian, F., Yang, D. C., Meng, Y. Q., Kong, L., Luo, J., et al. 2017. PlantTFDB 4.0: toward a central hub for transcription factors and regulatory interactions in plants. *Nucleic Acids Res,* 45**,** D1040-d1045

Josse, E. M., Gan, Y., Bou-Torrent, J., Stewart, K. L., Gilday, A. D., Jeffree, C. E., et al. 2011. A DELLA in disguise: SPATULA restrains the growth of the developing *Arabidopsis* seedling. *Plant Cell,* 23**,** 1337-51

Jubault, M., Lariagon, C., Simon, M., Delourme, R. & Manzanares-Dauleux, M. J. 2008. Identification of quantitative trait loci controlling partial clubroot resistance in new mapping populations of *Arabidopsis thaliana*. *Theor Appl Genet,* 117**,** 191-202

Julkowska, M. M., Klei, K., Fokkens, L., Haring, M. A., Schranz, M. E. & Testerink, C. 2016. Natural variation in rosette size under salt stress conditions corresponds to developmental

differences between *Arabidopsis* accessions and allelic variation in the LRR-KISS gene. *J Exp Bot,* 67**,** 2127-2138

Kang, K., Kong, K., Park, S., Natsagdorj, U., Kim, Y. S. & Back, K. 2011. Molecular cloning of a plant *N*-acetylserotonin methyltransferase and its expression characteristics in rice. *J Pineal Res,* 50**,** 304-9

Katori, T., Ikeda, A., Iuchi, S., Kobayashi, M., Shinozaki, K., Maehashi, K., et al. 2010. Dissecting the genetic control of natural variation in salt tolerance of *Arabidopsis thaliana* accessions. *J Exp Bot,* 61**,** 1125-38

Kijima, S. T., Staiger, C. J., Katoh, K., Nagasaki, A., Ito, K. & Uyeda, T. Q. P. 2018. *Arabidopsis* vegetative actin isoforms, *AtACT2* and *AtACT7*, generate distinct filament arrays in living plant cells. *Sci Rep,* 8**,** 4381

Koornneef, M., Alonso-Blanco, C. & Vreugdenhil, D. 2004. Naturally occurring genetic variation in *Arabidopsis thaliana*. *Annu Rev Plant Biol,* 55**,** 141-72

Koskela, M. M., Brunje, A., Ivanauskaite, A., Grabsztunowicz, M., Lassowskat, I., Neumann, U., et al. 2018. Chloroplast Acetyltransferase NSI Is Required for State Transitions in *Arabidopsis thaliana*. *Plant Cell,* 30**,** 1695-1709

Kudo, M., Kidokoro, S., Yoshida, T., Mizoi, J., Todaka, D., Fernie, A. R., et al. 2017. Double overexpression of DREB and PIF transcription factors improves drought stress tolerance and cell elongation in transgenic plants. *Plant Biotechnol J,* 15**,** 458-471

Lee, H. Y. & Back, K. 2018. Melatonin induction and its role in high light stress tolerance in *Arabidopsis thaliana*. *J Pineal Res,* 65**,** e12504

Lee, H. Y., Byeon, Y., Lee, K., Lee, H. J. & Back, K. 2014. Cloning of Arabidopsis serotonin N-acetyltransferase and its role with caffeic acid O-methyltransferase in the biosynthesis of melatonin in vitro despite their different subcellular localizations. *J Pineal Res,* 57**,** 418-26

Lee, H. Y., Byeon, Y., Tan, D. X., Reiter, R. J. & Back, K. 2015. *Arabidopsis* serotonin *N*-acetyltransferase knockout mutant plants exhibit decreased melatonin and salicylic acid levels resulting in susceptibility to an avirulent pathogen. *J Pineal Res,* 58**,** 291-9

Lee, H. Y., Lee, K. & Back, K. 2019. Knockout of *Arabidopsis* Serotonin *N*-Acetyltransferase-2 Reduces Melatonin Levels and Delays Flowering. *Biomolecules,* 9

Lee, K., Lee, H. Y. & Back, K. 2018. Rice histone deacetylase 10 and *Arabidopsis* histone deacetylase 14 genes encode *N*-acetylserotonin deacetylase, which catalyzes conversion of *N*-

acetylserotonin into serotonin, a reverse reaction for melatonin biosynthesis in plants. *J Pineal Res,* 64

Li, C., He, Q., Zhang, F., Yu, J., Li, C., Zhao, T., et al. 2019a. Melatonin enhances cotton immunity to *Verticillium* wilt via manipulating lignin and gossypol biosynthesis. *Plant J,* 100**,** 784-800

Li, C., Tan, D. X., Liang, D., Chang, C., Jia, D. & Ma, F. 2015. Melatonin mediates the regulation of ABA metabolism, free-radical scavenging, and stomatal behaviour in two *Malus* species under drought stress. *J Exp Bot,* 66**,** 669-80

Li, C., Zhao, Q., Gao, T., Wang, H., Zhang, Z., Liang, B., et al. 2018. The mitigation effects of exogenous melatonin on replant disease in apple. *J Pineal Res,* 65**,** e12523

Li, H., Chang, J., Chen, H., Wang, Z., Gu, X., Wei, C., et al. 2017a. Exogenous Melatonin Confers Salt Stress Tolerance to Watermelon by Improving Photosynthesis and Redox Homeostasis. *Front Plant Sci,* 8**,** 295

Li, H., Mo, Y., Cui, Q., Yang, X., Guo, Y., Wei, C., et al. 2019b. Transcriptomic and physiological analyses reveal drought adaptation strategies in drought-tolerant and -susceptible watermelon genotypes. *Plant Sci,* 278**,** 32-43

Li, W., Nguyen, K. H., Chu, H. D., Ha, C. V., Watanabe, Y., Osakabe, Y., et al. 2017b. The karrikin receptor KAI2 promotes drought resistance in *Arabidopsis thaliana*. *PLoS Genet,* 13**,** e1007076

Lu, R., Liu, Z., Shao, Y., Sun, F., Zhang, Y., Cui, J., et al. 2019. Melatonin is responsible for rice resistance to rice stripe virus infection through a nitric oxide-dependent pathway. *Virol J,* 16**,** 141

Mandal, M. K., Suren, H., Ward, B., Boroujerdi, A. & Kousik, C. 2018. Differential roles of melatonin in plant-host resistance and pathogen suppression in cucurbits. *J Pineal Res,* 65**,** e12505

Mcnamara, M. & Hennessy, R. 2010. THE GEOLOGY OF THE BURREN REGION, CO. CLARE, IRELAND. *Project NEEDN, The Burren Connect Project, 1st Edn.(Ennistymon: Clare County Council),*

Meng, Y., Shuai, H., Luo, X., Chen, F., Zhou, W., Yang, W., et al. 2016. Karrikins: Regulators Involved in Phytohormone Signaling Networks during Seed Germination and Seedling Development. *Front Plant Sci,* 7**,** 2021

Okazaki, M., Higuchi, K., Aouini, A. & Ezura, H. 2010. Lowering intercellular melatonin levels by transgenic analysis of indoleamine 2,3-dioxygenase from rice in tomato plants. *J Pineal Res,* 49**,** 239-47

Osborne, B., Black, K., Lanigan, G., Perks, M. & Clabby, G. 2003. Survival on the Exposed Limestone Pavement in the Burren: Photosynthesis and Water Relations of Three Co-Occurring Plant Species. *Biology and Environment: Proceedings of the Royal Irish Academy,* 103B(3)**,** 125-137

Park, S., Lee, D. E., Jang, H., Byeon, Y., Kim, Y. S. & Back, K. 2013. Melatonin-rich transgenic rice plants exhibit resistance to herbicide-induced oxidative stress. *J Pineal Res,* 54**,** 258-63

Petijova, L., Jurcackova, Z. & Cellarova, E. 2020. Computational screening of miRNAs and their targets in leaves of *Hypericum* spp. by transcriptome-mining: a pilot study. *Planta,* 251**,** 49

Pucciariello, C., Parlanti, S., Banti, V., Novi, G. & Perata, P. 2012. Reactive oxygen species-driven transcription in *Arabidopsis* under oxygen deprivation. *Plant Physiol,* 159**,** 184-96

Rahmati Ishka, M., Brown, E., Weigand, C., Tillett, R. L., Schlauch, K. A., Miller, G., et al. 2018. A comparison of heat-stress transcriptome changes between wild-type *Arabidopsis* pollen and a heat-sensitive mutant harboring a knockout of cyclic nucleotide-gated cation channel 16 (cngc16). *BMC Genomics,* 19**,** 549

Raithel, S., Johnson, L., Galliart, M., Brown, S., Shelton, J., Herndon, N., et al. 2016. Inferential considerations for low-count RNA-seq transcripts: a case study on the dominant prairie grass *Andropogon gerardii*. *BMC Genomics,* 17**,** 140

Re, D. A., Capella, M., Bonaventure, G. & Chan, R. L. 2014. *Arabidopsis AtHB7* and *AtHB12* evolved divergently to fine tune processes associated with growth and responses to water stress. *BMC Plant Biol,* 14**,** 150

Robinson, M. D., Mccarthy, D. J. & Smyth, G. K. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics,* 26**,** 139-40

Robinson, M. D. & Oshlack, A. 2010. A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biol,* 11**,** R25

Rosas, U., Mei, Y., Xie, Q., Banta, J. A., Zhou, R. W., Seufferheld, G., et al. 2014. Variation in *Arabidopsis* flowering time associated with cis-regulatory variation in CONSTANS. *Nat Commun,* 5**,** 3651

Sadak, M. S. & Bakry, B. A. 2020. Alleviation of drought stress by melatonin foliar treatment on two flax varieties under sandy soil. *Physiol Mol Biol Plants,* 26**,** 907-919

Schaarschmidt, S., Fischer, A., Zuther, E. & Hincha, D. K. 2020. Evaluation of Seven Different RNA-Seq Alignment Tools Based on Experimental Data from the Model Plant *Arabidopsis thaliana*. *Int J Mol Sci,* 21

Shi, H., Chen, K., Wei, Y. & He, C. 2016. Fundamental Issues of Melatonin-Mediated Stress Signaling in Plants. *Front Plant Sci,* 7**,** 1124

Shi, H., Chen, Y., Tan, D. X., Reiter, R. J., Chan, Z. & He, C. 2015a. Melatonin induces nitric oxide and the potential mechanisms relate to innate immunity against bacterial pathogen infection in *Arabidopsis*. *J Pineal Res,* 59**,** 102-8

Shi, H., Tan, D. X., Reiter, R. J., Ye, T., Yang, F. & Chan, Z. 2015b. Melatonin induces *class A1 heat-shock factors (HSFA1s)* and their possible involvement of thermotolerance in *Arabidopsis*. *J Pineal Res,* 58**,** 335-42

Simon, M., Loudet, O., Durand, S., Bérard, A., Brunel, D., Sennesal, F. X., et al. 2008. Quantitative trait loci mapping in five new large recombinant inbred line populations of *Arabidopsis thaliana* genotyped with consensus single-nucleotide polymorphism markers. *Genetics,* 178**,** 2253-2264

Simopoulos, A. P., Tan, D. X., Manchester, L. C. & Reiter, R. J. 2005. Purslane: a plant source of omega-3 fatty acids and melatonin. *J Pineal Res,* 39**,** 331-2

Sivitz, A. B., Hermand, V., Curie, C. & Vert, G. 2012. *Arabidopsis* bHLH100 and bHLH101 control iron homeostasis via a FIT-independent pathway. *PLoS One,* 7**,** e44843

Tsugama, D., Yoon, H. S., Fujino, K., Liu, S. & Takano, T. 2019. Protein phosphatase 2A regulates the nuclear accumulation of the *Arabidopsis* bZIP protein VIP1 under hypo-osmotic stress. *J Exp Bot,* 70**,** 6101-6112

Van Leeuwen, H., Kliebenstein, D. J., West, M. A., Kim, K., Van Poecke, R., Katagiri, F., et al. 2007. Natural variation among *Arabidopsis thaliana* accessions for transcriptome response to exogenous salicylic acid. *Plant Cell,* 19**,** 2099-110

Van Veen, H., Vashisht, D., Akman, M., Girke, T., Mustroph, A., Reinen, E., et al. 2016. Transcriptomes of Eight *Arabidopsis thaliana* Accessions Reveal Core Conserved, Genotype- and Organ-Specific Responses to Flooding Stress. *Plant Physiol,* 172**,** 668-689

Wan, J., Zhang, P., Wang, R., Sun, L., Ju, Q. & Xu, J. 2018. Comparative physiological responses and transcriptome analysis reveal the roles of melatonin and serotonin in regulating growth and metabolism in *Arabidopsis*. *BMC Plant Biol.,* 18**,** 362

Wang, M., Duan, S., Zhou, Z., Chen, S. & Wang, D. 2019. Foliar spraying of melatonin confers cadmium tolerance in *Nicotiana tabacum* L. *Ecotoxicol Environ* 170**,** 68-76

Wang, Q., An, B., Shi, H., Luo, H. & He, C. 2017. High Concentration of Melatonin Regulates Leaf Development by Suppressing Cell Proliferation and Endoreduplication in *Arabidopsis*. *Int J Mol Sci,* 18

Webb, D. A. 1961. Noteworthy Plants of the Burren: A Catalogue Raisonné. *Proceedings of the Royal Irish Academy. Section B: Biological, Geological, and Chemical Science,* 62**,** 117-34

Webb, D. A. & Scannell, M. J. 1983. *Flora of Connemara and the Burren,* CUP Archive.

Weeda, S., Zhang, N., Zhao, X., Ndip, G., Guo, Y., Buck, G. A., et al. 2014. *Arabidopsis* transcriptome analysis reveals key roles of melatonin in plant defense systems. *PLoS One,* 9**,** e93462

Wei, J., Li, D. X., Zhang, J. R., Shan, C., Rengel, Z., Song, Z. B., et al. 2018. Phytomelatonin receptor PMTR1-mediated signaling regulates stomatal closure in *Arabidopsis thaliana*. *J Pineal Res,* 65**,** e12500

Wintermans, P. C., Bakker, P. A. & Pieterse, C. M. 2016. Natural genetic variation in *Arabidopsis* for responsiveness to plant growth-promoting rhizobacteria. *Plant Mol Biol,* 90**,** 623-34

Yang, B., Song, Z., Li, C., Jiang, J., Zhou, Y., Wang, R., et al. 2018. RSM1, an *Arabidopsis* MYB protein, interacts with HY5/HYH to modulate seed germination and seedling development in response to abscisic acid and salinity. *PLoS Genet,* 14**,** e1007839

Zhang, N., Sun, Q., Li, H., Li, X., Cao, Y., Zhang, H., et al. 2016. Melatonin Improved Anthocyanin Accumulation by Regulating Gene Expressions and Resulted in High Reactive Oxygen Species Scavenging Capacity in Cabbage. *Front Plant Sci,* 7**,** 197

Zhang, X., Chen, Y., Wang, Z. Y., Chen, Z., Gu, H. & Qu, L. J. 2007. Constitutive expression of *CIR1 (RVE2)* affects several circadian-regulated processes and seed germination in *Arabidopsis*. *Plant J,* 51**,** 512-25

Zhang, Z., Hu, Q., Liu, Y., Cheng, P., Cheng, H., Liu, W., et al. 2019. Strigolactone represses the synthesis of melatonin, thereby inducing floral transition in *Arabidopsis thaliana* in an FLC-dependent manner. *J Pineal Res,* 67**,** e12582

Zhao, C., Hanada, A., Yamaguchi, S., Kamiya, Y. & Beers, E. P. 2011. The *Arabidopsis* Myb genes MYR1 and MYR2 are redundant negative regulators of flowering time under decreased light intensity. *Plant J,* 66**,** 502-15

Zia, S. F., Berkowitz, O., Bedon, F., Whelan, J., Franks, A. E. & Plummer, K. M. 2019. Direct comparison of *Arabidopsis* gene expression reveals different responses to melatonin versus auxin. *BMC Plant Biol,* 19**,** 567

Chapter 4: Melatonin restores swarming under ethanol treatment in *Pseudomonas aeruginosa* PAO1 but does not affect the growth of cadmium-tolerant plant growth-promoting rhizobacteria

4.1 Abstract

The communication between microorganisms and their hosts includes an array of signal molecules. Melatonin is an indole that is considered to be ancient due to conservation across all kingdoms. In the plant context, melatonin contributes to disease resistance against pathogenic microbes and enhanced plant growth upon colonization by plant growth promoting rhizobacteria (PGPRs). A few recent studies have shown that melatonin could potentially be a key signal in the interkingdom signalling. Assessing the response of microbes to melatonin in vitro without plants is an important first step in determining this. The effect of melatonin application was tested on the growth of recently identified plant-growth promoting rhizobacteria (PGPRs) in this study. The quorum-regulated swarming motility of the reference strain of the human and plant pathogen *Pseudomonas aeruginosa* PAO1 was also tested to further our understanding of melatonin as a signal. It was found that the growth of PGPRs was not affected by the melatonin treatments; however, melatonin treatment (10 µM) was able to significantly restore swarming motility upon low ethanol treatment (0.1% v/v). These initial findings are worthy of further investigation in terms of the conditions or mechanisms, whereby the PGPRs potentially respond to melatonin treatment and the underlying processes of melatonin-mediated modulation of motility behaviour of *P. aeruginosa* PAO1. Furthermore, the effect of melatonin on the microbes in interaction with the host plants needs to be investigated.

Keywords: Melatonin, PGPRs, *P. aeruginosa* PAO1, swarming

4.2 Introduction

The cross-kingdom signalling between microorganisms and their plant hosts involves a repertoire of small molecules such as secondary metabolites, hormones and quorum sensing (QS) signals (Hughes and Sperandio, 2008). The communications via these signals dictate the beneficial or the pathogenic nature of the host-microbial interaction. In the case of beneficial interactions, the plant-associated endo- and exophytic rhizospheric microbes,

commonly called plant growth promoting rhizobacteria (PGPR), play an important role. The benefits conferred by the plant-associated microbes in improving host fitness and stress tolerance are well characterized (Berendsen et al., 2012).

Mechanisms of action include utilising hormones such as the tryptophan-based indole derivative auxin (indole-3-acetic acid, IAA) through either microbial-production or homeostasis modulation in plants (Poupin et al., 2016). Metabolic production by microbes is attributed to potential communication and interaction with plant host (Brader et al., 2014). *Pseudomonas fluorescens* strain RRLJ 008 produces phenazines that play an integral role in suppressing the progression of fungal diseases in plants (Boruah and Kumar, 2002). Plant secondary metabolites, such as, rutin, phenolics and flavonoids, released as exudates may also act as chemo-attractants to recruit beneficial PGPRs for efficient host colonization by enhancing growth or flagellar motility such as swarming (De Weert et al., 2002, Singh et al., 2016) establishing a fruitful association benefiting both the parties (Doornbos et al., 2011). In fact, motility towards an exudate, is among the many important determinants of a successful colonisation by PGPRs or infection by pathogenic microbes (De Weert et al., 2002). One type of motility behaviour commonly exhibited by flagellated microbes is swarming. This is a rapid multicellular bacterial movement across a surface directed by rotating flagella (Yan et al., 2019). Bacteria that can swarm have been shown to efficiently colonise host roots. For example, swarming motility by the PGPR, *Bacillus subtilis SWR01* was shown to play a major role during tomato root colonisation by an increased colonisation efficacy of up to 79% as compared to the flagella-defective mutants unable to swarm which colonised only up to 15% (Gao et al., 2016).

In case of pathogenic interactions, secreted host secondary metabolites also play a role in interfering with bacterial quorum-sensing (QS) systems (Gao et al., 2003, Alagarasan and Aswathy, 2017). This is a sophisticated cell-to-cell communication employed by bacteria to enable virulence. Targeting the bacterial quorum-sensing regulated traits and machinery, which also includes swarming motility, is suggested to be a strategy by plants for fending off pathogenic microbes (Alagarasan and Aswathy, 2017). It has been previously reported that indole inhibits quorum sensing-controlled virulence factors and phenotypes such as swarming motility in a range of quorum-biosensors, *Chromobacterium violaceum* ATCC 12472, *Pseudomonas aeruginosa* and *Acinetobacter oleivorans* DR1(Chu et al., 2012, Kim and Park, 2013, Hidalgo-Romano et al., 2014). In light of this, the emerging role of melatonin in plant-microbe interactions is promising to study.

Melatonin (*N*-acetyl-5-methoxytryptamine) is an ancient tryptophan-derivative indoleamine that is conserved across many taxa such as mammals, plants, bacteria and fungi (Lerner et al., 1960, Dubbels et al., 1995, Hattori et al., 1995, Tilden et al., 1997, Rodriguez-Naranjo et al., 2012). Melatonin is classified as a potent antioxidant and a potential signalling molecule, based on its primary and secondary functions (acquired as a result of evolution) (Tan et al., 2010). As an antioxidant, melatonin detoxifies stress-induced reactive oxygen species (ROS) either by direct scavenging or indirectly activating anti-oxidant enzymatic activities which ultimately protects organisms from deleterious effects of oxidative stress. Melatonin is a much more efficient antioxidant compared to the classic antioxidants such as vitamin C and E, where one molecule of melatonin can remarkably scavenge up to 10 ROS (Tan et al., 2015).

In plants, melatonin acts as a growth regulator and alleviates different kinds of abiotic and biotic stresses mainly by its detoxification capability of ROS (Yin et al., 2013, Bajwa et al., 2014, Shi et al., 2015). Melatonin has conferred tolerance to a range of abiotic stress factors in plants such as salinity, temperature extremes and heavy metals such as cadmium and zinc (Arnao and Hernández-Ruiz, 2009, Bajwa et al., 2014, Shi et al., 2015, Li et al., 2017, Wang et al., 2019). The effect of melatonin on plant pathogenic microbes and stress tolerance in plants has been the subject of several studies in recent years (Lee et al., 2014, Mandal et al., 2018, Wei et al., 2018, Liu et al., 2019). Melatonin activates plant defences against *Pseudomonas syringae* pv. tomato DC3000 by inducing defence gene expression such as pathogenesis-related genes (*PR1* and *PR5*), plant defensin, *PDF1.2* and salicylic acid (SA)-related genes such as *isochorismate synthase* 1, ICS1 in *Arabidopsis thaliana* and tobacco (Lee et al., 2015). SA is a plant hormone and an internal defence signalling molecule which is required for the pattern-triggered immunity (PTI), effector-triggered immunity (ETI) and systemic acquired resistance (SAR) in plants (Delaney et al., 1994). Direct impact of melatonin on the motility behaviour of pathogenic microbes has also been reported to confer plant disease resistance (Chen et al., 2018). However, the underlying mode of action in directly affecting the suppression of plant pathogenesis is currently unknown.

Melatonin biosynthesis has been reported in cyanobacteria, algae (Byeon et al., 2013, Byeon et al., 2015); as well as various genera of bacteria such as *Bacillus*, *Pseudomonas*, *Agrobacterium* and *Rhodospirillum* (Manchester et al., 1995, Jiao et al., 2016, Ma et al., 2016). Melatonin production and the biosynthetic pathway have also been reported in commercially available baker's yeast (*Saccharomyces cerevisiae*) (Sprenger J, 1999,

Germann et al., 2016, Muniz-Calvo et al., 2019). Two recent reports have for the first time identified the in vitro melatonin synthesis capability in endophytic bacteria, *Bacillus amyloliquefaciens* SB-9, *Agrobacterium tumefaciens* CS-30 and *Pseudomonas fluorescens* RG11(Jiao et al., 2016, Ma et al., 2016). The endogenous melatonin synthesis was significantly enhanced upon root colonization by endophytic bacterium *Bacillus amyloliquefaciens* SB-9 in the host plant grapevine *(Vitis labruscana* 'summer black') roots under salt and drought stress (Jiao et al., 2016). These are also the only studies so far reporting the link between melatonin and PGPR and point to a novel mode of communication between endophytes and host plants via melatonin. This is similar to the communication observed for the commensal gut microbiome with the host gut tissues where melatonin was utilised as a signal to maintain the homeostasis of intestinal function to maintain the homeostasis of intestinal function (Mukherji et al., 2013). The swarming motility behaviour was affected by exogenous melatonin circadian-dependently in the commensal human gut bacterium, *Enterobacter aerogenes* (Paulose et al., 2016). However, relative to plants and animals, little knowledge exists regarding the roles of melatonin in microbes (Jiao et al., 2016, Liu et al., 2016, Ma et al., 2016, Paulose et al., 2016).

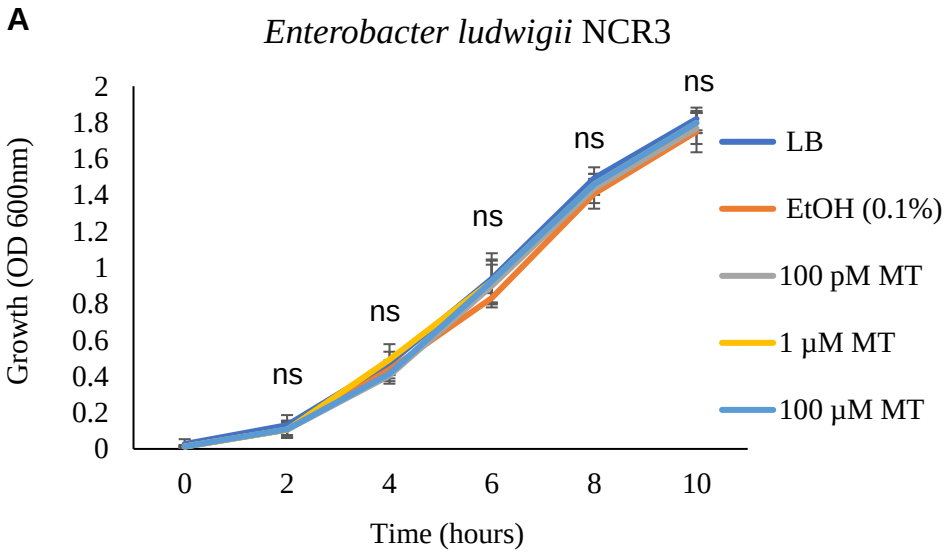
In this study, in vitro growth responses of a range of microbes, in terms of absorbance (OD 600nm) over time and swarming motility, were assessed.The PGPRs tested in this study were recently identified as cadmium-tolerant *Enterobacter ludwigii* NCR3, *Bacillus cereus* LCR12 and *Rhodococcus erythropolis* NSX2 (Egidi et al., 2016a, Egidi et al., 2016b, Egidi et al., 2016c). The human and plant pathogen *Pseudomonas aeruginosa* PAO1 was also tested. A range of concentrations of melatonin were applied in the growth medium to investigate whether these microbes are responsive to this ROS-scavenging molecule under stress. This is the first study to examine the effects of melatonin on these microbes. The knowledge can help to advance our understanding toward establishing melatonin as an important molecule for agronomic applications.

4.3 Results

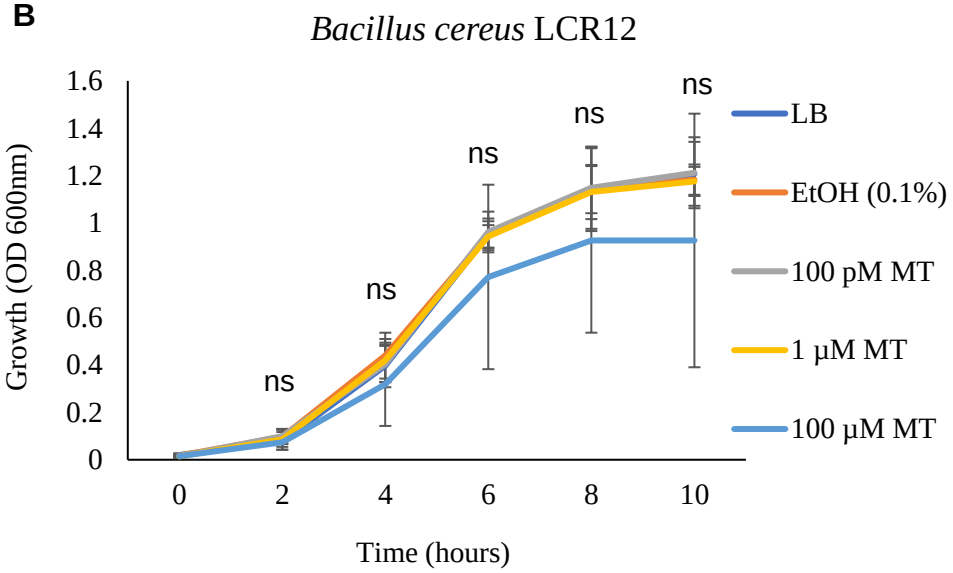
4.3.1 Effect of melatonin on growth of PGPRs and *Pseudomonas aeruginosa* PAO1 in response to melatonin concentrations

In order to assess whether the bacteria (PGPRs) and PAO1 are responsive to melatonin under ethanol treatment (EtOH 0.1% v/v), the growth of *E. ludwigii* NCR3, *B. cereus* LCR12 and *R. erythropolis* NSX2 was monitored spectrophotometrically over time in

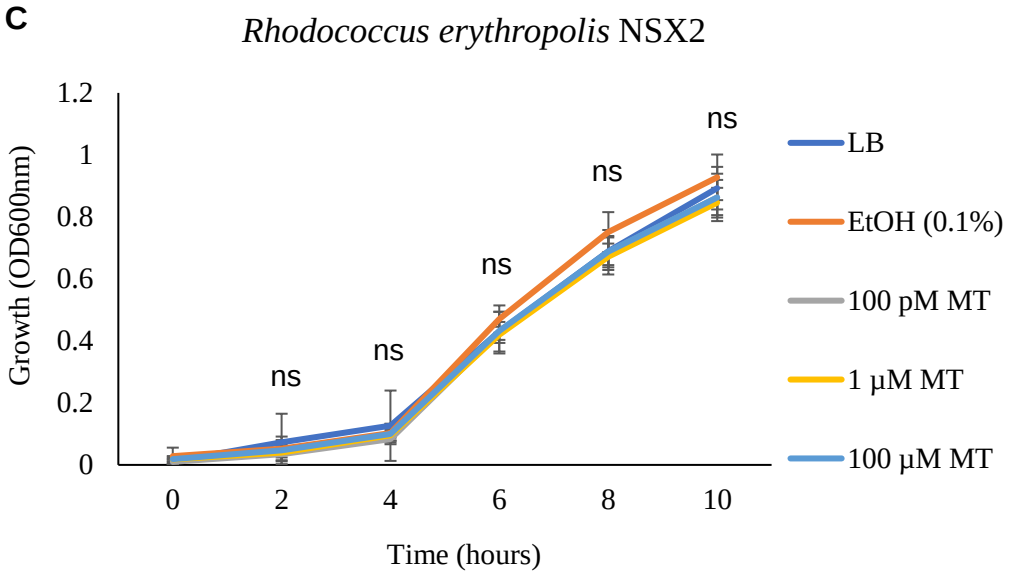
response to melatonin. Growth in response to three concentrations of melatonin (100 pM, 1 µM and 100 µM with standardised final ethanol concentration at (EtOH 0.1 % v/v) were compared to the ethanol-only treatment (EtOH 0.1% v/v) and culture medium with no ethanol Luria-Bertani (LB) broth. Ethanol was used as a solvent for dissolving melatonin as per the instructions by the manufacturer (M5250, Sigma Aldrich Pty. Ltd.). LB media was chosen because nutrient media such as LB, nutrient broth medium (NB) and Mueller Hinton Broth (MHB) have previously been used in studies reporting the effects of exogenous melatonin on microbes in vitro (Tekbas et al., 2008, Paulose et al., 2016, Chen et al., 2018, Chen et al., 2019). The concentration of 100 pM was chosen as it had previously shown to affect *E. aerogenes* (Paulose et al., 2016) and extended to 1 µM and 100 µM in order to observe any potential concentration-dependent effects of melatonin on the tested microbes. The effect of ethanol treatment alone was first evaluated. No inhibitory effect of ethanol treatment alone was observed on the growth of microbes as indicated by OD_{600} at all tested time-points, compared to the plain culture medium (LB), indicating that ethanol at 0.1% v/v is not stressful to the growth of the PGPRs and PAO1. The effect of melatonin addition under ethanol treatment was then assessed. It was observed that there were no statistically significant (p>0.05; one-way ANOVA) effect of melatonin treatment on growth of the PGPRs and PAO1 as indicated by the OD_{600} at all indicated time-points as compared to the ethanol-only treatment (EtOH 0.1% v/v) and plain culture medium (LB) (Figure 4.1).



B


Bacillus cereus LCR12
Growth (OD 600nm)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ns
ns
ns
ns
ns
0
2
4
6
8
10
Time (hours)
LB
EtOH (0.1%)
100 pM MT
1 µM MT
100 µM MT

C


Rhodococcus erythropolis NSX2
Growth (OD600nm)
1.2
1
0.8
0.6
0.4
0.2
0
ns
ns
ns
ns
ns
0
2
4
6
8
10
Time (hours)
LB
EtOH (0.1%)
100 pM MT
1 µM MT
100 µM MT

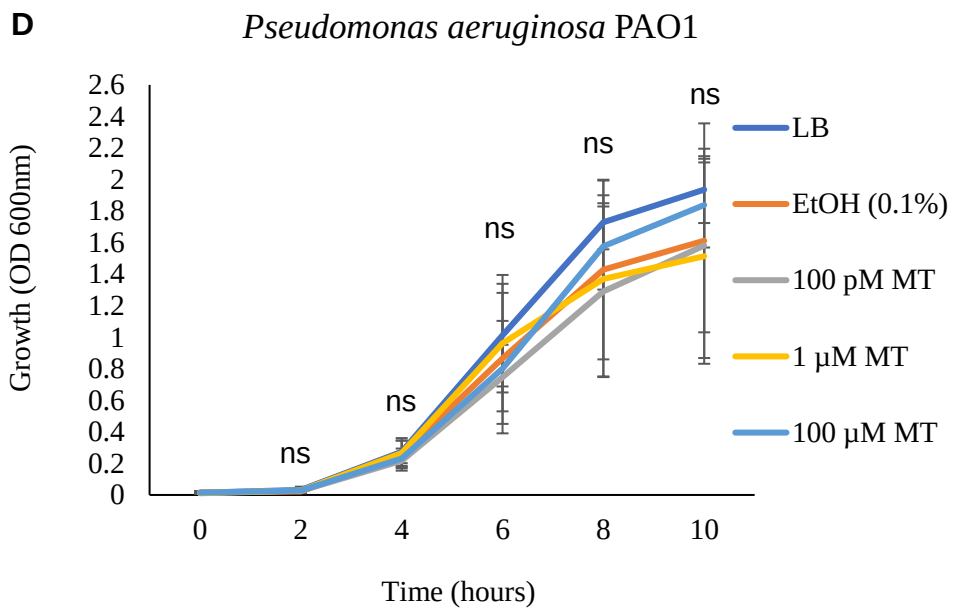


Figure 4.1: Effect of melatonin on the growth rate of PGPRs in response to melatonin concentrations. Bacterial growth was determined under optimum growth temperature (23°C for PGPRs and 37°C for PAO1) by assessing the absorbance (Optical density (OD) at 600 nm) over time (hours) for **(A)** *Enterobacter ludwigii* NCR3 **(B)** *Bacillus cereus* LCR12 **(C)** *Rhodococccus erythropolis* NSX2 **(D)** *Pseudomonas aeruginosa* PAO1 in the culture medium, Luria-Bertani (LB) broth and absence or presence of different concentrations of melatonin as indicated (each with a standardised final concentration of ethanol at 0.1% (v/v). Results are mean of absorbance with experiment conducted in triplicates (three biological replicates and nine technical replicates per strain). Error bars represent standard deviations. Statistical significance ($p < 0.05$) was determined in R (version R-3.5.2) by one-way analysis of variance (ANOVA) followed by post-hoc Tukey test for each time point. No statistical significance is indicated by the letters 'ns' (no significance).

4.3.2 Effect of melatonin on the swarming motility of *Pseudomonas aeruginosa* PAO1

The tested PGPRs in this study were not able to swarm under the swarming conditions indicating that they are not motile (Figure S4.1). That is the reason the effect of melatonin was tested specifically on the swarming motility behaviour of PAO1 to better understand the link between melatonin and QS (Figure 4.2). As no effects of melatonin were observed in the growth assays with previous concentrations, the concentration range was modified for swarm assay to include 1 nM and 10 µM. The maximum melatonin concentration (100 µM) was kept the same to avoid going into an inhibitory concentration range. To our surprise, ethanol treatment (EtOH 0.1% v/v) was found to be stressful to PAO1 by significantly decreasing the swarming pattern by 16.7% as compared to swarm media only

control (Figures 2.1, 2.2). Interestingly, this decrease in swarming pattern was restored by melatonin (10 µM). No significant inhibition of swarming motility was observed upon all the tested melatonin treatments as indicated by the swarming pattern and quantification of the surface area (Figures 2.1,2.2). In order to determine whether the concentrations did not have any negative effects such as anti-bacterial effect that would confound the analysis, the data on the growth of PAO1 tested over time with the highest melatonin concentration (100 µM) used in the swarming assay was investigated (Figure 4.1D). It confirmed that melatonin concentrations did not have any significant inhibitory effect on growth of PAO1 and any effect of melatonin on the swarming was solely due to its ability to affect swarming motility (Figure 2.3). Moreover, no differences in the growth rate upon ethanol treatment with other treatments were observed indicating that the stressful impact of ethanol can be regarded as low and specifically targets motility behaviour.

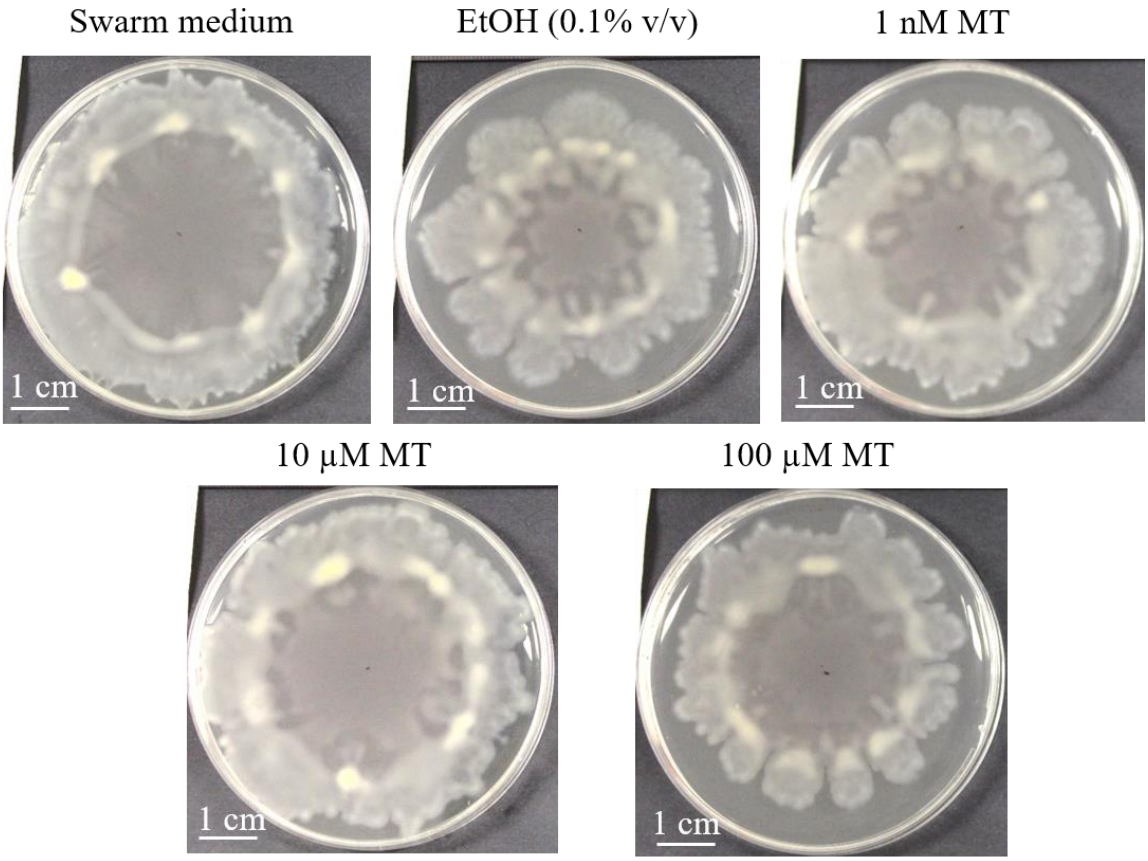


Figure 4.2: Swarm growth of *Pseudomonas aeruginosa* PAO1 in swarm medium only, ethanol (0.1% v/v) and treatments of melatonin (each with a standardised final concentration of ethanol (0.1% v/v). Representative images shown for swarming pattern after 24 hours incubation at 37 °C. Assay was conducted with three independent cultures. Scale bar = 1 cm.

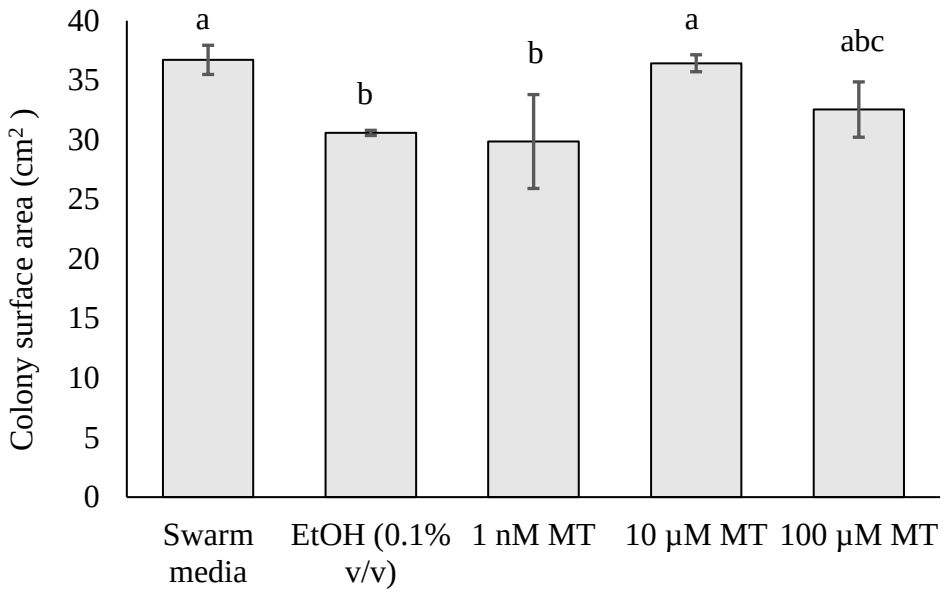


Figure 4.3: Swarm growth response (quantified as colony surface area (cm^2)) of *Pseudomonas aeruginosa* PAO1 to swarm media only, ethanol (EtOH 0.1% v/v) and treatments of melatonin (each with a standardised final concentration of ethanol (EtOH 0.1% v/v). Results are mean of colony surface area with experiment conducted in triplicates (three bio replicates and nine technical replicates per strain). Error bars represent standard deviations. Statistical significance ($p < 0.05$) was determined in R (version R-3.5.2) by one-way analysis of variance (ANOVA) followed by post-hoc Tukey. Different letters denote statistical significance.

4.4 Discussion

Soil microbes are continually in competition under nutrient-limiting conditions. Plant roots secrete and exude compound such as metabolites, organic acids, sugars and amino acids into the soil via rhizodeposition. These plant-derived compounds are used by microbes as substrates which greatly enhance the biomass and the activity of these microbes in the soil. In turn, plants benefit through selectively recruitment of beneficial microbes for efficient root colonization and confer multifarious beneficial effects such as enhanced growth and stress resistance (Bais et al., 2004, Doornbos et al., 2011).

Though it has not been established whether melatonin, a tryptophan-derived plant secondary metabolite, is part of the composition of root exudates, two recent studies have shown that beneficial endophytic bacteria are capable of producing melatonin and enhancing host plant fitness (Jiao et al., 2016, Ma et al., 2016). It is yet to be shown that plants as a part of this bilateral communication utilise melatonin to recruit beneficial

bacteria. Microbial growth at the host root is correlated with the ability to utilise root exudates (Toljander et al., 2007, Zhalnina et al., 2018).

Here, we report that two recently identified PGPRs were not responsive to melatonin treatment in vitro in terms of their growth capability over time (Figure 4.1). Recently, beneficial effects of melatonin toward plant beneficial bacteria such as *Pseudomonas fluorescence* RG11 and *Bacillus amyloliquefaciens* SB-9 have only been previously observed in vivo, i.e. during the association with their host plants (Ma et al., 2016). This can possibly indicate that melatonin exerts its action when PGPRs are in association with their host plants. This could be the reason that in this study there were no significant growth effects of melatonin on PGPRs in vitro in the absence of the host plant. Previous studies have reported the biocidal activities of exogenously applied melatonin (0.13 µM – 0.53 µM) and in vitro toward a range of human and plant pathogenic microbial species (Tekbas et al., 2008, Chen et al., 2019, Moustafa-Farag et al., 2019). However, there have also been cases where application of melatonin in vitro did not have any significant effect, for example, the growth and germination over time of green mould fungus, *Penicillium digitatum* on potato dextrose agar (PDA) growth medium was not affected by melatonin (50 µM) in vitro, but significant disease promoting effects of melatonin application applied as a soaking solution prior to fungal infection were only observed when the citrus fruit was infected with *P. digitatum* (Lin et al., 2019).

Similarly, melatonin (0.86 µM) did not show any anti-fungal effect against *Botrytis cinerea* in PDA plates (Wang et al., 2001), but melatonin infiltration (100 µM) led to disease resistance in *Botrytis cinerea*-infected tomato fruit (Liu et al., 2019). This is reasonable as melatonin treatment has shown to upregulate plant defence responses (Zia et al., 2019). The plant host might therefore be necessary to observe the effect of melatonin on PGPRs. In our study, the microbes were cultured and tested against melatonin in (LB) medium which is nutrient-rich. There are also few reports on the effect of melatonin on microbes where the culturing is undertaken in LB or other nutrient rich medium (Jiao et al., 2016, Paulose et al., 2016, Chen et al., 2018). A deliberate addition of L-tryptophan to the medium is required to study the production of tryptophan-derived compounds, for example, auxin by microorganisms (Ali et al., 2009, Erland and Saxena, 2019). Although LB medium contains beef and yeast extract as a source of amino acids and nitrogen, it does not contain the metabolic precursor of melatonin, L-tryptophan per se, so it is potentially unlikely that culturing of microbes in the nutrient rich medium would enable melatonin production. Although, this needs to be tested and confirmed by the quantification of melatonin. Moreover, analyses conducted by using minimal media usually give a limited picture in a

laboratory setting which cannot be extrapolated easily in in situ environment where tryptophan and other amino acids are already abundant through multiple sources such as in the form of root exudates (Moe, 2013).

Microbes rarely act alone in the rhizosphere. Large and complex bacterial and fungal communities surround the plants' roots (Philippot et al., 2013). Interestingly, it has been recently shown that microbial interactions significantly respond in terms of growth alteration (colony diameter) to chemicals in vitro when selected bacterial species forming the human lung microbiome were grown together on the same plate, as compared to when grown alone (Liu et al., 2017). Thus, a similar approach for assessing the growth behaviour of PGPRs in vitro in response to melatonin could provide more insights than using single strains. Moreover, melatonin application to soil infected with apple replant disease was shown to alter bacterial and fungal communities (Li et al., 2018). Similarly, secondary metabolites, coumarins, also altered root microbiome composition (Voges et al., 2019). It is thus possible that plants utilise the secondary metabolite, melatonin, to alter communities rather than single microbial strains that we used in our study. However, whether melatonin is secreted by plant roots needs to be tested.

Synthesis of melatonin by PGPRs has been recently reported (Jiao et al., 2016, Ma et al., 2016). It is also likely that the PGPRs in this study are capable of producing endogenous melatonin such that any external melatonin treatment did not have a significant effect. This hypothesis, however, remains speculative at this stage and requires further investigation. In this study, the quantification of melatonin was beyond the scope due to funding constraints. It is likely that associated PGPRs are cadmium tolerant due to the increased melatonin synthesis in these microbes as a result of bilateral communication between the host hyperaccumulator. In return these PGPRs deliver growth benefits to their hosts to balance the growth and stress tolerance. This has been recently demonstrated where *Burkholderia gladioli*, a heavy metal resistant PGPR, enhanced plant growth by reducing the bioavailability and uptake of cadmium in garden tomatoes (*Lycopersicon esculentum*) (Khanna et al., 2019). In this regard, a comparison of the tolerant PGPRs with a Cd-sensitive PGPR could provide more insights on to the effect of melatonin.

In plants, melatonin confers disease resistance by a variety of mechanisms which include activation of the plant hormone salicylic acid (SA) and mitogen activated protein kinases (MAPK) signalling cascades to upregulate expression of key defence related genes such as

plant defensin (*PDF1.2*) and pathogenesis-related gene *PR5* (Lee et al., 2014, Lee and Back, 2016, Mandal et al., 2018, Zia et al., 2019). More direct in vitro antibacterial effects of melatonin in *Xanthomonas oryzae* (*Xoo* strain PXO99) are observed in the form of inhibition of growth, biofilm formation and swimming motility (Chen et al., 2018). Transcriptome analysis of effect of melatonin on *Xoo* reveal that the underlying mechanisms of anti-bacterial effects are regulation of cell division, reduction of enzymatic activities related to metabolism and possibly depriving microbes of the iron in the environment by binding to it (Chen et al., 2018, Chen et al., 2019). Another possible mechanism of inhibition by melatonin could be targeting the quorum sensing system of the pathogenic microbes. This is the first study to explore this mechanism. QS is employed by bacteria for cell-to-cell communication which in turn activates a set of virulence determinants, such as gene expression of virulence genes, motility such as swarming and pigment production, for example, violacein (Koh et al., 2013). Recently, it was shown that caffeine possesses anti-quorum sensing properties by inhibiting the swarming motility in *Pseudomonas aeruginosa* PAO1 (Norizan et al., 2013). *P. aeruginosa* is a gram-negative, bacterium which is pathogenic to both animals and plants (Walker et al., 2004, De Bentzmann and Plesiat, 2011, Chahtane et al., 2018). In plants, *P. aeruginosa* PAO1 represses seed germination in *Arabidopsis thaliana* (Chahtane et al., 2018). However, a study has also shown that PAO1 exhibits commensal behaviour with *Arabidopsis* at theseedling stage by enhancing shoot fresh weight (approximately 1.75-fold) and lateral root numbers (approximately 2.8-fold) relative to uninoculated seedlings (Ortiz-Castro et al., 2011). From these two reports, it is clear that PAO1 is pathogenic or commensal dependent upon the plant developmental stage. However, it is unclear as to what other mechanisms or conditions are required for PAO1 to enable the switch from being pathogenic to beneficial to host plants. *Pseudomonas* spp. exhibit quorum sensing systems which are required for their virulence as well as beneficial growth promoting effects. Swarming motility in particular is activated by QS in *P. aeruginosa* (PAO1) (Heurlier et al., 2004). The experiment did not provide evidence that melatonin inhibited swarming motility in the current study. It indicates that quorum sensing system of the microbe might not be a potential target of melatonin to inhibit pathogenic infection (Figure 4.2-4.3). In fact, other mechanisms described above such as iron-binding to restrict pathogenesis as reported in the case of *X. oryzae* pv. *oryzae* could be more favourable (Chen et al., 2018). However, caution needs to be taken in interpreting the result as no additional QS molecules such as acyl-homoserine lactones were added to directly test the effect of melatonin on QS

system. This could be a future experimental approach for a detailed investigation of the link between melatonin and QS.

The finding in this study, that melatonin (10 µM) significantly restored the swarming motility which was otherwise inhibited by ethanol treatment is interesting (Figure 4.2 – 4.3). Firstly, it demonstrates that melatonin, at a certain concentration is capable of modulating the QS-regulated swarming motility behaviour in *P. aeruginosa* PAO1. Secondly, this effect is observable under ethanol treatment. It was surprising to see ethanol solvent control at a very low concentration (0.1% v/v) can affect the motility behaviour. However, our finding is partly in line with a recent study where sub-inhibitory concentration of ethanol (1 % v/v) repressed swarming motility in *P. aeruginosa* strain PA14 by a sophisticated mechanism of targeting the flagellar stators (Lewis et al., 2019). In the context of trans-kingdom signalling, it is probable that plants use melatonin to potentially recruit *P. aeruginosa* toward their roots by restoring the otherwise reduced swarming motility under ethanol stress. The benefit that plants would potentially obtain by this association would be enhanced growth promotion. Further investigation needs to be done to look at the effect of other abiotic stresses common in the rhizosphere such as heavy metals and salinity.

4.5 Conclusion

In this study, the effect of exogenous melatonin was tested in vitro toward a range of plant-associated microbes. While the microbes showed no response to melatonin in terms of their growth, *P. aeruginosa* PAO1 exhibited improved swarming motility upon melatonin treatment under low ethanol treatment. Further interaction studies are now required on the association of these microbes in the presence of their host plants both in vitro and in vivo. This will help to delineate the role of melatonin as a potential signal in interkingdom signalling. This knowledge is particularly important to reap agricultural benefits from melatonin such as improved crop yield and protection from stresses.

4.6 Materials and Methods

4.6.1 Bacterial strains and growth conditions

Bacterial strains used in this study are plant growth-promoting rhizobacteria (PGPRs), *Enterobacter ludwigii* NCR3, *Bacillus cereus* LCR12, *Rhodococcus erythropolis* NSX2

[49-51] and *Pseudomonas aeruginosa* PAO1 [84]. The microbes were kindly provided by Dr. Jennifer Wood, Prof. Ashley Franks laboratory (Department of Physiology, Anatomy and Microbiology, La Trobe University). All the microbes were routinely cultured on Luria-Bertani (LB) agar medium 1% tryptone (w/v) (Oxoid, LP0042, RG24 8PW), Basingstoke, United Kingdom), 0.5% yeast extract (w/v) (Oxoid, LP0021, RG24 8PW), Basingstoke, United Kingdom, 1% NaCl (w/v) (Chem-supply, SAO46, Gillman SA 5013, Australia), 1.5% agar (w/v) and overnight cultures were prepared with LB liquid and incubated in the growth chamber at optimum growth temperature at 28ºC (220 rpm). *P. aeruginosa* PAO1 was routinely grown on LB agar media and overnight cultures prepared in LB liquid with incubation at optimum growth temperature at 37 ºC (220 rpm) (Norizan et al., 2013).

4.6.2 Melatonin treatment

Melatonin (M5250, Sigma, Castle Hill, NSW) was initially dissolved in absolute ethanol (99.9% v/v) as per manufacturer's instructions to obtain stock concentrations (200 mM) which were further diluted to give the desired final concentrations as indicated for every experiment separately. Final concentration of ethanol as a solvent was 0.1% (v/v) and standardised across all melatonin treatments.

4.6.3 Bacterial growth analysis

A single colony from a freshly streaked plate was inoculated into 10 mL LB liquid at shaking 220 rounds per minute (rpm) in appropriate temperature as an overnight culture (<14 hours). 1:10 dilution of overnight culture was made in 200 µL of fresh LB liquid media in the round-bottomed 96-well microtiter plates (Greiner Bio-One, Item no. 650162, 28110 North Carolina, USA) i.e. 20 µL of overnight bacterial culture. To test the effect of melatonin on growth of bacteria, melatonin concentrations were added, i.e. 10 µL of melatonin plus 20 µL bacterial culture plus 170 µL fresh LB liquid media. Three biological replicates corresponding to three individual colonies with three technical replicates per colony were included in the experiment. Plates were shaken at 130 rpm at appropriate temperature and optical density (OD_{600} nm) was measured at every 2 hours interval for a period of 10 hours in Clariostar microplate reader (BMG Labtech, Victoria 3931, Australia).

4.6.4 Swarming motility assay

Swarming agar was prepared as follows: D-(+)-glucose (1% w/v) (SIGMA, St. louis, MO 63103, USA), Bacto peptone (0.5% w/v) (Oxoid, Basingstoke, United Kingdom), Yeast

extract (0.2% w/v) (Oxoid,) and Bactoagar (0.5% w/v), (BD, 214010, Sparks, MD 21152 USA) as described in [78]. Stock solutions of melatonin were added into the cooled autoclaved medium at 55 ºC to give final concentrations of 1 nM, 10 µM and 100 µM melatonin in final ethanol concentration of 0.1% in all the treatments. Equal volume of media (20 mL) was then poured into round Petri dishes (90 x 14 mm), (Pacific Lab, , Blackburn, VIC, 3130, Australia) and plates left to solidify for an hour. 2 µL of the overnight culture of *P. aeruginosa* PAO (adjusted to OD_{600nm}= 0.1) was spotted onto the centre of the agar plate and incubated at 37 ºC for 24 hours and plates were then photographed for any potential effect in swarming motility by melatonin. The swarming motility measured as colony surface area (cm^2) was quantified in ImageJ by adjusting the program settings to known scale unit based on the image scale bar which was kept standard for all the images. The outline of the surface coverage was then carefully traced by the freehand selection tool and the area generated by the analyse menu selection (Schindelin et al., 2012).

4.6.5 Statistical analysis

Analysis of variance (ANOVA) was conducted followed by post-hoc Tukey test in RStudio software (version R-3.5.2), wherever applicable.

4.7 Supplementary Information

4.7.1 Swarming motility test of microbes

Prior to investigating the effect of melatonin treatments, it was imperative to check the swarming ability of the tested microbes. While it is commonly known that *Pseudomonas aeruginosa* PAO1 exhibits motility behaviours (Norizan et al., 2013), no such information exists about the tested PGPRs in question. Since motility is one of the crucial traits for competitive colonisation of rhizospheres (Dietel et al., 2013, Gao et al., 2016), it was expected that the tested PGPRs would be motile and exhibit swarming motility. It was observed that PAO1 which was used as a positive control did exhibit swarming. However, the PGPRs failed to swarm under exact same conditions. Minimum bacterial growth was observed for the PGPRs at the point of inoculation. *Enterobacter ludwigii* NCR3 showed slightly more growth in colony after 24 hours incubation at 37°C but did not result in swarming (Figure S4.1). Optimum growth temperature for PGPR is 28°C. However, swarming assays require specific media and temperature conditions ranging from 30 – 37°C

(Norizan et al., 2013, Singh et al., 2016). It has been recently reported that melatonin affects swarming in *Enterobacter aerogenes* at 37°C (Paulose et al., 2016). Overall, the inability of motility by PGPRs, though unexpected was not surprising as lack of motility is not atypical for PGPRs. For example, in an earlier report both motile and non-motile strains of *Azospirillum lipoferum* have been isolated from the rhizosphere of rice and were equally able to colonise the roots, though the motile form was more efficient than the non-motile strain (Alexandre et al., 1996).

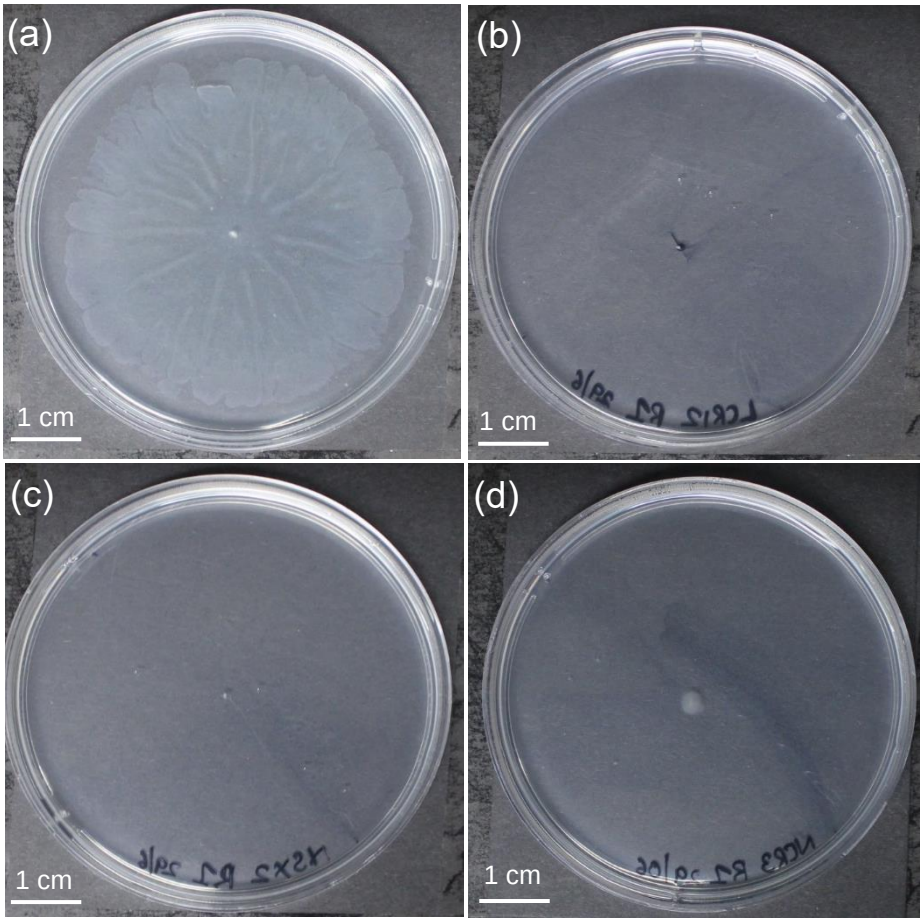


Figure S4.1: Swarm growth test of (a) *Pseudomonas aeruginosa* PAO1 (b) *Bacillus cereus* LCR12 (c) *Rhodococcus erythropolis* NSX2 and (d) *Enterobacter ludwigii* NCR3 under normal swarm media. Representative images shown. Assay was conducted with 3 independent cultures.

4.8 References

Alagarasan, G. & Aswathy, K. S. 2017. Shoot the Message, Not the Messenger-Combating Pathogenic Virulence in Plants by Inhibiting Quorum Sensing Mediated Signaling Molecules. *Front Plant Sci,* 8**,** 556

Alexandre, G. J., C., Faure, D. & Bally, R. 1996. Population dynamics of a motile and a non-motile *Azospirihm Zipoferum* strain during rice root colonization and motility variation in the rhizosphere. *FEMS Microbiol Ecol***,** 271-278

Ali, B., Sabri, A. N., Ljung, K. & Hasnain, S. 2009. Auxin production by plant associated bacteria: impact on endogenous IAA content and growth of *Triticum aestivum* L. *Lett Appl Microbiol,* 48**,** 542-7

Arnao, M. B. & Hernández-Ruiz, J. 2009. Chemical stress by different agents affects the melatonin content of barley roots. *J Pineal Res,* 46**,** 295-9

Bais, H. P., Park, S. W., Weir, T. L., Callaway, R. M. & Vivanco, J. M. 2004. How plants communicate using the underground information superhighway. *Trends Plant Sci,* 9**,** 26-32

Bajwa, V. S., Shukla, M. R., Sherif, S. M., Murch, S. J. & Saxena, P. K. 2014. Role of melatonin in alleviating cold stress in *Arabidopsis thaliana*. *J Pineal Res,* 56**,** 238-45

Berendsen, R. L., Pieterse, C. M. & Bakker, P. A. 2012. The rhizosphere microbiome and plant health. *Trends Plant Sci,* 17**,** 478-86

Boruah, H. P. & Kumar, B. S. 2002. Biological activity of secondary metabolites produced by a strain of *Pseudomonas fluorescens*. *Folia Microbiol (Praha),* 47**,** 359-63

Brader, G., Compant, S., Mitter, B., Trognitz, F. & Sessitsch, A. 2014. Metabolic potential of endophytic bacteria. *Curr Opin Biotechnol,* 27**,** 30-7

Byeon, Y., Lee, K., Park, Y. I., Park, S. & Back, K. 2013. Molecular cloning and functional analysis of serotonin *N*-acetyltransferase from the cyanobacterium *Synechocystis* sp. PCC 6803. *J Pineal Res,* 55**,** 371-6

Byeon, Y., Yool Lee, H., Choi, D. W. & Back, K. 2015. Chloroplast-encoded serotonin N-acetyltransferase in the red alga *Pyropia yezoensis*: gene transition to the nucleus from chloroplasts. *J Exp Bot,* 66**,** 709-17

Chahtane, H., Nogueira Fuller, T., Allard, P. M., Marcourt, L., Ferreira Queiroz, E., Shanmugabalaji, V., et al. 2018. The plant pathogen *Pseudomonas aeruginosa* triggers a DELLA-dependent seed germination arrest in *Arabidopsis*. *Elife,* 7

Chen, X., Sun, C., Laborda, P., He, Y., Zhao, Y., Li, C., et al. 2019. Melatonin treatments reduce the pathogenicity and inhibit the growth of *Xanthomonas oryzae* pv. *oryzicola*. *Plant Pathol,* 68**,** 288-296

Chen, X., Sun, C., Laborda, P., Zhao, Y., Palmer, I., Fu, Z. Q., et al. 2018. Melatonin Treatment Inhibits the Growth of *Xanthomonas oryzae* pv. *oryzae*. *Front Microbiol,* 9**,** 2280

Chu, W., Zere, T. R., Weber, M. M., Wood, T. K., Whiteley, M., Hidalgo-Romano, B., et al. 2012. Indole production promotes *Escherichia coli* mixed-culture growth with *Pseudomonas aeruginosa* by inhibiting quorum signaling. *Appl Environ Microbiol,* 78**,** 411-9

De Bentzmann, S. & Plesiat, P. 2011. The *Pseudomonas aeruginosa* opportunistic pathogen and human infections. *Environ Microbiol,* 13**,** 1655-65

De Weert, S., Vermeiren, H., Mulders, I. H., Kuiper, I., Hendrickx, N., Bloemberg, G. V., et al. 2002. Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonization by *Pseudomonas fluorescens*. *Mol Plant Microbe Interact,* 15**,** 1173-80

Delaney, T. P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., et al. 1994. A central role of salicylic Acid in plant disease resistance. *Science,* 266**,** 1247-50

Dietel, K., Beator, B., Budiharjo, A., Fan, B. & Borriss, R. 2013. Bacterial Traits Involved in Colonization of *Arabidopsis thaliana* Roots by *Bacillus amyloliquefaciens* FZB42. *Plant Pathol J,* 29**,** 59-66

Doornbos, R. F., Van Loon, L. C. & Bakker, P. a. H. M. 2011. Impact of root exudates and plant defense signaling on bacterial communities in the rhizosphere. A review. *Agron Sustain Dev,* 32**,** 227-243

Dubbels, R., Reiter, R. J., Klenke, E., Goebel, A., Schnakenberg, E., Ehlers, C., et al. 1995. Melatonin in edible plants identified by radioimmunoassay and by high performance liquid chromatography-mass spectrometry. *J Pineal Res,* 18**,** 28-31

Egidi, E., Wood, J. L., Aracic, S., Kannan, R., Mcdonald, L., Bell, C. A., et al. 2016a. Draft Genome Sequence of *Enterobacter ludwigii* NCR3, a Heavy Metal-Resistant Rhizobacterium. *Genome Announc,* 4

Egidi, E., Wood, J. L., Fox, E. M., Liu, W. & Franks, A. E. 2016b. Draft Genome Sequence of *Rhodococcus erythropolis* NSX2, an Actinobacterium Isolated from a Cadmium-Contaminated Environment. *Genome Announc,* 4

Egidi, E., Wood, J. L., Mathews, E., Fox, E., Liu, W. & Franks, A. E. 2016c. Draft Genome Sequence of *Bacillus cereus* LCR12, a Plant Growth-Promoting Rhizobacterium Isolated from a Heavy Metal-Contaminated Environment. *Genome Announc,* 4

Erland, L. a. E. & Saxena, P. 2019. Auxin driven indoleamine biosynthesis and the role of tryptophan as an inductive signal in *Hypericum perforatum* (L.). *PLoS One,* 14**,** e0223878

Gao, M., Teplitski, M., Robinson, J. B. & Bauer, W. D. 2003. Production of substances by *Medicago truncatula* that affect bacterial quorum sensing. *Mol Plant Microbe Interact,* 16**,** 827-34

Gao, S., Wu, H., Yu, X., Qian, L. & Gao, X. 2016. Swarming motility plays the major role in migration during tomato root colonization by *Bacillus subtilis* SWR01. *Biological Control,* 98**,** 11-17

Germann, S. M., Baallal Jacobsen, S. A., Schneider, K., Harrison, S. J., Jensen, N. B., Chen, X., et al. 2016. Glucose-based microbial production of the hormone melatonin in yeast *Saccharomyces cerevisiae*. *Biotechnol J,* 11**,** 717-24

Hattori, A., Migitaka, H., Iigo, M., Itoh, M., Yamamoto, K., Ohtani-Kaneko, R., et al. 1995. Identification of melatonin in plants and its effects on plasma melatonin levels and binding to melatonin receptors in vertebrates

Biochem Mol Biol Int, 35**,** 627-34

Heurlier, K., Williams, F., Heeb, S., Dormond, C., Pessi, G., Singer, D., et al. 2004. Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/RsmZ system in *Pseudomonas aeruginosa* PAO1. *J Bacteriol,* 186**,** 2936-45

Hidalgo-Romano, B., Gollihar, J., Brown, S. A., Whiteley, M., Valenzuela, E., Jr., Kaplan, H. B., et al. 2014. Indole inhibition of *N*-acylated homoserine lactone-mediated quorum signalling is widespread in Gram-negative bacteria. *Microbiology,* 160**,** 2464-73

Hughes, D. T. & Sperandio, V. 2008. Inter-kingdom signalling: communication between bacteria and their hosts. *Nat Rev Microbiol,* 6**,** 111-20

Jiao, J., Ma, Y., Chen, S., Liu, C., Song, Y., Qin, Y., et al. 2016. Melatonin-Producing Endophytic Bacteria from Grapevine Roots Promote the Abiotic Stress-Induced Production of Endogenous Melatonin in Their Hosts. *Front Plant Sci,* 7**,** 1387

Khanna, K., Jamwal, V. L., Gandhi, S. G., Ohri, P. & Bhardwaj, R. 2019. Metal resistant PGPR lowered Cd uptake and expression of metal transporter genes with improved growth and photosynthetic pigments in *Lycopersicon esculentum* under metal toxicity. *Sci Rep,* 9**,** 5855

Kim, J. & Park, W. 2013. Indole inhibits bacterial quorum sensing signal transmission by interfering with quorum sensing regulator folding. *Microbiology,* 159**,** 2616-25

Koh, C. L., Sam, C. K., Yin, W. F., Tan, L. Y., Krishnan, T., Chong, Y. M., et al. 2013. Plant-derived natural products as sources of anti-quorum sensing compounds. *Sensors (Basel),* 13**,** 6217-28

Lee, H. Y. & Back, K. 2016. Mitogen-activated protein kinase pathways are required for melatonin-mediated defense responses in plants. *J Pineal Res,* 60**,** 327-35

Lee, H. Y., Byeon, Y. & Back, K. 2014. Melatonin as a signal molecule triggering defense responses against pathogen attack in *Arabidopsis* and tobacco. *J. Pineal Res.,* 57**,** 262-8

Lee, H. Y., Byeon, Y., Tan, D. X., Reiter, R. J. & Back, K. 2015. *Arabidopsis* serotonin *N*-acetyltransferase knockout mutant plants exhibit decreased melatonin and salicylic acid levels resulting in susceptibility to an avirulent pathogen. *J Pineal Res,* 58**,** 291-9

Lerner, A. B., Case, J. D. & Takahashi, Y. 1960. Isolation of melatonin and 5-methoxyindole-3-acetic acid from bovine pineal glands. *J Biol Chem,* 235**,** 1992-7

Lewis, K. A., Baker, A. E., Chen, A. I., Harty, C. E., Kuchma, S. L., O'toole, G. A., et al. 2019. Ethanol Decreases *Pseudomonas aeruginosa* Flagellar Motility through the Regulation of Flagellar Stators. *J Bacteriol,* 201

Li, C., Zhao, Q., Gao, T., Wang, H., Zhang, Z., Liang, B., et al. 2018. The mitigation effects of exogenous melatonin on replant disease in apple. *J Pineal Res,* 65**,** e12523

Li, H., Chang, J., Chen, H., Wang, Z., Gu, X., Wei, C., et al. 2017. Exogenous Melatonin Confers Salt Stress Tolerance to Watermelon by Improving Photosynthesis and Redox Homeostasis. *Front Plant Sci,* 8**,** 295

Lin, Y., Fan, L., Xia, X., Wang, Z., Yin, Y., Cheng, Y., et al. 2019. Melatonin decreases resistance to postharvest green mold on citrus fruit by scavenging defense-related reactive oxygen species. *Postharvest Biol Tech,* 153**,** 21-30

Liu, A., Archer, A. M., Biggs, M. B. & Papin, J. A. 2017. Growth-altering microbial interactions are responsive to chemical context. *PLoS One,* 12**,** e0164919

Liu, C., Chen, L., Zhao, R., Li, R., Zhang, S., Yu, W., et al. 2019. Melatonin Induces Disease Resistance to *Botrytis cinerea* in Tomato Fruit by Activating Jasmonic Acid Signaling Pathway. *J Agric Food Chem,* 67**,** 6116-6124

Liu, T., Zhao, F., Liu, Z., Zuo, Y., Hou, J. & Wang, Y. 2016. Identification of melatonin in *Trichoderma* spp. and detection of melatonin content under controlled-stress growth conditions from *T. asperellum*. *J Basic Microbiol,* 56**,** 838-43

Ma, Y., Jiao, J., Fan, X., Sun, H., Zhang, Y., Jiang, J., et al. 2016. Endophytic Bacterium *Pseudomonas fluorescens* RG11 May Transform Tryptophan to Melatonin and Promote Endogenous Melatonin Levels in the Roots of Four Grape Cultivars. *Front Plant Sci,* 7**,** 2068

Manchester, L. C., Poeggeler, B., Alvares, F. L., Ogden, G. B. & Reiter, R. J. 1995. Melatonin immunoreactivity in the photosynthetic prokaryote *Rhodospirillum rubrum*: implications for an ancient antioxidant system. *Cell Mol Biol Res,* 41**,** 391-5

Mandal, M. K., Suren, H., Ward, B., Boroujerdi, A. & Kousik, C. 2018. Differential roles of melatonin in plant-host resistance and pathogen suppression in cucurbits. *J Pineal Res,* 65**,** e12505

Moe, L. A. 2013. Amino acids in the rhizosphere: from plants to microbes. *Am J Bot,* 100**,** 1692-705

Moustafa-Farag, M., Almoneafy, A., Mahmoud, A., Elkelish, A., Arnao, M. B., Li, L., et al. 2019. Melatonin and Its Protective Role against Biotic Stress Impacts on Plants. *Biomolecules,* 10

Mukherji, A., Kobiita, A., Ye, T. & Chambon, P. 2013. Homeostasis in intestinal epithelium is orchestrated by the circadian clock and microbiota cues transduced by TLRs. *Cell,* 153**,** 812-27

Muniz-Calvo, S., Bisquert, R., Fernandez-Cruz, E., Garcia-Parrilla, M. C. & Guillamon, J. M. 2019. Deciphering the melatonin metabolism in *Saccharomyces cerevisiae* by the bioconversion of related metabolites. *J Pineal Res,* 66**,** e12554

Norizan, S. N., Yin, W. F. & Chan, K. G. 2013. Caffeine as a potential quorum sensing inhibitor. *Sensors (Basel),* 13**,** 5117-29

Ortiz-Castro, R., Diaz-Perez, C., Martinez-Trujillo, M., Del Rio, R. E., Campos-Garcia, J. & Lopez-Bucio, J. 2011. Transkingdom signaling based on bacterial cyclodipeptides with auxin activity in plants. *Proc Natl Acad Sci U S A,* 108**,** 7253-8

Paulose, J. K., Wright, J. M., Patel, A. G. & Cassone, V. M. 2016. Human Gut Bacteria Are Sensitive to Melatonin and Express Endogenous Circadian Rhythmicity. *PLoS One,* 11**,** e0146643

Philippot, L., Raaijmakers, J. M., Lemanceau, P. & Van Der Putten, W. H. 2013. Going back to the roots: the microbial ecology of the rhizosphere. *Nat Rev Microbiol,* 11**,** 789-99

Poupin, M. J., Greve, M., Carmona, V. & Pinedo, I. 2016. A Complex Molecular Interplay of Auxin and Ethylene Signaling Pathways Is Involved in *Arabidopsis* Growth Promotion by *Burkholderia phytofirmans* PsJN. *Front Plant Sci,* 7**,** 492

Rodriguez-Naranjo, M. I., Torija, M. J., Mas, A., Cantos-Villar, E., Garcia-Parrilla Mdel, C., Tilden, A. R., et al. 2012. Production of melatonin by *Saccharomyces* strains under growth and fermentation conditions.

. *J Pineal Res,* 53**,** 219-24

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. 2012. Fiji: an open-source platform for biological-image analysis. *Nat Methods,* 9**,** 676-82

Shi, H., Tan, D. X., Reiter, R. J., Ye, T., Yang, F. & Chan, Z. 2015. Melatonin induces *class A1 heat-shock factors (HSFA1s)* and their possible involvement of thermotolerance in *Arabidopsis*. *J Pineal Res,* 58**,** 335-42

Singh, A., Gupta, R. & Pandey, R. 2016. Rice Seed Priming with Picomolar Rutin Enhances Rhizospheric *Bacillus subtilis* CIM Colonization and Plant Growth. *PLoS One,* 11**,** e0146013

Sprenger J, H. R., Fuhrberg B, Han S.-Z. 1999. Melatonin and other 5-methoxylated indoles in yeast: presence in high concentrations and and dependence on tryptophan availability. *Cytologia,* 64**,** 209-213

Tan, D. X., Hardeland, R., Manchester, L. C., Paredes, S. D., Korkmaz, A., Sainz, R. M., et al. 2010. The changing biological roles of melatonin during evolution: from an antioxidant to signals of darkness, sexual selection and fitness. *Biol Rev Camb Philos Soc,* 85**,** 607-23

Tan, D. X., Manchester, L. C., Esteban-Zubero, E., Zhou, Z. & Reiter, R. J. 2015. Melatonin as a Potent and Inducible Endogenous Antioxidant: Synthesis and Metabolism. *Molecules,* 20**,** 18886-906

Tekbas, O. F., Ogur, R., Korkmaz, A., Kilic, A. & Reiter, R. J. 2008. Melatonin as an antibiotic: new insights into the actions of this ubiquitous molecule. *J Pineal Res,* 44**,** 222-6

Tilden, A. R., Becker, M. A., Amma, L. L., Arciniega, J. & Mcgaw, A. K. 1997. Melatonin production in an aerobic photosynthetic bacterium: an evolutionarily early association with darkness. *J Pineal Res,* 22**,** 102-6

Toljander, J. F., Lindahl, B. D., Paul, L. R., Elfstrand, M. & Finlay, R. D. 2007. Influence of arbuscular mycorrhizal mycelial exudates on soil bacterial growth and community structure. *FEMS Microbiol Ecol,* 61**,** 295-304

Voges, M., Bai, Y., Schulze-Lefert, P. & Sattely, E. S. 2019. Plant-derived coumarins shape the composition of an *Arabidopsis* synthetic root microbiome. *Proc Natl Acad Sci U S A,* 116**,** 12558-12565

Walker, T. S., Bais, H. P., Deziel, E., Schweizer, H. P., Rahme, L. G., Fall, R., et al. 2004. *Pseudomonas aeruginosa*-plant root interactions. Pathogenicity, biofilm formation, and root exudation. *Plant Physiol,* 134**,** 320-31

Wang, H. X., Liu, F. & Ng, T. B. 2001. Examination of pineal indoles and 6-methoxy-2-benzoxazolinone for antioxidant and antimicrobial effects. *Comp Biochem Physiol C Toxicol Pharmacol,* 130**,** 379-88

Wang, M., Duan, S., Zhou, Z., Chen, S. & Wang, D. 2019. Foliar spraying of melatonin confers cadmium tolerance in *Nicotiana tabacum* L. *Ecotoxicol Environ* 170**,** 68-76

Wei, Y., Chang, Y., Zeng, H., Liu, G., He, C. & Shi, H. 2018. RAV transcription factors are essential for disease resistance against cassava bacterial blight via activation of melatonin biosynthesis genes. *J Pineal Res,* 64

Yan, J., Monaco, H. & Xavier, J. B. 2019. The Ultimate Guide to Bacterial Swarming: An Experimental Model to Study the Evolution of Cooperative Behavior. *Annu Rev Microbiol,* 73**,** 293-312

Yin, L., Wang, P., Li, M., Ke, X., Li, C., Liang, D., et al. 2013. Exogenous melatonin improves *Malus* resistance to *Marssonina* apple blotch. *J Pineal Res,* 54**,** 426-34

Zhalnina, K., Louie, K. B., Hao, Z., Mansoori, N., Da Rocha, U. N., Shi, S., et al. 2018. Dynamic root exudate chemistry and microbial substrate preferences drive patterns in rhizosphere microbial community assembly. *Nat Microbiol,* 3**,** 470-480

Zia, S. F., Berkowitz, O., Bedon, F., Whelan, J., Franks, A. E. & Plummer, K. M. 2019. Direct comparison of *Arabidopsis* gene expression reveals different responses to melatonin versus auxin. *BMC Plant Biol,* 19**,** 567

Chapter 5: General Discussion

5.1 Overview

Global agricultural production currently faces challenges posed by climate change (Leng, 2017, Ray et al., 2019, Sultan et al., 2019). For example, the recent occurrence and the severity of the impacts witnessed by drought in regions of Australia are reported as likely to be the worst in 800 years (Freund et al., 2017). Global warming has also led to an increase in the concurrence of stress factors such as drought, invertebrate pests and pathogenic infections (Bebber et al., 2013). Current threats to food supply and livelihoods include devastations caused by wheat yellow rust (*Puccinia striiformis* f. sp. *tritici*), especially in regions of India and Pakistan, and pest infestation by the desert locust (*Schistocerca gregaria*) across East Africa, Asia and the Middle East (Ali et al., 2017). While it is logical (and perhaps the only resort) to use established synthetic fungicides and insecticides in the event of an outbreak emergency, more preventive measures than curative are required for long term sustainable agriculture and to ensure food security. The year of 2020 is declared by the United Nations as the 'International Year of Plant Health'. There is an increasing need now more than ever for developing innovative agricultural practices, such as use of plant-based bio-stimulants (Francesca et al., 2020).

In the past 25 years, research on melatonin in plants has advanced rapidly. Melatonin improves plant tolerance to several abiotic stressors, such as temperature extremes, drought, salinity, heavy metals, as well as to biotic stressors in the form of insects, bacterial and fungal pathogenic infections (Bajwa et al., 2014, Shi et al., 2015, Zhao et al., 2015, Zhang et al., 2017, Nehela and Killiny, 2018, Li et al., 2019). This has resulted in interest in potential use of melatonin in agricultural practices to negate the deleterious impacts of environmental stresses to improve crop yield. However, it is first essential to understand the phenotypic responses and underlying molecular mechanisms of melatonin action in plants and in vitro growth responses of soil microbes.

Differential effects on gene expression by melatonin and auxin treatments

A parallel comparison of exogenous root treatments of melatonin versus the structurally similar indole plant hormone, auxin, demonstrated that these two molecules have distinctly different effects on phenotype and gene expression in leaves of the model plant *Arabidopsis thaliana* (Chapter 2) (Zia et al., 2019). The parallel comparison of both the treatments under the exact same set of experimental conditions was needed. The outcome of these experiments resolves the conflicting reports in the literature regarding auxin-like

functions of melatonin in regulating plant growth. Notably, melatonin and auxin have only one commonality in their structures i.e. the indole moiety. Structure-activity relationships indicate that melatonin is not likely to be an auxin itself as it does not contain all the other molecular parts in its structure that are required for a functional auxinic activity (Katekar, 1979). Auxin and mitochondrial retrograde signalling are antagonistic to enable growth-defence trade-off (Ivanova et al., 2014). On the contrary, melatonin enables growth-defence trade-off in a different manner. Exogenous melatonin (100 µM) root treatments resulted in plants going into 'survival mode' by downregulating the expression of photosynthesis-related genes to enable induction of biotic defence-related genes (Chapter 2). This phenomenon was observed with melatonin treatments of 100 µM, but not observed when the higher concentration (400 µM) was applied in the same ecotype (Col-0), or in other ecotypes (Bur-0 and Hn-0) indicating that this is potentially a melatonin treatment-dependent effect (Chapter 3). Nevertheless, the biotic stress defence signalling genes activated by melatonin (400 µM) were induced across ecotypes and treatments (Chapter 3). The finding that melatonin enables growth-defence trade-offs provides a foundation in melatonin's action mechanism. However, several questions remain to be answered. Further work is needed to understand the timing of when and how melatonin releases the growth-defence switch to maintain an equal balance and not compromise the growth (or crop yield in plants of agricultural interest) for longer duration (Brown, 2002). The use of melatonin application to activate defences constitutively, in anticipation of a pathogen attack might also induce fitness costs at a higher rate as compared to defence induction in an event of an actual pathogenic encounter. Although, the fitness costs resulting from trade-off between defence and growth (for example, developmental impacts of melatonin on flowering, seed production etc.) would have to be investigated on a case by case basis. The theory suggests that, although plants investing in defensive compounds grow at a slower rate, they may suffer less damage upon a pathogenic infection compared to plants that activate defences only upon pathogen perception (Perrin and Sibly, 1993).

Local habitat adaptation

The transcriptional effects of melatonin in Col-0 in Chapter 2 provided a baseline to extend the study to include other ecotypes of *Arabidopsis*. Chapter 3 documented that the effects of melatonin on transcriptome of *Arabidopsis* ecotypes (Col-0, Bur-0 and Hn-0) are both common as well as specific to each ecotype, further indicating a potential role

for melatonin in providing adaptive benefits in *Arabidopsis* to local habitats. In the ecotype studies, stress-response genes and transcription factors that regulate stress responses activated (to both biotic and abiotic stresses) by melatonin were observed. Differences in endogenous melatonin concentrations could be the cause of alteration in expression responses to melatonin. However, that might not always be the case, as previously shown for ecotypes varying in auxin response which did not significantly differ in their endogenous auxin levels (Delker et al., 2010). Further work should therefore look at quantifying endogenous melatonin levels in the tested ecotypes to investigate any potential link with variation in gene expression. The disruption in the expression of the melatonin biosynthetic gene *AtSNAT1* encoding a key enzyme serotonin *N*-acetyltransferase in the melatonin biosynthetic pathway resulted in 111 differentially expressed genes as compared to wild-type (Col-0) under control and 374 differences compared to Col-0 in response to melatonin. This indicates that the AtSNAT1 plays essential roles in governing melatonin-mediated responses (Chapter 3). It is also important to note that the expression of *AtSNAT1* was not reflective of the variation in phenotypic and transcriptomic variation among the Col-0, Bur-0 and Hn-0. A further detailed expression analysis of both melatonin biosynthetic iso-genes *AtSNAT1* and *AtSNAT2* could give more insights into natural variation. The experiments were focused on a single time point analysis of melatonin exposure in *Arabidopsis* genotypes (Chapter 3). Further research is required to explore the temporal effects of melatonin and whether that can be a contributing factor to the observed natural variation. A time course of treatments was beyond the budget for the current study. Moreover, while this thesis (Chapter 2 and 3) investigated the systemic effect of melatonin action (applying melatonin through roots and analysing effects in rosette leaves), organ-, tissue- or cell-specific effects of melatonin would provide further insights.

Response of agricultural soil microbes to melatonin – probing the potential role of melatonin in interkingdom signalling

Establishing that natural variation occurs in *Arabidopsis* ecotypes in response to melatonin led to a preliminary investigation into the melatonin responsiveness of agricultural soil microbes (*Pseudomonas aeroginosa* PAO1, *Bacillus cereus* LCR12, *Enterobacter ludwigii* NCR3 and *Rhodococcus erythropolis* NSX2) (Chapter 4). The in vitro growth rates of PGPRs (*B. cereus* LCR12, *E. ludwigii* NCR3 and *R. erythropolis* NSX2) were not affected by exposure to melatonin in vitro. However, the swarming

growth of the pathogen, *P. aeroginosa* PAO1, was significantly enhanced by melatonin (100 µM) under ethanol treatment. The interactions of microbes in the presence of plant host(s) would have to be determined to observe the potentially marked effect of melatonin in cultivation scenarios. Natural variation in response to PGPR inoculations has been reported in *Arabidopsis* (Wintermans et al., 2016). Once the colonisation ability of the tested PGPRs in this study is established with *Arabidopsis* ecotypes Col-0, Bur-0 and Hn-0 (tested in Chapter 3), then it would be useful to test the role of melatonin during plant-microbe interactions. Overall, the findings in Chapter 4 indicate that the effect of melatonin in vitro in microbial responses is not pronounced in absence of the host. Further work is required to investigate if melatonin is secreted by *Arabidopsis* roots as an exudate to recruit beneficial microbes as has been shown for indoleamine, auxin (indole-3-acetic acid) or if it solely acts as a signalling molecule in the inter-kingdom signalling.

5.2 Implications of melatonin studies for agriculture and human health

The findings in this thesis establish the effect of melatonin with distinct modes of action compared to structurally similar plant hormone, auxin and conserved and unique changes across diverse genotypes of *Arabidopsis* species. The results also provide an indication of the melatonin-responsiveness of a soil microbe in vitro. Based on the findings, it can be concluded that the effects of melatonin are distinct across genotypes within a single plant species and future plant-melatonin research studies should take that into consideration to obtain a complete picture of the mode/s of action of melatonin in the context of crop production. This is relevant as the intended potential use of melatonin in future agricultural practices is for a broad range of species with a potential use in crop rotation practices.

Melatonin plays essential roles in humans such as regulating circadian rhythms, boosting immune system, especially in diseases such as cancer, and improving overall health (Carrillo-Vico et al., 2013, Zisapel, 2018). The synthesis of essential amino acid tryptophan (precursor of melatonin) is not sufficient in humans and they must obtain it from other sources such as plant-based diets (Peuhkuri et al., 2012). Human clinical trials on melatonin administration have reported that melatonin as a supplement is generally considered safe for consumption with minimal to no side effects when taken as per the prescribed and recommended dosages (Sletten et al., 2018). Plant-based diets rich in melatonin have shown to have beneficial health effects in humans (Sae-Teaw et al., 2013, Iriti and Varoni, 2015). In fact, health benefits of traditional Mediterranean diets such as

lowered risk of chronic-degenerative diseases have been partly attributed to the high melatonin content present in the food groups such as grapes, yoghurt, ricotta cheese and olive products (Iriti and Varoni, 2015). Therefore, increased melatonin content in edible plants and fruits due to exogenous melatonin application has potential to not only enhance plant fitness but also promote overall human health (Sae-Teaw et al., 2013). Melatonin can be applied as a seed coat, foliar spray, root irrigating solution or as a soaking solution for post-harvest vegetables (Yin et al., 2013, Wei et al., 2015, Gao et al., 2018, Wang et al., 2019). Currently, a major limiting factor in the potential use of melatonin in future agricultural practices is the high cost of melatonin production. The melatonin chemical powder, which was used in the experiments for this thesis and is available solely for scientific research purposes, costs Australian Dollars (AUD) $152 per gram (M5250, Sigma Aldrich Pty. Ltd. Australia). For this reason, research in phyto-melatonin is now seeing a rise in studies identifying melatonin-rich plant extracts (Arnao and Hernández-Ruiz, 2018, Perez-Llamas et al., 2020). This is to enable an integrated system such as using bioreactor technology for large-scale propagation of melatonin-rich plant germplasm. In fact, in vitro clonal propagation of melatonin-rich medicinal plants, such as Huang-qin (*Scutellaria baicalensis*) and St. John's wort (*Hypericum perforatum*) cv. 'New Stem' and cv. 'Anthos', has already been demonstrated (Li et al., 2000, Zobayed and Saxena, 2003). Furthermore, production of melatonin by yeast (*Saccharomyces cerevisiae*) through a glucose-based strategy has also been demonstrated (Germann et al., 2016). Once these processes for melatonin production are optimised they might be relatively cost-effective. Cheaper and natural sources of melatonin will result in more realistic testing, e.g. under pot or field conditions. This will lead to research that can be conducted on a field scale rather than high-cost laboratory assays.

5.3 References

Ali, S., Rodriguez-Algaba, J., Thach, T., Sorensen, C. K., Hansen, J. G., Lassen, P., et al. 2017. Yellow Rust Epidemics Worldwide Were Caused by Pathogen Races from Divergent Genetic Lineages. *Front Plant Sci,* 8**,** 1057

Arnao, M. B. & Hernández-Ruiz, J. 2018. Phytomelatonin, natural melatonin from plants as a novel dietary supplement: Sources, activities and world market. *J Funct Foods,* 48**,** 37-42

Bajwa, V. S., Shukla, M. R., Sherif, S. M., Murch, S. J. & Saxena, P. K. 2014. Role of melatonin in alleviating cold stress in *Arabidopsis thaliana*. *J Pineal Res,* 56**,** 238-45

Bebber, D. P., Ramotowski, M. A. & Gurr, S. J. 2013. Crop pests and pathogens move polewards in a warming world. *Nat Clim Chang,* 3**,** 985

Brown, J. K. 2002. Yield penalties of disease resistance in crops. *Curr Opin Plant Biol,* 5**,** 339-44

Carrillo-Vico, A., Lardone, P. J., Alvarez-Sanchez, N., Rodriguez-Rodriguez, A. & Guerrero, J. M. 2013. Melatonin: buffering the immune system. *Int J Mol Sci,* 14**,** 8638-83

Delker, C., Poschl, Y., Raschke, A., Ullrich, K., Ettingshausen, S., Hauptmann, V., et al. 2010. Natural variation of transcriptional auxin response networks in *Arabidopsis thaliana*. *Plant Cell,* 22**,** 2184-200

Francesca, S., Arena, C., Hay Mele, B., Schettini, C., Ambrosino, P., Barone, A., et al. 2020. The Use of a Plant-Based Biostimulant Improves Plant Performances and Fruit Quality in Tomato Plants Grown at Elevated Temperatures. *Agronomy,* 10

Freund, M., Henley, B. J., Karoly, D. J., Allen, K. J. & Baker, P. J. 2017. Multi-century cool- and warm-season rainfall reconstructions for Australia's major climatic regions. *Clim Past,* 13**,** 1751-1770

Gao, H., Lu, Z., Yang, Y., Wang, D., Yang, T., Cao, M., et al. 2018. Melatonin treatment reduces chilling injury in peach fruit through its regulation of membrane fatty acid contents and phenolic metabolism. *Food Chem,* 245**,** 659-666

Germann, S. M., Baallal Jacobsen, S. A., Schneider, K., Harrison, S. J., Jensen, N. B., Chen, X., et al. 2016. Glucose-based microbial production of the hormone melatonin in yeast *Saccharomyces cerevisiae*. *Biotechnol J,* 11**,** 717-24

Iriti, M. & Varoni, E. M. 2015. Melatonin in Mediterranean diet, a new perspective. *J Sci Food Agric,* 95**,** 2355-9

Ivanova, A., Law, S. R., Narsai, R., Duncan, O., Lee, J. H., Zhang, B., et al. 2014. A Functional Antagonistic Relationship between Auxin and Mitochondrial Retrograde Signaling Regulates *Alternative Oxidase1a* Expression in *Arabidopsis*. *Plant Physiol,* 165**,** 1233-1254

Katekar, G. F. 1979. Auxins: on the nature of the receptor site and molecular requirements for auxin activity. *Phytochemistry,* 18**,** 223-233

Leng, G. 2017. Evidence for a weakening strength of temperature-corn yield relation in the United States during 1980-2010. *Sci Total Environ,* 605-606**,** 551-558

Li, H., Murch, S. J. & Saxena, P. K. 2000. Thidiazuron-induced de novo shoot organogenesis on seedlings, etiolated hypocotyls and stem segments of Huang-qin. *Plant Cell Tiss Org* 62**,** 169-173

Li, T., Wu, Q., Zhu, H., Zhou, Y., Jiang, Y., Gao, H., et al. 2019. Comparative transcriptomic and metabolic analysis reveals the effect of melatonin on delaying anthracnose incidence upon postharvest banana fruit peel. *BMC Plant Biol,* 19**,** 289

Nehela, Y. & Killiny, N. 2018. Infection with phytopathogenic bacterium inhibits melatonin biosynthesis, decreases longevity of its vector, and suppresses the free radical-defense. *J Pineal Res,* 65**,** e12511

Perez-Llamas, F., Hernandez-Ruiz, J., Cuesta, A., Zamora, S. & Arnao, M. B. 2020. Development of a Phytomelatonin-Rich Extract from Cultured Plants with Excellent Biochemical and Functional Properties as an Alternative to Synthetic Melatonin. *Antioxidants (Basel),* 9

Perrin, N. & Sibly, R. M. 1993. Dynamic models of energy allocation and investment. *Annu Rev Ecol Evol S,* 24**,** 379-410

Peuhkuri, K., Sihvola, N. & Korpela, R. 2012. Dietary factors and fluctuating levels of melatonin. *Food Nutr Res,* 56**,** 10.3402/fnr.v56i0.17252

Ray, D. K., West, P. C., Clark, M., Gerber, J. S., Prishchepov, A. V. & Chatterjee, S. 2019. Climate change has likely already affected global food production. *PLoS One,* 14**,** e0217148

Sae-Teaw, M., Johns, J., Johns, N. P. & Subongkot, S. 2013. Serum melatonin levels and antioxidant capacities after consumption of pineapple, orange, or banana by healthy male volunteers. *J Pineal Res,* 55**,** 58-64

Shi, H., Tan, D. X., Reiter, R. J., Ye, T., Yang, F. & Chan, Z. 2015. Melatonin induces *class A1 heat-shock factors (HSFA1s)* and their possible involvement of thermotolerance in *Arabidopsis*. *J Pineal Res,* 58**,** 335-42

Sletten, T. L., Magee, M., Murray, J. M., Gordon, C. J., Lovato, N., Kennaway, D. J., et al. 2018. Efficacy of melatonin with behavioural sleep-wake scheduling for delayed sleep-wake phase disorder: A double-blind, randomised clinical trial. *PLoS Med,* 15**,** e1002587

Sultan, B., Defrance, D. & Iizumi, T. 2019. Evidence of crop production losses in West Africa due to historical global warming in two crop models. *Sci Rep,* 9**,** 12834

Wang, M., Duan, S., Zhou, Z., Chen, S. & Wang, D. 2019. Foliar spraying of melatonin confers cadmium tolerance in *Nicotiana tabacum* L. *Ecotoxicol Environ* 170**,** 68-76

Wei, W., Li, Q. T., Chu, Y. N., Reiter, R. J., Yu, X. M., Zhu, D. H., et al. 2015. Melatonin enhances plant growth and abiotic stress tolerance in soybean plants. *J Exp Bot,* 66**,** 695-707

Yin, L., Wang, P., Li, M., Ke, X., Li, C., Liang, D., et al. 2013. Exogenous melatonin improves *Malus* resistance to *Marssonina* apple blotch. *J Pineal Res,* 54**,** 426-34

Zhang, S., Zheng, X., Reiter, R. J., Feng, S., Wang, Y., Liu, S., et al. 2017. Melatonin Attenuates Potato Late Blight by Disrupting Cell Growth, Stress Tolerance, Fungicide Susceptibility and Homeostasis of Gene Expression in *Phytophthora infestans*. *Front Plant Sci,* 8**,** 1993

Zhao, H., Xu, L., Su, T., Jiang, Y., Hu, L. & Ma, F. 2015. Melatonin regulates carbohydrate metabolism and defenses against *Pseudomonas syringae* pv. tomato DC3000 infection in *Arabidopsis thaliana*. *J Pineal Res,* 59**,** 109-19

Zia, S. F., Berkowitz, O., Bedon, F., Whelan, J., Franks, A. E. & Plummer, K. M. 2019. Direct comparison of *Arabidopsis* gene expression reveals different responses to melatonin versus auxin. *BMC Plant Biol,* 19**,** 567

Zisapel, N. 2018. New perspectives on the role of melatonin in human sleep, circadian rhythms and their regulation. *Br J Pharmacol,* 175**,** 3190-3199

Zobayed, S. M. A. & Saxena, P. K. 2003. In vitro-grown roots: a superior explant for prolific shoot regeneration of St. John's wort (*Hypericum perforatum* L. cv 'New Stem') in a temporary immersion bioreactor. *Plant Sci,* 165**,** 463-470