

“धान में मेलाटोनिन मध्यस्थ सूखे की सहनशीलता
को आणविक मार्ग द्वारा समझने”

**“Deciphering the molecular pathway of
melatonin mediated drought tolerance in rice”**

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**DIVISION OF PLANT PHYSIOLOGY
ICAR – INDIAN AGRICULTURAL RESEARCH INSTITUTE
NEW DELHI – 110012**

2018

**“DECIPHERING THE MOLECULAR
PATHWAY OF MELATONIN MEDIATED
DROUGHT TOLERANCE IN RICE”**

A Thesis

by

OM PRAKASH GHIMIRE

**Submitted to the Faculty of Post Graduate School,
ICAR- Indian Agricultural Research Institute, New Delhi
in partial fulfilment of the requirements
for the award of the degree of**

MASTER OF SCIENCE

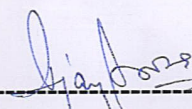
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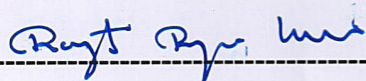
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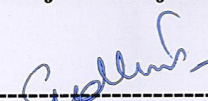
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This is to certify that the thesis entitled “**Deciphering the molecular pathway of melatonin mediated drought tolerance in rice**” submitted to the Faculty of the Post-Graduate School, ICAR - Indian Agricultural Research Institute, New Delhi, in partial fulfilment of the requirements for the award of **MASTER OF SCIENCE in PLANT PHYSIOLOGY**, embodies the results of bonafide research work carried out by **Mr. OM PRAKASH GHIMIRE, Roll No. 20904** under my guidance and supervision, and that no part of this thesis has been submitted for any other degree or diploma.

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Acknowledgement

Faith is to believe what you do; the reward of this faith is to see what you believe. I thank heavenly God for great health, supportive family and always being by my side for making this manuscript possible.

Foremost, I would express my sincere gratitude with reverence to my Chairperson **Dr. Ajay Arora, Principal Scientist**, Division of Plant Physiology, ICAR-IARI, New Delhi, for his sagacious guidance, intellectual stimulation, invaluable support and constant inspiration. His scholarly suggestions, sincere guidance, sustained interest, valuable help and affectionate behaviour have been a fountain of great inspiration to me. He nurtured my capabilities and gave me ample freedom to work under him. His positive attitude and hardworking nature will always inspire me throughout my life.

It is a great privilege for me to express my esteem and profound sense of gratitude to my Co-Chairperson **Dr. Viswanathan Chinnusamy, Head and Principal Scientist**, Division of Plant Physiology, ICAR-IARI for his constant encouragement and continuous support and guidance throughout the course and research work. I also want to deliver my kind thanks to **Dr. Madan Pal Singh, Professor and Dr. V. P. Singh, Emeritus Professor**, Division of Plant Physiology, ICAR-IARI, New Delhi, for their encouragement, immense knowledge and valuable motivation throughout the course and research work.

My sincere gratitude also goes to my advisory committee members: **Dr. Sudhir Kumar, Scientist**, Division of Plant Physiology and **Dr. Ranjeet Ranjan Kumar, Senior Scientist**, Division of Biochemistry ICAR-IARI, New Delhi for their precious suggestions and guidance. I would like to deliver thanks to **Dr. Jasdeep Chatrath Padaria**, Principal Scientist, Division of Molecular Biology and Biotechnology, ICAR-IARI, New Delhi for providing the molecular lab facilities and her valuable suggestions.

I sincerely acknowledge and thank faculty members; **Dr. Bhupindar Singh, Dr. Anjali Anand, Dr. Renu Pandey, Dr. Pramod Kumar, Dr. Rakesh pandey, Dr. Dhandapani Raju, Dr. Lekshmy Sathy Nair and Dr. Archana Kumari Watts** for their whole hearted support during the course of my work.

My thanks also go to all the members of Abiotic Stress Laboratory, Division of Plant Physiology for their support and help during my research work. My special thanks go to **Mr Santosh Kumar V.V.** who has supported and guided me throughout the research work.

I would like to thank my classmates **Dipankar, Birendra, Pandurang, Nisha** and **Priya** and batchmates **Joshna, Amit, Dipro, Sanjay, Niraj and Rahul** with whom I started my journey at IARI and had a wonderful time. I also deliver thanks to my beloved seniors; **Dr. Krishna Kumar, Gurumurthy, Gajendra Rathore, Rajeev Kumar, Omotayo Komolafe, Karan Singh Dhami, Hemant Ghimerey, Albert Maibam, Harindar Viswakarma, Rahul Banerjee, Anu Kumari, Bhoopesh Punera** and **Rishav Kumar** for their motivation and support.

I also want to express my deep thanks to my cousin **Lilanath Sapkota** and Swedes guardian **Anette Axelsion** for their continuous support and motivation because of them I am able to achieve this success in my life. I also want to thank my friends **Kishor GC** and **Navraj Pandey** who continuously motivated and supported me throughout my life.

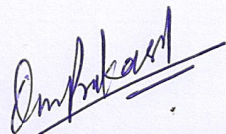
I am overwhelmed with joy to avail this rare opportunity to acknowledge my beloved mother **Mrs. Devkala Ghimire** and beloved father **Mr. Bhimlal Sharma** for their effort in moulding me to be a good person with good ideas in the society for providing the best education they could afford and by supporting me during the tough situation throughout my journey. Similarly, I will utilize this privilege to remember and acknowledge my **late father**; Late **Mr. Tilakram Ghimire**, who inspired and motivate me throughout my childhood and I learnt a lot from his life.

I sincerely apologize to all those who helped me, whose name I could not mention here. Indeed, it is not for lack of gratitude, but lack of space, I appreciate them all.

Finally I sincerely appreciate **Department and Agriculture and Research Education, Ministry of Agriculture, Government of India**, for providing scholarship opportunity under "Nepal Aid Fund Scheme" to me and other colleagues all through the period of my degree.

"Even if your kindness won't be acknowledged by anyone, remember that someone in heaven smile's for what you've done "Be Good Do Good".

Date : 02/02/2019
Place : New Delhi


(OM PRAKASH GHIMIRE)



**Dedicated to my Grand father
and my beloved parents**

Rice is the staple crop of more than 60% population of world and also in south eastern Asia. Asia consumes and produces about 90 % of all rice grown in the world. Rice is extensively grown in India as staple food crops which occupy the 43 million of hectares of land. In Indian agriculture, rice shares the larger portion and 70% of population depends upon on the rice and for 120-150 million rural people, it is source of livelihood. It accounts for 43 % of total production of food grain and 55 % of cereals production in the country. In 2016/17, India produced 104.32 million ton of rice in 43 million ha (Ministry of Commerce and Industry-MOCI, 2017). There are various constraints for rice production from ecological to edaphic factors. The accelerated production of rice is the main success of The Green Revolution movement in various countries. Both abiotic and biotic factors affect the growth and development of rice ultimately affecting its productivity. Further, reduced yield and quality of rice due to climate change is major concern for world food security. Most of the rice cultivars have been grown in flood irrigated areas which makes rice vulnerable to soil water deficit as compared to other cereals like maize and wheat. It is reported that one kilogram of rice needs 5000 litres of water to produce (IRRI). Thus, this huge demand of water makes rice vulnerable to water deficit. So drought is the most detrimental stress for production of rice in rain-fed areas (Pandey and Bhandari, 2009).). Growing population has raised the pressure on the food production. So improvement of water use efficiency has drawn the world wide attention and the focus has shifted to the identification of cultivars that demonstrate increased yield under drought stress conditions. Thus, bio prospecting of rice cultivars for better adaptation to various climates has been initiated across the world.

Drought stress is one of the major constraints for rice cultivation and production in water limited conditions (Bernier et al., 2008; Mishra et al., 2015) such rice cultivation in upland areas. In rainfed area, the low land and upland has 31% and 11% area of global rice cultivation respectively (Murty et al., 2001; Kamoshita et al., 2008). Evenson et al. (1996) reported 18 million tonnes average annual global reduction of production of rice due to water deficit. During drought stress, dry weight accumulation reduces in plants and its life cycle shortens which leads to yield

reduction (Blum, 2005; Kamoshita et al., 2008). Vegetative growth just before flowering also reduces the yield by suppressing the growth of leaves and storage organs (McDonald et al., 2003; Okami et al., 2015). During flowering, drought stress reduces the pollen viability and stigma receptivity as well as seed set and grain formation (Barnabas et al., 2008, Boonjung and Fukai, 1996). Reproductive organ failures like ovary and pollen along with inhibition of pollen germination and fertilization (Satake and Yoshida, 1978; Ekanayake et al., 1990) have been recorded in drought stress (Saini et al., 1983, 1997). The spikelet fertility reduction and panicle exertions are the major effects of drought stress on grain yield (Jiet al., 2005).

Thus, drought is challenging aspects in present and coming time for rice production. It has been reported that in rice, severe drought stress at grain filling reduce yield by 60 % (Basnayake et al., 2006) and at reproductive stage reduce yield up to 94.8 % (Lafitte et al., 2006).

Therefore, understanding and developing the drought tolerance mechanism in rice is important aspects to look in rice production. Since the population is increasing rapidly, we need to double our rice production by 2030. So it's a big challenge to double the production with prone of water deficit and other abiotic and biotic stresses in rice. In India, more than 45% rice production area is under rainfed situation. So it's very important issues to develop the drought tolerant varieties. There are several strategies to increase the drought tolerance. Scientists all over the India are working on it to promote the drought tolerance in rice. In current time, research in Melatonin has come up with some effective results in drought tolerance mechanism in crop plants (Arnao and Hernández-Ruiz, 2006; Wei et al., 2015).

Melatonin is a low molecular weight indoleamine that ubiquitously present in plants (Erland et al., 2015). Its molecular formula is N-acetyl-5-methoxytryptamine. Melatonin was first identified as an important animal hormone which function as important multiple role in biological processes like antioxidative activity, circadian rhythms, seasonal reproductive physiology, sleep, sexual behavior control, mood response, temperature biohomeostasis, retinal physiology, and enhancement of immunology. In plants, Melatonin is found in different organs like seeds, roots, stems, shoots, leaves, flowers, bulbs and fruits (Arnao and Hernández-Ruiz, 2014). Its content ranges from pico gram to micro gram. Melatonin has been reported to have function in many physiological processes which respond to environmental

stress further redirecting plant growth and development, protecting developing tissues from damage in stress (Murch et al. 2009; Erland et al. 2015; Reiter et al. 2015). Melatonin acts as potent antioxidant for scavenging the reactive oxygen species (ROS) under environmental stress (Tan et al. 2007, 2015). Several reports revealed that melatonin interacts with different signaling pathways such as calcium/calmodulin, ubiquitination and cyclic adenosine monophosphate (cAMP) (Reiter et al. 1993; Hardeland 2009; Tan et al. 2015; Sanchez-Barcelo et al. 2016). Its amphiphilic property helps to drive into the cell membrane easily and goes into cell compartments which help for faster signaling and responses in different stress (Shida et al., 1994). In plants, several reports have been published to establish its roles in plant development, stress tolerance and as a growth promoter and rooting agent, free radical scavenger and abiotic and biotic stress tolerance factors (Hernández-Ruiz and Arnao, 2008; Zhang et al., 2014). Li et al. (2014) reported that Melatonin treatment increased the drought tolerance level in both drought contrasting *Malus prunifolia* and *M. hupehensis* plants by reducing the expression of *MdNCED3* and upregulating expression of catabolic genes, *MdCYP707A1* and *MdCYP707A2* ultimately reducing ABA level in drought-stressed plants. Similarly it was reported that Melatonin treated seed of cucumber rapid variation in ABA level in germination stage by up regulating ABA catabolic genes like CYP707A1 and CYP707A2 and down regulating ABA biosynthesis genes like NCED2 (Zhang et al., 2014). Comparative transcriptomic analysis showed that exogenous melatonin treatment in bermuda grass down regulated the ABA signaling genes like RCAR3, LEA protein and transcription factors like AREB3, RABs, etc. (Shi et al., 2014) while up regulated the PP2C related genes and other ABA independent transcription factors. Similarly, Ma et al. (2018) revealed that Melatonin suppressed the leaf senescence induced by water deficit by up-regulating genes for cytokinin and Melatonin signaling and responses and by down-regulating chlorophyll-degradation genes and enzyme activities in bent grass seedlings, thus suppressing senescence. It was also reported that Melatonin induced heat tolerance by reducing the senescence by up regulating cytokinin biosynthesis genes and down regulating the cytokinin degrading genes and ABA biosynthesis genes (Zhang et al., 2017). Melatonin promotes the biotic and abiotic stress tolerance by inducing the CBF/DREB1s transcripts under water deficit stress in Arabidopsis (Shi et al., 2015). The transcriptome study revealed that exogenous Melatonin induces the expression of DREB transcription

factors (Zhang et al., 2014). Melatonin increased the expression of DREB2A transcription factor for enhancing the drought tolerance (Ma et al., 2018). It was revealed by transcriptomic analysis that exogenous Melatonin down regulated the expression of NAC transcription factors (Zhang et al., 2014, Liang et al., 2015).

From the above literatures, it is very clear that Melatonin enhances the drought tolerance. But there is still ambiguity about the mechanisms through which Melatonin confers drought tolerance. Most of the research in this area are conducted at seedling stages of the rice but the gap is still there during reproductive development, whether Melatonin is playing any role in favor of drought tolerance or not. Moreover, it has yet to be identified that the Melatonin mediated drought tolerance works through ABA-dependent or ABA-independent pathway under drought condition.

To test the above hypothesis, we have targeted some of the physiological and biochemical traits which are marker for drought stress. In molecular traits, we have targeted Melatonin biosynthesis genes, ABA biosynthesis and signaling genes, ABA-dependent transcription factors, ABA-independent transcription factors promoting drought tolerance and cytokinin biosynthesis genes for drought tolerance which may be regulated by Melatonin. The research title of the thesis is **“Deciphering the molecular pathway of melatonin mediated drought tolerance in rice”**. Our present study was planned on the basis of following objectives:

1. Physiological and biochemical elucidation of melatonin-mediated drought tolerance mechanism in rice by using non transgenic and transgenic line of ABA receptor PYL6.
2. Molecular expression of melatonin mediated ABA-dependent and ABA-independent genes promoting drought tolerance in non-transgenic and transgenic rice.

Chapter 2

REVIEW OF LITERATURE

Rice is the staple crop of more than 60% population of world and also in south eastern Asia. Asia consumes 90% of total rice produced in world. 43 millions of hectares of land are under rice cultivation in India. 70 % of Indian population has rice as staple food and it is livelihood of more than 120 million populations. So rice is major crop in Indian agriculture. It accounts for 43 % of total food grain and 55 % of cereals produced. In 2016-17, India produced 104.32 million tonnes of rice in 43 million ha (Ministry of Commerce and Industry-MOCI, 2017). Rice has approximately 22 species of genus *Oryza*. It is semi aquatic plants which belong to grass family. Most common rice species used for human consumption are *O. sativa* and *O. glaberrima*. *O. sativa* was first domesticated in southeast Asia like Mynmar, Thialand, Vietnam and China around 8000 to 15000 year ago (OCED,1999; Normile, 2004). *O. glaberrima* was domesticated by people living in the flood plains of the Niger River in Africa about 3000 years ago from its wild ancestor *Oryza barthii* (Porteres, 1979, 1999). Today, rice is cultivated all over the world. *O. sativa* is widely cultivated in Asia, Europe, America, the Middle East, and Africa and *O. glaberrima* is limited to Africa which is being rapidly replaced by *O. sativa*.

There are various constraints for rice production from ecological to edaphic factors. The accelerated production of rice is the main success of The Green Revolution movement in various countries. Both abiotic and biotic factors affect the growth and development of rice ultimately affecting its productivity. Further, reduced yield and quality of rice due to climate change is major concern for world food security. Most of the rice cultivars have been grown in flood irrigated lands which make rice more sensitive to water deficit stress as compared to other cereals It is reported that 5000 litres of water is needed to produce 1 KG rice (IRRI 2005) Thus, this huge demand of water makes rice vulnerable to water deficit. Thus, water deficit is the most severe stress for rice cultivation in rainfed areas (Pandey and Bhandari, 2009). Growing population has increased the pressure on the food production. So improvement of water use efficiency has drawn the world wide attention and the focus has shifted to the identification of cultivars that demonstrate

increased yield under drought stress conditions. Thus, bio prospecting of rice cultivars for better adaptation to various climates has been initiated across the world.

Water is the most crucial component of life. Photosynthesis, nutrition uptake and transportation require water as well as canopy cooling needs sufficient water (Farooq et al., 2009). Even though plants are sessile in nature they can respond to environmental changes. The ability to tolerate water deficit stress determines their survival tendency. Plant needs water for transpiration through which stomata opens and CO₂ uptake occurs through atmosphere and photosynthesis happens. The root water uptake and xylem water transport is driven by transpiration. The opening and closing mechanism of stomata regulates the CO₂ uptake and transpiration. During unfavorable environment conditions like drought stress plants regulate the water loss by regulating the stomata as well as sacrificing the CO₂ uptake and photosynthesis phenomenon.

2.1. Drought stress

Water deficit in plants results from insufficient soil water availability to meet the demand of a particular plant or canopy at a particular time, resulting in a change in plant water status. This dynamic representation involves the root capacity to supply water to the shoots (Lobet et al., 2014) and plant's transpiration rate, which combines evaporative demand and shoot characteristics (Mott and Parkhurst, 1991). Drought is one of the major hindrances for rice cultivation and production in water limited conditions (Bernier et al., 2008; Mishra et al., 2015). During drought stress, dry weight accumulation reduces in plants and its life cycle shortens which leads to yield reduction (Blum, 2005; Kamoshita et al., 2008). Vegetative growth just before flowering also reduces the yield by suppressing the growth of leaves and storage organs (McDonald et al., 2003; Okami et al., 2015). During flowering, drought stress reduces the pollen viability and stigma receptivity as well as seed set and grain formation (Barnabas et al., 2008, Boonjung and Fukai, 1996). Reproductive organ failures like ovary and pollen along with inhibition of pollen germination and fertilization (Satake and Yoshida, 1978; Ekanayake et al., 1990) have been recorded in drought stress (Saini et al., 1983, 1997). The spikelet fertility reduction and panicle exertions are the major effects of drought stress on grain yield (Ji et al., 2005). Thus, drought is challenging aspects in present and coming time for rice production. It has been reported that in rice, severe drought stress at grain filling

reduce yield by 60 % (Basnayake et al., 2006) and at reproductive stage reduce yield up to 94.8 % (Lafitte et al., 2006).

2.2. Drought impact on growth

Drought stress causes the loss of turgor pressure and cell shrinking due to decrease in water status. Turgor cell expansion is inhibited when there is loss of turgor pressure which limits the plant growth. Stem length of rice is reduced due to reduced cell growth in drought (Petropoulos et al., 2008; Sankar et al., 2008). Similarly when there is reduced in the cell growth, there is reduction in leaf expansion (Ren et al., 2007) and the leaf size diminishes and there is reduction in the transpiration but in some cases it leads to abscission of leaves. (Giovannelli et al., 2007; Gu et al., 2007). Cell volume reduction increases the solutes concentration in cells which decreases the water potential. Many plants increase water uptake by root growth to deeper or lateral side to maintain a low osmotic pressure in drought (Djibril et al., 2005; Jaleel et al., 2008; Trachsel et al., 2010).

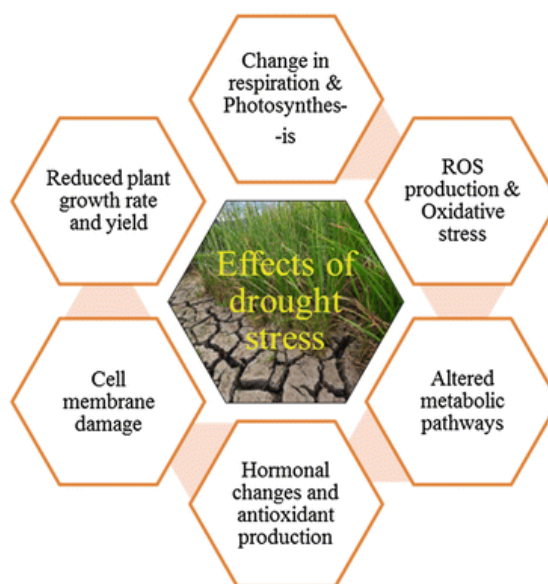


Fig. 2.1 Effects of drought stress in rice

2.3. Drought impact on physiological and biochemical factors

Plants rapidly respond to their changing environment and adapt by undergoing different phenomenal changes. It involves different physiological modifications like wilting of leaf, leaf area reduction, and abscission of leaves, root growth stimulation and changes in relative water content. Similarly it involves

different biochemical changes like electrolytic leakage, ROS generation and free radicals accumulation which disrupt the homeostasis of cells by reacting with lipids and protein which results in the peroxidation of lipids, protein damage, damage of membranes and enzyme inactivation thus affecting the viability of cells (Bartels and Sunkar, 2005). Plant hormones like abscisic acid (ABA), induces leaf stomata closure and reduces water loss through decrease in transpiration and photosynthetic rate thus, improving the water-use efficiency of plants.

Thus understanding and developing the drought tolerance mechanism in rice is important aspects to look in rice production. Since the population is increasing rapidly, we need to double our rice production by 2030. So it's a big challenge to double the production with prone of drought and other abiotic and biotic issues in rice.

2.4. Drought stress impact on photosynthetic performance

2.4.1. Photosynthesis

Photosynthesis is the main metabolic process which determines the crop production and is affected by water deficit and drought stress. It is well documented that drought stress causes the reduction in the rate of photosynthesis of rice (Ji et al, 2012; Lauteri et al, 2014; Yang et al, 2014). CO₂ diffusional limitation due to early closure of stomata is the major components limiting photosynthesis thus reducing photosynthetic enzyme activity, the biochemical components of formation of triose-phosphate and decreasing efficiency of PSII. Conductance of stomata and mesophyll showed decrease response to drought (Centritto et al, 2009).

2.4.2. PS II Activity

PSII activity is necessary for providing reducing power and ATP. When PSII activity crosses the limit of balance tradeoff, there is excess reduction of photosynthetic electron transport chain which triggers the ROS formation. Therefore the balance between PSII activity and photo assimilates demand must be maintained. It is reported that in rice drought reduces the PSII activity in the leaf (Pieters and Souki, 2005) which can be possibly due to di polypeptide degradation leading to inactivation of the PSII reaction center (Lauteri et al, 2014).

2.4.3. Photosynthetic pigments

Drought stress changes the total content of chlorophyll (Sestak et al., 1986) contained within protein-pigment complexes of thylakoid membranes. They are the first structures to be damaged under stress conditions (Synkova, 2000). Drought stress generates the ROS which damages the chloroplasts leading to the reduction in the chlorophyll (Smirnoff, 1995). Drought stress caused the changes in chlorophyll a and b ratio and also reduction in the carotenoids (Anjum et al., 2003; Farooq et al., 2009). Chlorophyll absorbs the light and transfer the light energy to the photosystem reaction center thus producing reducing power. Other pigments like carotenoids have additional roles in chloroplast photosystem structure, light harvesting and photo protection. They help plants to fight against the drought stress and its adverse effects. Drought brings the decrease in content of chlorophyll and quantum yield of PSII in rice. (Pirdashti et al, 2009; Cha-um et al, 2010; Sikuku et al, 2012; Ha, 2014; Maisura et al, 2014).

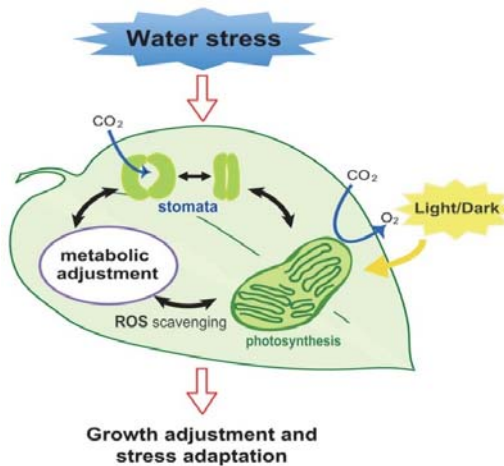


Fig: 2.2. Plant response to water stress (Osakabe et al., 2014).

2.2.1. Stomatal Opening

When two guard cells which surround the stomata lose the turgor pressure, there is closing of stomata (Outlaw, 2003). ABA induces the stomatal closure under drought stress and other environmental stresses like cold, osmotic pressure, CO₂ concentration, and salt stress (Nilson and Assmann, 2007; Kim et al., 2010). Secondary messengers, who are important for stress signalling like such as Ca²⁺, H₂O₂ and NO are also important signals for stomatal closure (Atkinson et al., 1990;

Zhang et al., 2001; Neill et al., 2002; Garcia-Mata and Lamattina, 2009). Similarly loss of turgor pressure loss in passive way results in stomatal closure.

2.5. Drought stress impact on Reactive Oxygen Species production and Antioxidative capacity

2.5.1 Reactive oxygen species (ROS)

The ROS generation is a important drought responsive factor in plants (Miller et al., 2010). ROS such as singlet oxygen ($^1\text{O}_2$), hydrogen peroxide (H_2O_2), and superoxide anion (O_2^-) are toxic byproducts of stress metabolism and also act as important signal transduction molecules which depend upon their cellular amount (Miller et al., 2010). ROS acts as important stress signalling molecules at different concentration from low to moderate which triggers the stress responsive adaptations. ROS at high level causes oxidative damage of lipids, proteins and DNA leading to retarded growth and eventually cell death (Hou et al., 2009). The hydroxyl radical ($\bullet\text{OH}$), which is also the most electrophilic one is the most reactive free radical among ROS. The hydroxyl radical ($\bullet\text{OH}$) can damage almost any molecule near its formation site, including lipids, proteins and DNA. This radical is responsible for the most important oxidative damage to DNA. During drought stress, CO_2 fixation limitation will reduce the regeneration of NADP^+ , thus leading to excessive reduction of electrons in photosynthetic reactions. Similarly, it is also reported that increased photorespiration causes more than 70 % of total H_2O_2 produced during drought stress (Noctor et.al, 2002).

2.5.2. Lipid peroxidation

Lipid peroxidation is a continuous reaction induced by free radicals which leads to cell membrane damage by altering the unsaturated fatty acids. Under drought stress, malondialdehyde content which is the last product of peroxidation shows the intensity of membrane lipid peroxidation and disrupts the cellular function under drought stress. Drought stressed rice seedlings showed significant enhancement in the lipid peroxide level (Sharma and Dubey, 2005).

2.5.3. Antioxidants

An efficient antioxidative system (non enzymic and enzymic antioxidants) is needed for scavenging or detoxification of excess ROS (Noctor and Foyer, 1998). The enzymic antioxidants include superoxide dismutase (SOD), catalase (CAT),

guaiacol peroxidase (GPX), enzymes of ascorbate-glutathione (AsA-GSH) cycle such as ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Noctor and Foyer, 1998) while non enzymic antioxidants include carotenoids, tocopherols and phenolics which are indicator of stress response. Maintenance of higher antioxidant capacity to detoxify the toxic ROS is important character of stress tolerance in plants (Zaefyzadeh et al, 2009; chen et al, 2002). There are several transgenic lines developed by altering the antioxidants which induce tolerance to oxidative stress (Allen et al., 1997; Faize et al., 2011). Multiple expressions of many antioxidant enzymes can be more powerful than the individual expression of enzymes for increasing the tolerance to environmental stress (Lee et al., 2007).

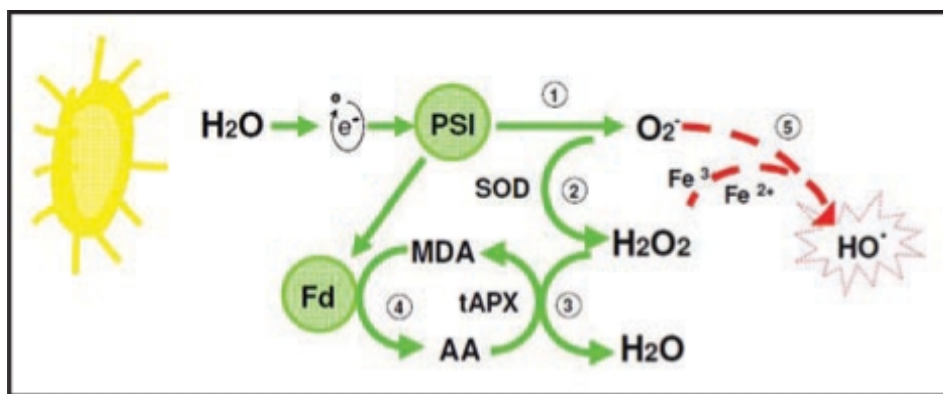


Fig: 2.3. Mehler-peroxidase reaction (Cruz de Carvalho, 2008)

2.5. Osmotic Adjustment

Osmotic adjustment is accumulation of solutes due to decrease in osmotic potential in different stress (Steponkus et al., 1982; Turner, 1986; Munns, 1988). Osmotic adjustment helps in the turgor maintenance and cell volume during drought, and has been important character of drought tolerance of crops (Turner and Jones, 1980; Yang et al., 1983; Morgan, 1984). Osmolytes include amino acids like free proline, various soluble sugars like sucrose and raffinose, and metabolites like galactinol and myo-inositol. Proline is one of the important osmolytes which plays as protective role under various environmental stress conditions (Verbruggen and Hermans, 2008). Kemble and MacPherson (1954) reported first time that free proline accumulation during water stress. Proline accumulation helps in better adaptation and drought tolerance (Vajrabhaya et al., 2001). Proline concentration changes when rice

faces drought stress (Sheela and Alexallder, 1995; Mostajeran and Rahimi-Eichi, 2009; Bunnag and Pongthai, 2013; Kumar et al., 2014; Lum et al., 2014; Maisura et al., 2014). Proline also plays important role as a metal chelator and a signaling molecule under stress condition (Hayat et al., 2012). During drought stress, Proline accumulation promotes tolerance by increasing antioxidant activity. Under drought stress, increased proline content can be taken as marker for variety selection which can resist the harsh environment conditions (Fahramand et al., 2014).

2.6. Drought stress impact on molecular aspects in rice

Molecular response to different environmental stresses includes perception of various stress and its signal transduction to internal parts of cells, expression of various genes and finally changing the levels of metabolics by enzyme activity (Agarwal et al., 2006). Thus, the stress inducible genes not only protect cells from stress production of metabolic proteins but regulate the downstream signal transduction.

2.7.1 Absciscic acid (ABA)

ABA is stress hormone which has important regulatory function in different physiological and developmental process like maturation of embryo, development of seeds. It also induces the dormancy of seeds and buds as well as it has important role in seed germination, root growth. It regulates the fruit ripening in different non climacteric fruits. In stress condition, it regulates stomatal aperture and activates many stress inducible genes (Agarwal and Jha, 2010). Environmental stresses like drought, salinity, cold, heat and wound triggers the ABA level. ABA signaling has major role in controlling many stress-adaptation responses such as the activation of genes responsible for osmotic adjustment, ion compartmentalization, root hydraulic conductivity under drought, shoot growth regulation, reducing transpiration and reducing the loss of water due to wilting in plants (Verslues and Zhu, 2005; Pospíšilová et al., 2009). ABA regulates many stress responsive genes during osmotic and drought stress (Ingram and Bartels, 1996).

2.7.2 ABA biosynthesis

ABA is derived from β -carotene (C-40) through an oxidative cleavage reaction in plastids. The first step of ABA biosynthesis pathway starts with Zeaxanthin epoxidase (ZEP) catalysis of conversion of zeaxanthin and

antheraxanthin to all trans-violaxanthin. C15 intermediated product, Xanthoxin is created when all-trans-violaxanthin is converted to 9-cis-violaxanthin or 9'-cis-neoxanthin by the 9-cis-epoxy carotenoid dioxygenase (NCED). Then xanthoxin from plastid, it is converted to ABA in cytosol by abscisic aldehyde oxidase (Nambara and Marion-Poll, 2005; Shah et al., 2016).

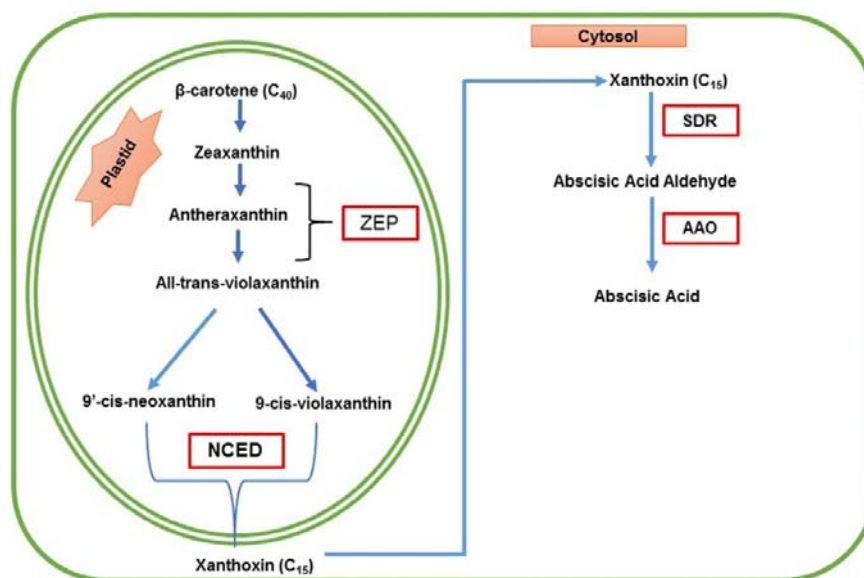


Fig: 2.4. ABA biosynthesis pathway

NCED (9-cis-epoxy carotenoid dioxygenase) is the key enzyme for biosynthesis of ABA (Tan et al., 1997). ABA biosynthesis involves 5 *nced* genes in Arabidopsis and rice (Iuchi et al., 2000; Schwartz et al., 2003; Saika et al., 2007; Zhu et al., 2009). During water deficit, NCED3 regulates the ABA synthesis. NCED5 also contributes, together with NCED3, for ABA production during water stress tolerance (Frey et al., 2012). NCED4 is up-regulated by salt stress (Welsch et al., 2008).

2.7.3 ABA Signaling

Absciscic acid is a stress hormone which has important role in various plant stresses. Understanding of ABA signaling mechanism is very important for plant improvement in different environmental stresses like drought stress. Pyrabactin resistance (PYR)/pyrabactin resistance like (PYL)/regulatory component of ABA receptors (RCAR), protein phosphatase 2C (PP2C) and (Sucrose non-fermenting) SNF1-related protein kinase 2 (SnRK2) are there three main components of ABA signaling. ABA inhibits PP2C Activated SNRK2 further phosphorylates the

downstream signaling proteins like transcription factors which ultimately do the expression of ABA responsive genes (Ma et al., 2009; Park et al., 2009).

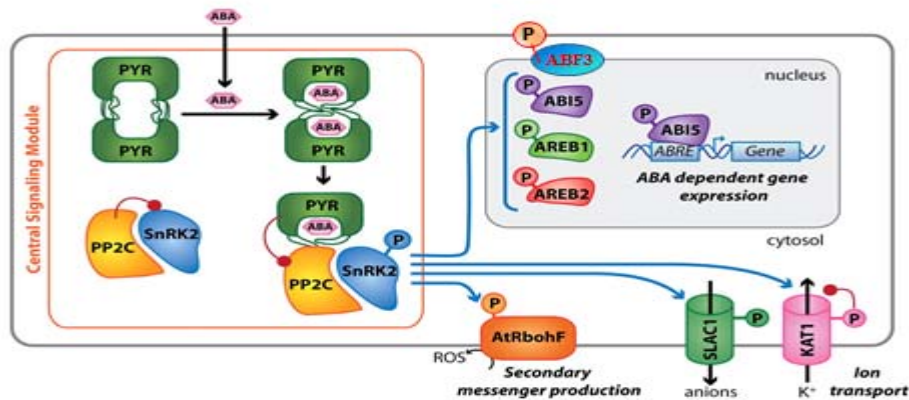


Fig: 2.5. The core ABA signaling pathway: Recent progress in understanding early ABA signal transduction has led to the construction of a PYR/RCAR–PP2C–SnRK2 signal transduction model. In the absence of ABA, PP2Cs inhibit protein kinase (SnRK2) activity through removal of activating phosphates. ABA is bound by intracellular PYR/PYL dimers, which dissociate to form ABA receptor–PP2C complexes. Complex formation therefore inhibits the activity of the PP2C in an ABA-dependent manner, allowing activation of SnRK2s. Several SnRK2 targets have been identified both at the plasma membrane and in the nucleus, resulting in control of ion channels, secondary messenger production, and gene expression. Red connections on *left* indicate an inhibitory interaction (Hubbard, 2010).

2.7.4 ABA Receptors

After being established as ABA receptors, the function of PYL in expression of stress responsive genes and regulation of ABA sensing and its signaling route has been a matter of interest for different research group thus, it is being extensively studied in Arabidopsis and functionally characterized in rice (Ma et al., 2009; Kim et al., 2011; Kim et al., 2014; Tian et al., 2015; Yu et al., 2016; Lenka et al., 2018). The function of PYLS in stress response mediated by ABA is important for understanding and improving the stress tolerance in rice. It is reported that pRDA:29A PYL transgenic lines exhibit decrease in water loss by transpiration and acceleration of leaf senescence, decreased in membrane damage, reduction of oxidative damage, increase of water use efficiency, and finally, increased survival rates in extreme drought conditions (Zhao et al., 2016). Similarly over expression of PYL5/RCAR5 causes ABA hypersensitivity and also water deficit tolerance

(Santiago et al., 2009; Kim et al., 2014). PYL4 overexpressing transgenic plants also showed improved drought tolerance (Pizzio et al., 2013) and PYL3 (Lenka et al., 2018).

2.7.5 Cytokinin

Naturally available cytokinins are the derivatives of adenine with different substitutions to the adenine ring at N⁶ position. Among them, the most common class of cytokinins is with isoprenoid side chains which include the most abundant cytokinin in Arabidopsis, trans-zeatin (tZ). Zeatin occurs in cis and trans form in higher plants. Sometimes, cytokinins are found as ribosides, attached with a ribose sugar and ribotides, attached with a ribose moiety containing a phosphate group along with free base. Further, it is also reported that cytokinins conjugate with sugar moieties. Cytokinins regulate a different growth and developmental phenomenon. It also suppresses the degradation of chlorophyll and senescence of leaves (Choi and Hwang, 2007; Lim et al., 2007). Cytokinin is synthesized by the attachment of the isopentenyl moiety of isopentenyl pyrophosphate to the N⁶-position of adenine molecule by the enzyme Isopentenyl Pyrophosphate Transferase (IPT). IPT reaction has substrate of adenine nucleotide of trna or an adenine of ATP, ADP or AMP. The main biosynthesis enzymes involve in CK synthesis are isopentenyltransferases (ipt), riboside 5'-monophosphate phosphoribohydrolase, and cytochrome P450 monooxygenases while involve in degrading are cytokinin oxidase/dehydrogenases (CKXs) (Sakakibara et al., 2006). Cytokinin has two main components (His-Asp) which is phosphorelay pathway. It includes histidine kinases (HKs), histidine phosphotransfer (HPs) and response regulators. B-RRs activate the A-RR primary response genes which activate the downstream signaling of cytokinin (Ha et al., 2012).

Cytokinins are involved in regulating biological activities like cell division, organogenesis, meristematic activities, and gametophyte development (Kieber, 2002). Organ-specific and stress-inducible production of cytokinin is considered more preferable. Cytokinins play important role in stress tolerance in plants. Rivero et al (2007) proved that transgenic tobacco plant expressing ipt gene with stress inducible promoter showed that it improved the tolerance to drought stress. It also showed the high RWC and retained rate of photosynthesis. In Arabidopsis, AHK1

and AHK3 transgenics showed delay in leaf senescence in cytokinin dependent manner through the phosphorylated cytokinin response regulators under drought condition (Kim et al., 2006; Wohlbach et al., 2008; Nguyen et al., 2016).

The root specific and shoot specific expression of Cytokinins has different role in stress tolerance. Root-specific expression of *CKX* in tobacco and *Arabidopsis* showed enlargement of root system in both well growing conditions and restricted water supply (Werner et al., 2010). Cytokinin-deficient plants showed more tolerance to salt stress through enhanced growth of primary roots and more tolerant to drought due to increased cell membrane integrity (Nishiyama et al., 2011). Similarly, shoot specific expression of *IPT* gene of *Agrobacterium tumefaciens* with promoters like *PSAG12*, *PSARK*, *rd29A*, or *HSP* increased tolerance to different stresses like drought and flooding in *Arabidopsis* (Zhang et al., 2000), creeping bentgrass (*Agrostis stolonifera*) (Merewitz et al., 2010, 2011a, 2011b, 2011c), rice (*Oryza sativa*) (Peleg et al., 2011), and tobacco (*Nicotiana tabaccum*) (Rivero et al., 2007, 2010). *PSAG39:IPT* expression in rice led to early flowering and advanced heading date improved plants against mild water deficit and also increase grain yield during drought stress (Zou et al., 2007; Liu et al., 2010). Furthermore *IPT* over expression increased the plant performance under submerging and water logging conditions (Huynh et al., 2005). It was also reported that *IPTs* Transgenic increased CKs level, thus shows increased plant growth and flowering prolonging, and increased chlorophyll synthesis, better photosynthesis, better maintenance of intracellular water content and transpiration rate, as well as postponed leaf senescence and faster post-drought recovery stage (Guo et al., 2010).

Thus, Cytokinin-mediated stress responses in plants is regulated by concentrations of Cytokinins (O'Brien and Benkova, 2013) while constitutive reduction and overproduction of Cytokinins result tolerance to various environmental stresses. Manipulation of concentration of Cytokinins can be better strategy for stress tolerance (Rivero et al., 2007; Werner et al., 2010; Nishiyama et al., 2011; Ha et al., 2012).

Cytokinin and ABA interactions are well reported in non-stress conditions like during seed germination. *ARR4*, *5*, and *6* proteins interact with and inhibit the function of the transcription factor *ABI5*, a positive regulator of ABA signaling

(Wang et al., 2011). Similarly it is reported that ABI4 induced the expression of ARR5 in lateral root formation (Shkolnik-Inbar and Bar-Zvi, 2010). These findings support the cross-talk of ABA and Cytokinins exists in stress conditions. ABA mediated stomatal closure in under drought condition involves the production of nitric oxide and this process was inhibited by cytokinins by direct interaction with and reduction of NO molecule, acting as a scavenger (Liu et al., 2013). Thus, this evidence showed that cytokinin itself is directly involved in modulating the ABA response.

2.7.6 ABA-dependent and ABA-independent pathway under drought

Most of the plant's stress responses are mediated through Abscissic acid (ABA). Based on the evidence of ABA response, drought responsive genes can be divided into two groups: ABA-dependent and ABA-independent genes (Yamaguchi-Shinozaki and Shinozaki, 2005).

2.7.6.1 ABA dependent pathway

A cis-element analysis showed that many ABA-dependent drought-related genes contain a conserved ABA-responsive element (ABRE) with ACGT cores in their promoter regions (Uno et al., 2000). These genes are regulated by ABRE binding proteins-AREB or ABRE binding factors-ABFs (Uno et al., 2000; Fujita et al., 2009), ABI5 (ABA insensitive 5), MYB (myeloblastosis), MYC (myelocytomatosis), NACs, and ERF.

ABA and drought stress induce AREB1/ABF2, AREB2/ABF4 and ABF3 (Fujita et al., 2013). it has been proven by the overexpression study that AREB/ABFs have positive regulation on ABA signaling in water deficit condition (Kang 2002; Kim et al., 2004; Furihata et al., 2006; Fujita et al., 2009). Thus AREB/ABFs are important transcription factors controlling ABA dependent drought responsive gene expression

Several reports revealed that genes with NAC domain like NAM, CUC2 and NAP have important role in plant development. *OsNAC* genes have big space in rice genome with large gene family. Each *OsNAC* genes has specific pattern according to various organs which correlates its diverse and important function in development of rice. Various factors like drought, salinity, hormones like ABA and methyl

jasmonate induce the expression of NAC genes which shows it has role in tolerance of abiotic stress. NAC gene overexpression can be important key tool for achieving various stress tolerance in plants .

2.7.6.2 ABA independent pathway

DREB (dehydration responsive element binding factors) Transcription factors

DREBs act mostly on ABA independent manner which regulate many stress-inducible gene expression. DREBs play important role in abiotic stress tolerance by interacting with DRE/CRT cis element which is found in different abiotic stress responsive genes. There is 9 bp conserved core sequence (5' TACCGACT 3') in DRE which was reported in the promoter region of *rd29A*, drought-responsive gene (Yamaguchi-Shinozaki and Shinozaki, 1993).

DRE binding proteins were reported first time from *Arabidopsis*. They are CBF1 (CRT binding factor1), DREB1A and DREB2A (Stockinger et al., 1997; Liu et al., 1998). DREB1/CBF expression is induced by cold, while the *DREB2* expression are induced by dehydration, high-salinity, and heat stresses (Liu et al., 1998; Shinwari et al., 1998; Nakashima et al., 2000). Similarly osmotic stress induce CBF4/DREB1D, DREB1E/DDF2 and DREB1F/DDF1 which revealed that there is important interaction between DREB1 and the DREB2 (Haake et al., 2002; Nakashima et al., 2009).

Drought and salinity stress induced expression of DREB2 highlight their role in stress-responsive gene expression. Out of eight DREB2s only *DREB2A* and *DREB2B* expression under drought were reported from *Arabidopsis* (Liu et al., 1998). *DREB2* homologous genes are already isolated from cereal crops like rice (Lata et al., 2011). Osmoprotectants like proline, soluble sugars, galactinol and myo-inositol are elevated by DREB transcription factors (Gilmour et al., 2000; Shiqing et al., 2005; Ito et al., 2006) thus improving the drought stress tolerance and leaf water status in plants. DREB1/CBF target includes TFs, phospholipase C, LEA proteins and sugar transport proteins.

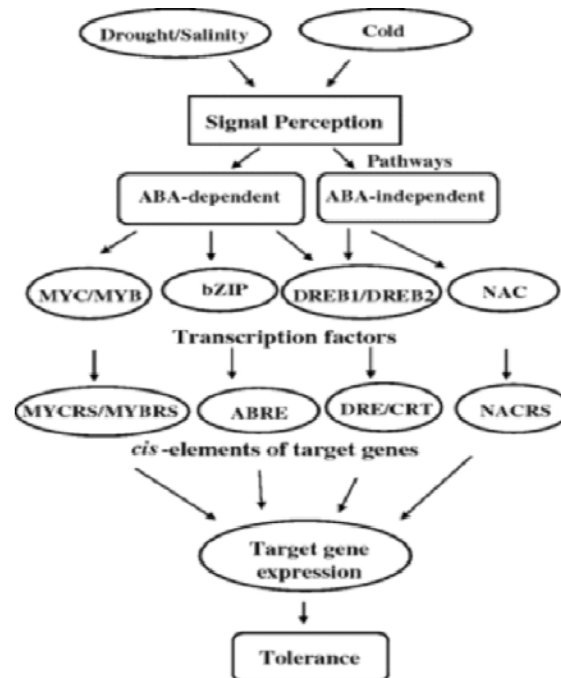


Fig: 2.6. A schematic representation of stress signal perception and gene expression via ABA-dependent and independent pathways at cellular level in plants (Lata and Prasad, 2011)

It also targets protein related to osmoprotectant biosynthesis and protease inhibitors (Fowler and Thomashow, 2002; Maruyama et al., 2004). Thus, DREBs can be important strategy for crop improvement providing osmotolerance to the plants under stress conditions (Hussain et al., 2011).

2.7.1.1 Crosstalk of ABA dependent and ABA independent pathway

The possibility of cross talk between ABA-dependent and ABA-independent pathway lies during drought tolerance (Yoshida et al., 2015). ABA signaling component, SnRK2s regulate the expression of both AREB/ABFs and DREBs under drought condition suggesting the role of SnRK2s in convergence of both these pathways together (Fujita et al., 2013). Recently it has been reported that ABA-dependent proteins AREB1/ABF2, AREB2/ABF4 and ABF3 can interact with the ABA-independent proteins DREB2A, DREB1A and DREB2C for bringing drought tolerance (Lee et al., 2010; Kim et al., 2011). ANAC096 has been reported to be ABA independent but it also interacts with the tf's like ABF2 and ABF4 to regulate drought responsive gene expression (Xu et al., 2013). Oh et al (2005) reported that a

receptor kinase gene, *Hsp70* and *PP2Ca* were activated by transgenic ABF3 and CBF3 in *Arabidopsis* suggesting ABA-dependent and ABA-independent pathways converge, at least in part, into these common target genes. Thus these results support the possibility of cross talk between the ABA dependent and ABA independent pathway which can be utilized to engineering and breeding the crops for broad spectrum drought stress and other stress tolerance in crops.

2.7. Melatonin

Melatonin is a low molecular weight indoleamine that ubiquitously present in plants (Erland et al., 2015). Its molecular formula is N-acetyl-5-methoxytryptamine. Melatonin was first identified as an important animal hormone which function as important multiple role in biological processes like antioxidative activity, circadian rhythms, seasonal reproductive physiology, sleep, sexual behavior control, mood response, temperature biohomeostasis, retinal physiology, and enhancement of immunology. In plants, Melatonin is found in different organs like seeds, roots, stems, shoots, leaves, flowers, bulbs and fruits (Arnao and Hernández-Ruiz, 2014). Its content ranges from pico gram to micro gram. Melatonin has been reported to have function in many physiological processes which respond to environmental stress further redirecting plant growth and development, protecting developing tissues from damage in stress (Murch et al. 2009; Erland et al. 2015; Reiter et al. 2015). Melatonin acts as potent antioxidant for scavenging the reactive oxygen species (ROS) under environmental stress (Tan et al. 2007, 2015). Several reports revealed that melatonin interacts with different signaling pathways such as calcium/calmodulin, ubiquitination and cyclic adenosine monophosphate (cAMP) (Reiter et al. 1993; Hardeland 2009; Tan et al. 2015; Sanchez-Barcelo et al. 2016).

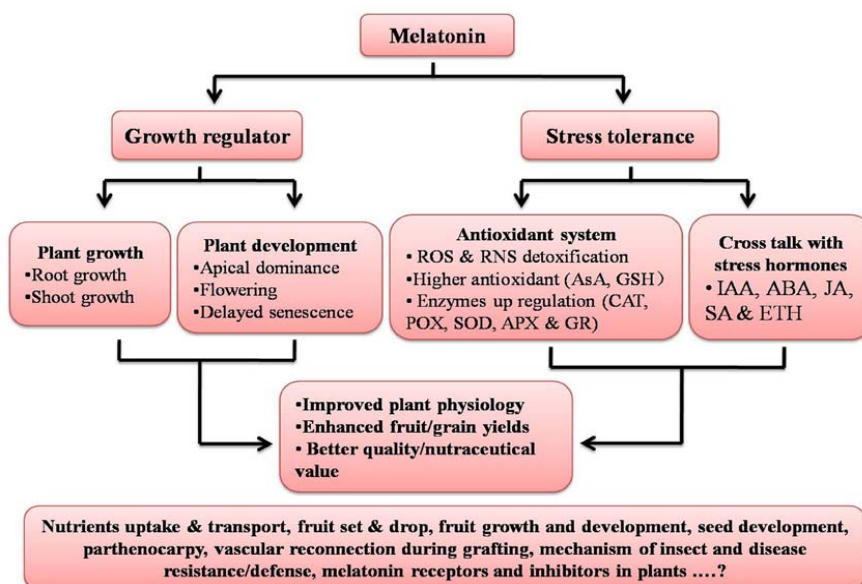


Fig: 2.7. Role of Melatonin in plant. ROS, Reactive oxygen species; RNS, Reactive nitrogen species; AsA, Ascorbic acid; GSH, Glutathione; CAT, Catalase; POX, Peroxidase; SOD, Superoxide dismutase; APX, Ascorbate peroxidase; GR, Glutathione reductase; IAA, Indole-3-acetic acid; ABA, Absciscic acid; JA, Jasmonic acid; SA, Salicylic acid; and ETH, Ethylene (Nawaz et al., 2016)

steps required for melatonin biosynthesis are described below.

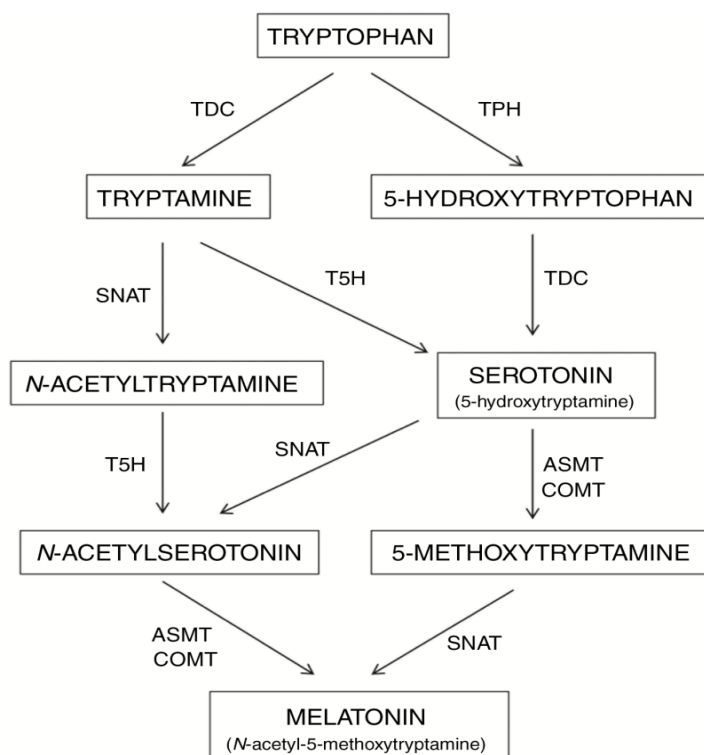


Fig: 2.8. Melatonin Biosynthesis pathway in plant; T5H-tryptophan 5-hydroxylase; TDC-tryptophan decarboxylase; SNAT-serotonin N-acetyltransferase; ASMT-acetylserotoninmethyltransferase; and COMT-caffeic acid O-methyltransferase (Arnao and Hernandez, 2017)

The first step of conversion of tryptophan into tryptamine is done by tryptophan decarboxylase (TDC). TDC has been cloned from different plant genome like rice, pepper, etc (Di Fiore et al., 2002; Kang et al., 2008; Park et al., 2009).

The second step includes the conversion of tryptamine into serotonin with hydroxylation of C5 position of tryptamine by Tryptamine 5 Hydroxylase, which is Cytochrome P450 enzyme (Fujiwara et al., 2010). Rice has constitutive expression of T5H enzyme (Kang et al., 2007).

Aryl alkyl amine N-acetyl transferase (AANAT) and N-acyl serotonin methyl transferase (ASMT) are the final two enzymes on the melatonin biosynthetic pathway. In green alga, *Chlamydomonas reinhardtii*'s AANAT has been cloned but still its analogs in higher plants is missing (Okazaki et al., 2009). Serotonin N-acetyl transferase (SNAT) genes have been cloned in rice catalyze conversion of serotonin into N-acetyl serotonin. ASMT, also known as HIOMT (hydroxyl indole-O-methyl transferase), has been extracted from rice via *Escherichia coli* overexpression of its heterolog (Kang et al., 2013). Being the last step enzyme, ASMT is the rate-limiting enzyme. Subcellular localization revealed that ASMT lies in cytoplasm and SNAT protein found in chloroplasts (Byeon et al., 2014). Gene annotation and analysis of metabolic pathway network database (Rice Cyc) in rice have revealed the abiotic and biotic stress mediated induction of TDC (Dharmawardhana et al., 2013). Transgenic approaches for melatonin biosynthesis pathway promises the drought tolerance. TDC overexpression in rice exhibits delayed senescence of leaves (Byeon et al., 2014, Kang et al., 2009), while RNAi suppression of TDC promotes senescence process by reducing the serotonin levels. SNAT can be alternative for AANAT for the last step enzyme in melatonin biosynthesis in rice (Kang et al., 2013). Overexpression of AANAT in rice increased the seminal root elongation and same time it also increases the seedling growth and herbicide resistance (Byeon and Back, 2014; Park et al., 2013; Park and Back, 2012). Similarly, overexpression of ovine AANAT in tomato plant created the apical dominance loss and enhancing drought tolerance (Wang et al., 2014).

2.7.2. Role of melatonin in stress tolerance in plants

Exogenous melatonin induces the formation of adventitious root along with formation of root primordia from cells of pericycle. It modifies the adventitious or later root formation distribution patterns which are key factors for the drought stress tolerance. Cui et al., (2017) reported that exogenous melatonin increased antioxidant capacity and decreased the damage of cell membrane in wheat seedlings and helps to maintain the intact grana lamella in chloroplast during the drought stress in wheat seedlings thus promoting drought tolerance. It helps to maintain the cell turgor balance and water holding capacity by increasing the water potential which leads to improvement of photosynthesis and photosystem efficiency. Exogenous melatonin also increased the total antioxidant capacity by decreasing the level of oxidants like H_2O_2 and superoxide O_2^- . Similarly Wang et al. (2012) reported that exogenous melatonin delayed drought and dark -induced leaf senescence in apple by retaining photosystem II (PSII) function and delaying chlorophyll reduction under drought in apple. Similarly it was observed that down-regulation of SAG12 by melatonin treatment during dark-induced senescence. Melatonin slowed chlorophyll loss in leaves in barley seedlings (Arnao and Hernández-Ruiz, 2009). Thus, these evidences suggest that melatonin might be the broad-spectrum stress tolerance agent (Tan et al., 2000; Allegra et al., 2003).

2.7.3. Role of melatonin in drought tolerance

2.8.3.1 Relative water content

RWC is correlated with the soil water level which is important character of drought stress (Sarker et al., 1999; Merah, 2001). RWC

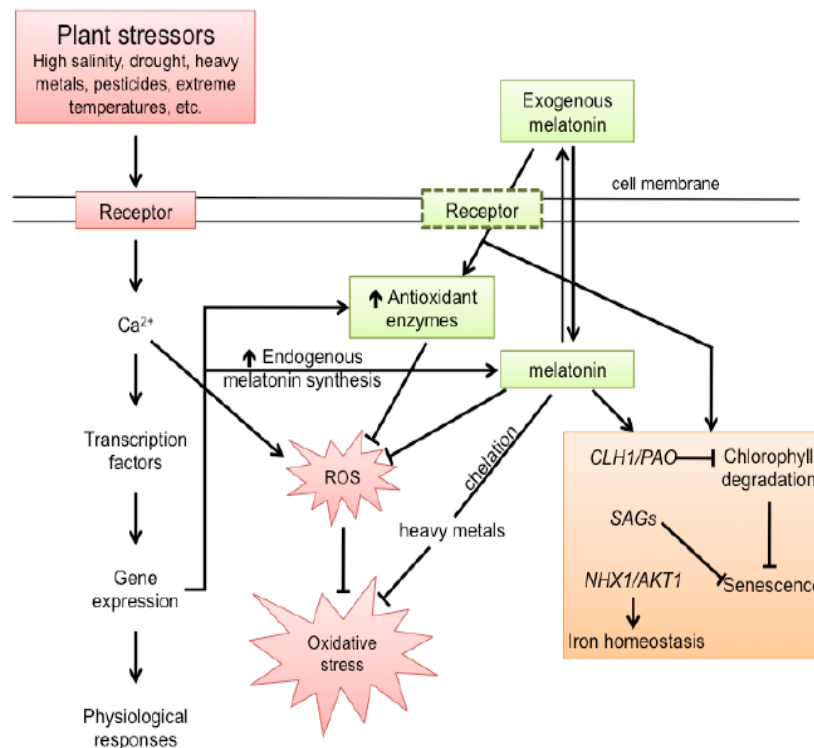


Fig: 2.9. Exogenous melatonin confers stress tolerance. Stress induced the production of reactive oxygen species (ROS). The exogenous melatonin application protects against ROS. Melatonin receptors (hyphenated box) in the cell membrane which are yet to be identified. CLH1/PAO = chlorophyll degradation-related genes; NAX1/AKT1 ion channel-related genes; SAGs = senescence associated genes (Reiter et al., 2015)

decreases immediately when there is water deficit exposed to plants (Grover et al., 2004). RWC was proposed by Sinclair and Ludlow 1985 as a better water status indicator. Previous reports have shown that melatonin treatment in drought significantly improve leaf water status by decreasing water potential (Ye et al., 2016), higher turgor pressure (Meng et al., 2014; Cui et al., 2017) and higher RWC (Turk et al., 2014).

2.8.3.2 Photosynthesis

Imbalance tradeoff between light harvest and its consumption, drought stress inhibits the photosynthetic activity (Foyer and Noctor, 2000). Stomatal limitation is the major indicator of the reduction in the photosynthesis in water deficit stress (Cornic, 2000). Drought stress decreases the chlorophyll content by affecting the

chlorophyll biosynthesis thus damaging the photosynthetic apparatus and reducing the photosynthesis which acts as non stomatal limitation of drought stress (Iturbe Ormaetxe et al., 1998). Thus it was generally accepted as major factor for photosynthesis reduction can be stomatal limitation as well as non stomatal limitation in water deficit stress (Farooq et al., 2009). Drought stress reduces the photosynthetic performance of rice (Ji et al., 2012; Yang et al., 2014). It has been reported that that melatonin application significantly maintained the photosynthesis and conductance of stomata under water deficit condition in wheat (Cui et al., 2017), maize (Fleta-Soriano et al., 2017), apple (Wang et al., 2013; Li et al., 2014), rapeseed (Li et al., 2015) and alfalfa (Antonioni et al., 2017).

2.8.3.3 Photosynthetic pigments

Drought causes loss of or reduces pigments biosynthesis which results in the light harvest reduction and less generation of reducing power thus limiting the source of energy for photosynthesis. Photosynthetic pigments amount shows the relation with yield and plant biomass (Jaleel et al., 2009). Decrease in chlorophyll content in drought stressed rice is well reported (Pirdashti et al., 2009; Cha-um et al., 2010; Sikuku et al., 2012; Ha et al., 2014; Maisura et al., 2014). Melatonin application significantly increased chlorophyll level in ryegrass (Zhang et al., 2017), wheat (Li et al., 2018), watermelon (Nawaz et al., 2018), Kiwi fruit (Liang et al., 2018) and carotenoids content (Arnao and Hernández-Ruiz, 2014, 2015) under stress condition. Melatonin application delayed the leaf senescence by delaying the chlorophyll degradation (Wang et al., 2012; Zhang et al., 2013; Liang et al., 2018).

2.8.3.4 Photosystem II activity

PSII activity is crucial in providing reducing power NADPH and ATP. When the PSII activity surpasses the demand, there is over reduction of electrons thus forming ROS. Therefore, balance between photochemical activity and the demand for photosynthetic assimilates are required for proper functioning of photosynthetic apparatus. Water stress affects the activity of PSII (He et al., 1995, Giardi et al., 1996). Under drought stress, PSII activity in rice flag leaf is severely impaired (Pieters and Souki, 2005). Melatonin application maintained significantly higher PSII activity under drought stress in maize (Ye et al., 2016; Fleta Soriano et al., 2017), wheat (Cui et al., 2017), apple (Wang et al., 2012; Li et al., 2014), and rye grass

(Zhang et al., 2017). Zhou et al., 2016 reported that melatonin application elevated the PSII quantum yield and coefficient of photochemical quenching thus mitigating the photosynthetic electron chain damage in PSII by stabilizing the D1 proteins which repair of PSII.

2.8.3.5 Stomata opening

ABA is responsible for the closure of stomata and inducing the tolerance to the osmotic stress and drought and cold stress (Nilson and Assmann, 2007; Kim et al., 2010). It is reported that melatonin reopened the closed stomata under water deficit in apple (Li et al., 2014).

2.8.3.6 Reactive oxygen species

Generation of ROS is important biochemical process for stress response in plants (Miller et al., 2010). In stress signaling, ROS behaves as secondary messengers from low to medium level which trigger the defense and adaptive response in the plants. ROS at high level causes oxidative damage of lipids, proteins and DNA leading to in retarded growth and eventual cell death (Hou et al., 2009). Previous studies have reported that melatonin application during drought stress reduces oxidative stress by reducing the level of ROS (Li et al., 2014; Wang et al., 2017, Antoniou et al., 2017, Cui et al., 2018).

2.8.3.7. Lipid peroxidation

Lipid peroxidation is a continuous reaction induced by free radicals which leads to cell membrane damage by altering the unsaturated fatty acids. Poly unsaturated fatty acids which are main components of membrane lipids are susceptible to peroxidation thus resulting in the damage of the membranes and disrupt the cellular function under drought stress. Malondialdehyde (MDA) content, end product of peroxidation determines the level of peroxidation in membranes under stress condition. Drought stressed rice seedlings showed significant enhancement in the lipid peroxide level (Sharma and Dubey, 2005). Melatonin ameliorated the drought stress induce membrane damage by reducing the lipid peroxidation activity (Antoniou et al., 2017). Similarly it was observed that melatonin improved the membrane damage in heat stress (Deng et al., 2017)

2.8.3.8 Antioxidants

Efficient antioxidative system is needed for excess ROS scavenging (Noctor and Foyer, 1998). In enzymatic systems, super oxide dismutase (SOD) catalyzes super oxide dismutation to hydrogen peroxide (Smironoff, 1993). Catalase (CAT) and ascorbate

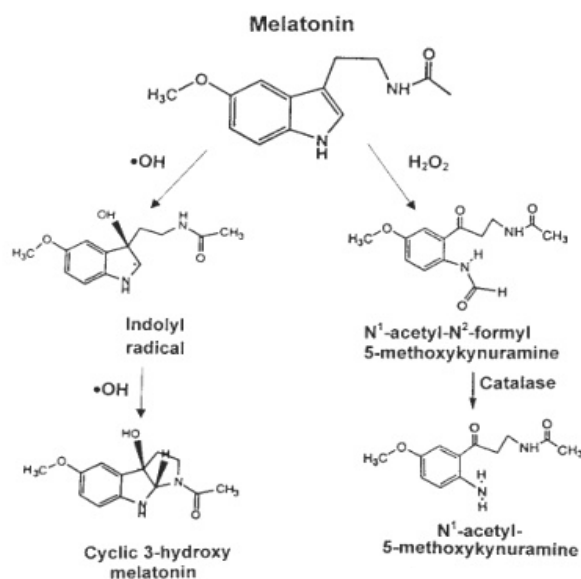


Fig:2.10. Melatonin pathway for scavenging reactive oxygen species (Reiter et al., 2007)

peroxidase (POX) can break down H_2O_2 (Larson et al., 1988). However drought stress interrupts the function of the scavenging enzymes systems thus leading to lipid peroxidation and consequent membrane damage and other cellular damages (Jagtap and Bhargava, 1995; Dat et al., 1998). Maintenance of a high antioxidant capacity is important character for increased tolerance of plants. It is reported that melatonin systemically ameliorated damaged done by drought stress by increasing the activity of SOD, CAT and POX (Antoniou et al., 2017; Cu et al., 2017; Liang et al., 2018; Li et al., 2018).

2.8.3.9. Osmotic adjustment: Proline

Osmotic adjustment is accumulation of osmolytes in the cell which decreases in osmotic potential (Steponkus et al., 1982; Turner, 1986). Osmotic adjustment assists in the turgor maintenance and cell volume which has been marked as

important character for drought tolerance of crops (Turner and Jones, 1980; Yang et al., 1983; Morgan, 1984). Proline is one of the important osmolytes which has positive role in drought tolerance (Kemble and Mac-Pherson, 1954; Vajrabhaya et al., 2001; Verbruggen and Hermans, 2008). Proline also shows the role of metal chelator (Hayat et al., 2012). Level of Proline exceeds over all the amino acids under water deficit in plants. It is reported that melatonin significantly increased the proline content under different stress condition (Turk et al., 2014; Ye et al., 2016; Zhang et al., 2017; Liang et al., 2018) through which plants acquire tolerance.

2.8.3.10 Endogenous melatonin content

Melatonin level in plants increases with various stress conditions which is believed to marker for stress resistance. It has been seen as exogenous melatonin application ameliorates the negative effects of stress. Exogenous melatonin application increased the endogenous melatonin content (Zhang et al., 2017; Wang et al., 2017; Ahammed et al., 2018; Ke et al., 2018). It is also observed that melatonin induced the melatonin biosynthesis genes (Park et al., 2013; Zheng et al., 2018; Zhao et al., 2018; Ni et al., 2018). Similarly TDC overexpression enhanced the level of serotonin while RNAi suppression lowers the level of serotonin (Byeon et al., 2014). Exogenous melatonin application enhanced the biosynthesis gene expression in apple (*MbT5H1*, *MbAANAT3* and *MbASMT9*) and increased melatonin production (Zheng et al., 2017).

2.5.2. Interaction of Melatonin and ABA for drought tolerance

Li et al. (2014) reported that melatonin treatment increased the drought tolerance level in tolerant *Malus prunifolia* and sensitive *M. hupehensis* plants by reducing the expression of *MdNCED3* and up regulating expression of catabolic genes, *MdCYP707A1* and *MdCYP707A2* ultimately reducing ABA level in drought-stressed plants. Direct scavenging of H₂O₂ by melatonin and enhancing antioxidant enzymes activities can synergistically work together to re-open the stomata and improve their function under drought stress. Similarly it was reported that Melatonin treated seed of cucumber shows rapid decrease in the level of ABA in germination early stage by up regulating ABA catabolic genes like *CYP707A1* and *CYP707A2* and down regulating ABA biosynthesis genes like *NCED2* (Zhang et al., 2014). Countering the above evidences, it was reported that foliar and rhizospheric

treatment of melatonin in barley under combined drought and cold stress increases endogenous ABA level in leaves (Li et al., 2016). At the same time (Fu et al., 2017) reported that exogenous treatment of melatonin under cold stress in *Elymus nutans* increases the ABA level.

Previous study showed that high salt, dehydration or ABA treatment induce the AREB1/ABF2, AREB2/ABF4, and ABF3 transcription factors in vegetative tissues (Riechmann et al., 2000; Yoshida et al., 2010). It was reported that transgenic ABF3 confers tolerance to drought stress, chilling, freezing, high temperature, (Kim et al., 2004). Transgenic ABP9 improved photosynthetic capacity under water deficit and heat shocks in *Arabidopsis* (Zhang et al., 2008). Comparative transcriptomic analyses revealed that exogenous melatonin application in Bermuda grass down regulated the ABA signaling genes like RCAR3, LEA protein and transcription factors like AREB3, RABs, etc. (Shi et al., 2014) while up regulated the PP2C related genes and other ABA independent transcription factors.

2.5.3. Interaction of Melatonin and Cytokinin for drought tolerance

Cytokinins has important role in wide range of growth and development in plants. It also suppresses degradation of chlorophyll and senescence of leaf (Choi and Hwang, 2007; Lim et al., 2007). Cytokinins are involved in regulating biological activities like cell division, organogenesis, meristematic activities, and gametophyte development (Kieber, 2002). Organ-specific and stress-inducible production of Cytokinin is considered more preferable. Cytokinins play important role in stress tolerance in plants. Rivero et al (2007) proved that transgenic tobacco plant expressing *ipt* gene with stress inducible promoter showed that it improved the tolerance to drought stress. In *Arabidopsis*, AHK1 and AHK3 transgenics showed delay in leaf senescence in cytokinin dependent manner through the phosphorylated cytokinin response regulators under drought condition (Kim et al., 2006; Wohlbachet al., 2008; Nguyen et al., 2016). Exogenous melatonin application have positive effects on plant stress tolerance in various plant species (Wang et al., 2013, 2012; Zhang et al., 2014; Shi et al., 2015). Similarly, Maet al. (2018) revealed that melatonin suppressed the leaf senescence induced by drought by up-regulating genes for Cytokinin and Melatonin signaling and responses and by down-regulating chlorophyll-degradation genes and enzyme activities in bent grass seedlings thus

suppressing senescence. Also it was reported that melatonin induces heat tolerance by reducing the senescence through up regulating cytokinin biosynthesis genes and down regulating the cytokinin degrading genes and ABA biosynthesis genes (Zhang et al., 2017).

2.7.4. Melatonin and DREB transcription factors for drought tolerance

DREB protein plays key role in stress tolerance in ABA dependent manner by stress responsive gene expression under different environmental stress in plants. It was reported that OsDREB1C and OsDREB2A were highly expressed among five DREB gene during drought stress and their over expression cause drought tolerance (Dubouzet et al., 2003). Similarly transgenic rice overexpressing OsDREB1F and OsDREB1G induces drought tolerance in significant level (Wang et al., 2008, Chen et al., 2008). Melatonin promotes the biotic and abiotic stress tolerance by inducing the CBF/DREB1s transcripts under water deficit stress in Arabidopsis (Shi et al., 2015). The transcriptome study reveals that exogenous melatonin enhances the DREB transcription factor expression (Zhang et al., 2014). Melatonin increases the expression of DREB2A transcription factor for enhancing the drought tolerance (Ma et al., 2018).

2.7.5. Melatonin and NAC transcription factors for drought tolerance

NAC genes are the largest transcription factors families in plants present in large range of species (Souer et al., 1996; Aida et al., 1997). They have crucial role in expression regulation of different transcription factors related to the stress response. NAC genes regulate the key developmental and apical meristem formation process in the pants senescence of leaves, development of seeds, embryo and fibre and cell division (Kim et al., 2006; Ko et al., 2007; Kim et al., 2007; Guo et al., 2006; Breeze et al., 2011; Duval et al., 2002). It is reported that NAC works in ABA dependent and ABA independent pathways for various abiotic stress responses. Overexpression of SNAC3 transcription factor in rice exhibits tolerance of drought by scavenging the reactive oxygen species through ABA independent pathway (Fang et al., 2015). Similarly, overexpression of OsSNAC1 confers drought tolerance by increasing the stomata closure (Hu et al., 2006). It was revealed by transcriptomic analysis that exogenous Melatonin down regulated the expression of NAC transcription factors (Zhang et al., 2014, Liang et al., 2015).

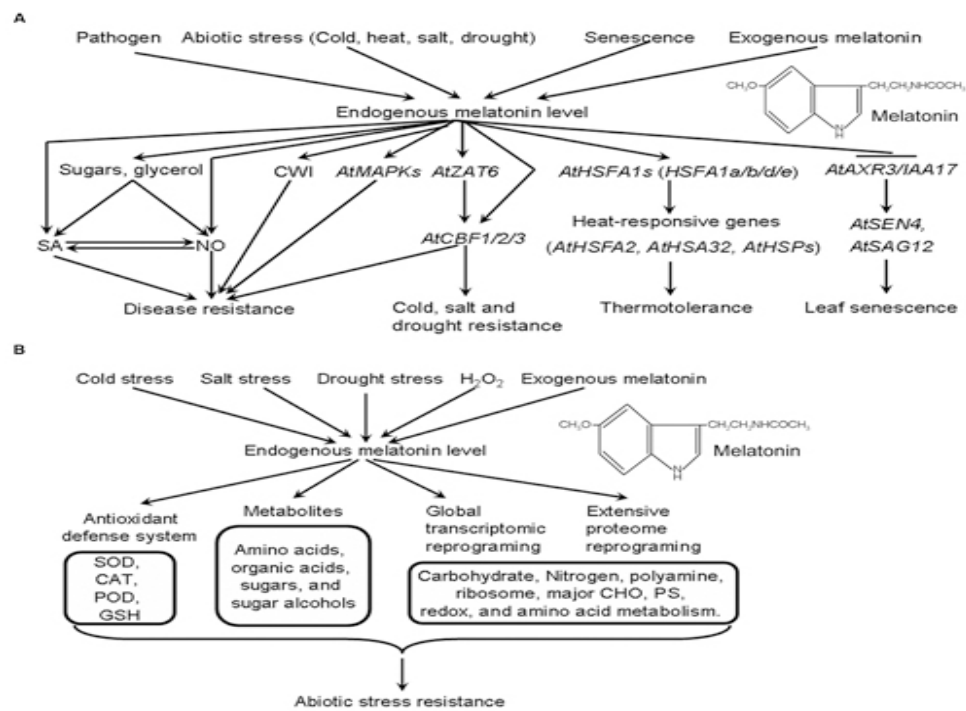


Fig: 2.11. Hypothetical model explaining melatonin-mediated stress responses in *Arabidopsis* (A) and bermuda grass (B) (Shi et al., 2015)

Chapter 3

MATERIALS AND METHODS

3.1 Experimental site and planting materials

The planting of the material was done in the transgenic glass house of Division of Plant Physiology, ICAR-IARI, New Delhi. Transgenic lines of Pusa Sugandh 2 viz: Overexpressed line and RNAi line of ABAR6 ABA receptor PYL6 under constitutive and stress inducible RD29A promoter were obtained from Dr Viswanathan C. lab. Thus, non-transgenic (NT), overexpression of ABAR6 (ABAR6ox) and RNAi of ABAR6 (ABAR6-Ri) were used for study. Soil was collected from rice field of ICAR-IARI farm for pot culture. Surface soil of 0-30 cm was collected followed by air drying and sieving through 2 mm size sieve. Total 60 pots with each pot single seedling were grown and 20 pots for each line were used.

3.2 Plant culture

The transgenic seeds were sown in lab condition and treated with antibiotics to confirm the transgenic line. After 21 days, seedlings were transplanted to the pot under controlled condition with temperature and relative humidity 30°C and 70% RH respectively at Division of Plant Physiology, ICAR Indian Agricultural Research Institute, New Delhi during Kharif, 2017. Twenty one days old seedlings were transplanted two seedlings per pot and later thinned to one seedling/pot after establishment. Nitrogen was applied as urea @ 120 kg ha⁻¹, Phosphorus was applied as SSP @ 60 kg ha⁻¹ as P₂O₅ and potassium as muriate of potash @ 40 kg ha⁻¹ as K₂O during transplanting.

3.3 Experimental design

The experimental design was based on randomized complete block design (RCBD) concept. For each treatment three biological replicates were grown in three different pots which were used for physiological/chemical/molecular_for destructive analysis.

3.4 Drought treatment

The rice plants were grown on pot inside controlled conditions of transgenic glass house. The average daily temperature and humidity at ICAR-IARI, New Delhi (28° 38' 23" N latitude and 77° 09' 27" E longitude with elevation 228.61 meters

above mean sea level were 30°C and 70% RH respectively. Drought treatments to plants were given at maximum tillering stage, 65 days after sowing. The initial pot weights were measured before starting the drought stress and it was measured 3 times in between drought period. Drought was given for 20 days by withholding water completely. Soil water potential was measured by tensiometer. Soil water content was measured by gravimetric method and loss of pot weight basis. The three different approaches were used to quantify the moisture stress in the pot. The average temperature and relative humidity and light were 30°C, 70% relative humidity and 600-700 lux, respectively.

3.5 Exogenous Melatonin treatment

The Melatonin treatment was standardized based on the previous preliminary study and plant response to the dose of 50 µM to 500 µM. 200 µM Melatonin was used for the treatment in in this experiment along with water as mock treatment. Each plant was treated with 50 ml solution. Melatonin was first mixed in 0.5 mL of ethanol then diluted with water to makeup required volume since melatonin is not finely soluble in water. The application was done in the morning with every alternate day for 5 days.

3.6 Determination of soil and leaf water status

- a. **Soil moisture content (SMC)** was measured by gravimetric method. Soil sample was collected at top 15 cm and bottom at 10 cm from each pot and its fresh weight (FW) was taken. The soil samples were hot air oven dried and dry weight (DW) was recorded. The SMC was evaluated by formula

$$\text{SMC (\%)} = [(\text{Soil FW} - \text{Soil DW}) / \text{Soil DW}] \times 100.$$

- b. **Relative water content (RWC)** was measured in fully opened flag leaf (Barrs and Weatherley, 1962). After taking FW, leaves were made full turgid by hydrating. After 4 h, turgid weight (TW) was taken and leaves were kept for drying and dry weight (DW) was taken. RWC was evaluated by the formula

$$\text{RWC (\%)} = [(\text{FW} - \text{DW}) / (\text{TW} - \text{DW})] \times 100.$$

3.7 Photosystem II activity

Pulse Amplitude Modulated (PAM) Chlorophyll fluorescence was measured FluoroPen FP100 growth chamber (Photo System Instruments, Czech Republic), on 30 min dark-adapted leaves. Light energy yield of PSII reaction center were measured by saturation pulse method as described by Schreiber et al. (1986). The measuring light intensity was $<0.1 \text{ mmol m}^{-2} \text{ s}^{-1}$ of blue light. A 0.8 s saturating blue light pulse with an intensity of $8000 \text{ mmol m}^{-2} \text{ s}^{-1}$ was used to estimate the maximum fluorescence signal over time.

3.8 Photosynthesis (IRGA-system)

Photosynthesis rate was evaluated with an Infra-red gas Analyzer (IRGA), LI-6400 system (LICOOR USA). The flag leaf of the main tiller was used 3 days after stress from 1000 to 1200 hrs during the day time. Air temperature during measurement was 33°C and ambient CO_2 was 410 ppm. The light intensity was fixed at $1000 \text{ } \mu\text{mol m}^{-2}\text{s}^{-1}$. LI-COR (LI-6400) works with the law that hetero-atomic molecules (CO_2 , H_2O , etc.) absorb IR radiation at specific wavelength. The system has a CO_2 analyzer which is a non-dispersive infra-red type calibrated for measurements of $0\text{-}2000 \text{ } \mu\text{l l}^{-1}$, a system console and leaf chamber. Photosynthesis rate was measured in open system IRGA where air flow moves through a controlled atmosphere surrounding the leaf placed in the gas exchange chamber to maintain CO_2 level of the air in steady-state (Long et al., 1996). The system console consists of data logging, storage, calculations and communication. The IRGA sensor comprises of leaf chamber, fans and the intake and exhaust parts from the analyzer. There were three technical replication of each biological replication for each treatment. The net photosynthesis was calculated using ΔCO_2 , flow rate and other components such as the enclosed leaf area, the volume of enclosure and temperature and photosynthesis rate was expressed as $\mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$.

3.9 Chlorophyll content

Chlorophyll estimation was done based on the principle explained by Hiscox and Israelstam (1979). The principle of this method is based on the incubation of leaf tissues in DMSO and extracting the chlorophyll and taking the reading of absorption of light by chlorophyll extracts. DMSO increases plasma membrane permeable thereby, causing the leaching out of the pigments (Hiscox and Israelstam, 1979).

Absorbance at 663 nm and 645 nm was taken for solution containing the leaf tissue for measurement of chlorophyll. Total chlorophyll content was measured by method of Arnon (1949). Test tubes containing the 50 mg fresh leaf samples were kept in dark at 65 degree Celsius for 4 hours. Absorbance readings were taken at room temperature using DMSO as blank. The values thus obtained were in $\mu\text{g/ mL}$ of extract (solvent). Values in mg/ g fresh weight were obtained by multiplying the above values with $V/ W \times 1000$, where V is volume of extract and W is dry weight of sample.

Total chlorophyll content ($\text{mg g}^{-1}\text{DW}$) = $[(20.2 \times A_{645}) + (8.02 \times A_{663}) \times (V / W) \times 1000$

Chlorophyll a content ($\text{mg g}^{-1}\text{DW}$) = $[(12.7 \times A_{663}) - (2.69 \times A_{645})] \times (V / W) \times 1000$

Chlorophyll b content ($\text{mg g}^{-1}\text{DW}$) = $[(22.9 \times A_{645}) - (4.68 \times A_{663})] \times (V / W) \times 1000$

Total Carotenoid content ($\text{mg g}^{-1}\text{DW}$) = $[A_{470} + (0.114 \times A_{663}) - (0.638 - A_{645})] \times (V / W) \times 1000$

Where, A_{470} = Absorbance values at 470 nm

A_{645} = Absorbance values at 645 nm

A_{663} = Absorbance values at 663 nm

3.10 Melatonin content estimation (Byeon and Back, 2014)

Rice seedlings (0.1 g) were ground to powder in liquid nitrogen. The powdered samples (0.2 g) were extracted with chloroform/methanol (30:1) for overnight at 4°C. After centrifugation at 13,500 g for 10 min, the chloroform fractions were decanted and evaporated to dryness and then dissolved in 100 μL of 50% methanol. Aliquots of 20 μL were taken to the HPLC having fluorescence detector system (Waters, Milford, MA, USA). The samples were separated on an Atlantis C18 column (Waters; 3.9×150 mm) with samples were separated by C18 column. Elution profile was 37% v/v methanol in water at flow rate 0.22 ml/min. Melatonin was detected at an excitation of 280 nm and emission of 348 nm.

3.11 ABA estimation

Abscisic acid in the leaf sample is estimated by using HPLC, following the methods of Zeevart (1980).

Preparation of sample:

Leaf samples (1 g) collected, frozen in liquid nitrogen, pulverized and stored at -20 °C until used. Frozen samples were taken three times with 10 ml of 80 % v/v acetone (80 ml acetone, 1 ml glacial acetic acid and 100 mg of 2, 6 di-tert-butyl 4-methyl phenol in a total volume of 100 ml) and collected in a 100 ml volumetric flask. The tissue residue was then homogenized with pestle and mortar with acetone (80 % v/v). The homogenate filter was done by using the Whatmann No. 1 paper. The filtrate along with the extracts made earlier, were transferred to the boiling flask of rotary flash vacuum evaporator for removing the acetone. As the acetone was evaporated, the lipid soluble material was deposited on the walls of the boiling flask (round bottomed). This was dissolved in 1% acetic acid solution and the amber coloured aqueous solution was transferred into small vials (10 ml). Before injecting the sample into HPLC, the samples were filtered with 0.45 mm Millipore filter using 2.5 ml plastic syringe.

Assay

Conditions of assay:

Detector: A variable wavelength 150 UV-visible detector.

Injector: Rheodyne injector (20 µl loop).

Column/stationary phase: The column used is µBondapak TM/C18 P/N 841/6 S/N. The stationary phase consisted of a Lichrosorb C-18.

Mobile phase: 1 % acetic acid in 95 % methanol solution attached to the pump.

Flow rate: 2.5 ml/min

Wavelength: Wavelength was set at 265 nm before injecting the sample.

Instrument is set on injection mode and 20 µl of standard ABA (10 ppm)/or sample solution injected into it and then the instrument is switched on to operation mode. Data on retention time and area of the peak recorded by the integrator is collected. The peak belonging to ABA identified based on its specific retention time, using the data registered by the standard ABA.

The concentration of ABA present in the sample is calculated by calculating the area under the peak by the formula as described in the principal of the instrument ($A = \frac{1}{2} b \times h$), and comparing it with the standard curve prepared with known

concentrations of ABA. Standard curve is prepared by plotting concentrations on X-axis and respective areas (of the peak) of the standard ABA on Y-axis.

3.12 Lipid peroxidation

Lipid peroxidation was measured as the thiobarbituric acid reactive substances (TBARS), according to the method of Heath and Packer (1968).

Reagents

- i. Trichloroacetic acid solution: Trichloroacetic acid (TCA) solution (0.1 %) was prepared by dissolving 0.1 g TCA in double distilled water and volume made to 1000 ml with it.
- ii. Thiobarbituric acid reagent: Thiobarbituric acid (TBA) 0.5 g was dissolved in 20 % trichloro acetic acid (TCA) (prepared by dissolving 20.0 g TCA in 100 mL distilled water) and volume was made up to 100.0 mL with 20 % TCA solution.

TBARS estimation

Leaf samples 0.5g were completely homogenized by using 10 ml 0.1% TCA and centrifuge at 15000 g for 15 min at 4 degree Celsius of 0.5 % thiobarbituric acid (TBA) in 20 % TCA was added to 1 mL aliquot of the supernatant. The mixture was heated at 95°C for 30 min in the water bath and then cooled under room temperature. The homogenate was centrifuged @ 10000 g for 10 min at 4°C. and reading taken at 532 nm. The TBARS content was calculated according to its extinction coefficient ($155 \text{ mM}^{-1}\text{cm}^{-1}$). The values for non-specific absorbance at 600 nm were subtracted.

3.13 Hydrogen peroxide estimation

Hydrogen peroxide content was estimated following the method described by Rao et al. (1997).

Reagents

- i. Titanium reagent: One-gram titanium dioxide and 10.0 g potassium sulphate were digested in 150.0 mL conc. sulphuric acid over a hot plate for 4 h. The digested mixture was diluted to 500-600 mL and stirred with a magnetic stirrer cum heater at 70-80°C till a clear transparent solution obtained, diluted to 1.5 liter and store in dark brown bottle (Teranishi *et al.*, 1974).

- ii. Acetone: Analytical grade reagent
- iii. Liquid ammonia: Analytical grade reagent

Estimation

One g leaf material was grinded to fine powder with the help of liquid nitrogen, followed by addition of 10.0 mL cooled acetone. Mixtures filtered and add 4.0 ml of titanium reagent and 5.0 mL liquid ammonia solution to make the precipitation of hydroperoxide titanium. Reaction mixture is centrifuged at 10000 g for 10 min at 4°C. Precipitate is dissolved in 10.0 mL of 2.0 M H₂SO₄ and then centrifuged. Supernatant is read at 415 nm against reagent blank in UV-visible spectrophotometer. Concentration of H₂O₂ was measured by using the standard curve of known concentration ($y = 0.124 x$) and was expressed as $\mu\text{mol g}^{-1}$ dry weight.

3.14 Proline estimation (Bates et al., 1973)

Reagents

- Acid-ninhydrin: Prepared by warming 1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid, with agitation, until dissolved. Keep cool (stored at 4°C) the reagent remains stable 24 hours.
- Extraction buffer: 3% (w/v) aqueous sulfosalicylic acid.
- Glacial acetic acid
- Toluene
- Proline

Procedure:

The chromophore-containing toluene (1 ml, upper phase) is warmed to room temperature and its optical density is measured at 520 nm using toluene as a blank. The proline concentration is determined from a standard curve using D-Proline

3.15 Antioxidants: Superoxide dismutase (SOD), Catalase (CAT) and Total peroxidase (POX)

Estimation of SOD activity

Superoxide dismutase activity was analyzed by the method of Dhindsa et al. (1981). The principle is nitro blue tetrazolum based blue color formation of

formazone and O_2^- radical, which absorbs at 560 nm light. SOD decreases this absorbance due to reduction in the formation of O_2^- radical by SOD.

Reagents:

1. Methionine (200.0 mM)
2. Nitroblue tetrazolium chloride (NBT) (2.25 mM)
3. EDTA (3.0 mM)
4. Riboflavin (60.0 μ M)
5. Sodium carbonate (1.5 M)
6. Phosphate buffer (100.0 mM, pH 7.8)

Solution A: Potassium dihydrogen phosphate 6.80 g was dissolved in water and the volume was made up to 500.0 mL with double distilled water.

Solution B: Di-potassium hydrogen phosphate 8.71 g was dissolved in water and the volume was made up to 500.0 mL with double distilled water.

8.5 mL of solution A and 91.5 mL of solution B was mixed and final pH was adjusted with the help of pH meter.

Grinding media:

Grinding media is prepared by mixing phosphate buffer of 0.1 M (pH 7.5), containing 0.5 mM EDTA (for SOD, CAT, GR and POX). EDTA 0.0186 g was dissolved in phosphate buffer 0.1 M, pH 7.5 (made by mixing 16.0 mL of solution A and 84.0 mL of solution B and final pH was adjusted with the help of pH meter) and volume was made to 100.0 mL with the buffer.

Enzyme extraction

Enzyme was extracted by grinding 1 g of leaf sample using liquid nitrogen by grinding with 10 mL extraction buffer. Homogenate was passed through cheese cloth having 4 layers and filtrate was centrifuged at 15000 g for 20 min at 4⁰C and the supernatant was used for enzyme assay.

Enzyme assay

Superoxide dismutase activity was measured by taking the note of decrease in OD of formazone. Three mL final reaction mixture was prepared by mixing the following reagents:

Reaction mixture:

- i. 13.33 mM methionine (0.2 mL of 200.0 mM)
- ii. 75.0 μ M nitroblue tetrazolium chloride (NBT) (0.1 mL of 2.25 mM)
- iii. 0.1 mM EDTA (0.1 mL of 3.0 mM)
- iv. 50.0 mM phosphate buffer (pH 7.8) (1.5 mL of 100.0 mM)
- v. 50.0 mM sodium carbonate (0.1 mL of 1.5 M)
- vi. 0.1 mL enzyme
- vii. 0.9 to 0.95 mL of water (to make a final volume of 3.0 mL)

One unit of enzyme activity was taken as that amount of enzyme, which reduced the absorbency reading to 50 % in comparison with tubes lacking enzyme. The enzyme activity was expressed as units per mg protein per hour.

Estimation of catalase (CAT) Activity

Catalase activity was estimated following the method described by Aebi et al. (1984).

Reagents:

- i. Hydrogen peroxide (75 mM): Hydrogen peroxide (30 %, 775 μ L) was dissolved in double distilled water and the volume was made up to 100 mL.
- ii. Phosphate buffer (100 mM, pH 7.0)

Enzyme assay

Final reaction mixture of 3 mL was prepared with the following reagents:

- i. 1.5 mL of 100 mM Potassium phosphate buffer, pH 7.0 (50.0 mM)
- ii. 50 μ L enzyme
- iii. 950 μ L water
- iv. Then hydrogen peroxide (0.5 mL) was added to start the reaction.

The decrease in absorbance was recorded for 1 min at 240 nm. Enzyme activity was calculated as per extinction coefficient of hydrogen peroxide ($36.5 \text{ M}^{-1}\text{cm}^{-1}$). Enzyme activity was expressed as μmol hydrogen peroxide reduced per minute per mg of protein.

Estimation of peroxidase (POX) activity

The total peroxidase activity was estimated following the method described by Castillo et al. (1984).

Reagents:

- i. 100 mM Phosphate buffer (pH 6.1)
- ii. 12 mM Hydrogen Peroxide: Hydrogen peroxide (30 %, 124 μL) was dissolved in double distilled water and the volume was made upto 100 mL.
- iii. 96 mM Guaiacol: Analytical grade guaiacol (1.07 mL) was dissolved in distilled water and the volume was made up to 100 mL.

Enzyme assay

Final reaction mixture of 3 mL was prepared with the following reagents :

- i. 1 mL of 100 mM potassium phosphate buffer, pH 6.1 (50 mM)
- ii. 0.5 mL of 96 mM guaiacol (16 mM)
- iii. 0.5 mL of 12 mM hydrogen peroxide (2 mM)
- iv. 0.1 mL enzyme
- v. 0.4 mL water and the total volume were made up to 3 mL.

Absorbance due to the formation of tetra-guaiacol was recorded at 470 nm and enzyme activity was calculated as per extinction coefficient of its oxidation product, tetra-guaiacol, i.e. $26.6 \text{ mM}^{-1}\text{cm}^{-1}$. Enzyme activity was expressed as μmol tetra-guaiacol formed per minute per mg of protein.

3.16 Stomatal aperture measurement

Plant tissue SEM preparation

Primary Fixation: Immerse sample in 2.8% glutaraldehyde in 0.1M Hepes buffer, pH 7.2 (with 0.02% Triton X-100), for several hours at room temperature or overnight at 4°C .

Primary Wash: 3 washes (each 5 to 10 minute duration) in 0.1 M Hepes buffer, pH 7.2

Dehydration: 1 x 10 min. in 25% ethanol1 x 10 min. in 50% ethanol1 x 10 min. in 70% ethanol1 x 10 min. in 85% ethanol1 x 10 min. in 95% ethanol2 x 10 min. in 100% ethanol1 x 10 min. in 100% ethanol (EM grade)

Critical Point Dry: This automated process takes approximately 40 minutes to complete.

Mounting: Mount the sample onto metal stub with double sided carbon tape.

Sputter Coating: Finally, apply a thin layer of metals (gold and palladium) over the sample using an automated sputter coater. This process takes about 10 minutes.

3.17 Expression profiling of selected genes by Quantitative RT-PCR

a. Selection of candidate genes and primer designing

Nucleotide sequences for candidate genes were obtained from National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). The Basic Local Alignment Search Tool (Altschul *et al.*, 1990, <http://www.ncbi.nlm.nih.gov/BLAST/>) was used to identify the homologs of candidate genes. Primers were designed manually, and quality, GC % and T_m were analysed by using Oligoanalyzer 3.0 tool (<http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/> Integrated DNA Technologies, Coralville, IA 52241, USA). Sequences of gene specific forward and reverse primers employed are described below.

b. Primer list:

Table 3.1 List of primers used for expression analysis

S N	Gene	Sequence 5' to 3'	
1	OsUBI5	Forward	GAAGCACAAGCACAAGAAGGTG
		Reverse	CTGGTTGTAGACGTAGGTGAG
2	OsASMT1	Forward	CAGCAGCCGATGTTGTG
		Reverse	GCATCCCTCGCTGAGATT
3	OsASMT2	Forward	TCATTCCAGCAGCCGAT
		Reverse	CCGGGATTGCCTCTTTG
4	OsASMT3	Forward	GAGTTCATTCCAGCAGCC
		Reverse	GCTGAGATTGCCTCCTTG
5	OsTDC3	Forward	CGGAGTACCTCAAGAACCA
		Reverse	TGACCATCCAGAGCTTGAG
6	OsNAC2	Forward	GTCCAAGAACGAGTGGGT
		Reverse	CTCGCCATCTCCATCGT
7	OsNAC6	Forward	GAAGGCCCTCGTCTTCTA
		Reverse	ACCTGAGGCTGTTCTTCT
8	OsNAC10	Forward	GCAGCGTAGAGCATCATC
		Reverse	TTGATCCCTCGGGTTCTT
9	OsNCED5	Forward	GCTCGACAGGGAGAAGA
		Reverse	CACAGGTGGAAGCAGAAG
10	OsPYL6	Forward	GAGAGCCACGTCCTCAGCTTC
		Reverse	CCGAAGAAACACATCCATC
11	OsNCED3	Forward	CCCTCCCAAACCATCCAAACCGA
		Reverse	GTGAGCATATCCTGGCGTCGTGA

12	OsLEA3	Forward	TTCCTCGGCGAGAAGAC
		Reverse	GGCAGAGTCCTTGGTGTA
13	OsABF2	Forward	TGCCTTACCCATTCGACAC
		Reverse	GACTCCCTGTTCTTGATCATCC
14	OsDREB1B	Forward	TGCCTCAACTTCGCCGACTTC
		Reverse	TGGCTTCTTCTTCGTCGCCAT
15	OsDREB1F	Forward	ATCCGCAGGAAGGCACGAGC
		Reverse	TCATCGTCGTCGTCGATGGC
16	OsDREB1 A	Forward	TGAGTGACATGGGCTGGGACC
		Reverse	TGGCATCGGAAGCCAGAAAAG
17	OsDREB2 A	Forward	CTGAGATCCGTGAACCAAAC
		Reverse	TGCTGTGGGACCATAACAT
18	OsDREB2B	Forward	GGTTGCTGAAATTCGGGAG
		Reverse	TGGCTGCCTCGTCATAA
19	ITP7	Forward	CGGGATCCATGACCAGCGTTGCCACCAG
		Reverse	GTA CTGCGACATGGTGGGGTGAGGTACCCC
20	IPT9	Forward	CGGGATCCTGACCGGAATGGCCACCTC
		Reverse	CCGAGCTCGAACACCGAGAGTGCAAACAGT G
21	OsLOGL1	Forward	TCATGCCCACCGAGATA
		Reverse	ATGAAGGCATCGGATTGG
22	OsLOGL2	Forward	GTATATGGAGGAGGGAGTGTAG
		Reverse	GCATGAGAGTCTTGGGAATG

3.18 Isolation of plant RNA and cDNA synthesis

Total RNA was extracted from flag leaf tissues by using TRIzol® reagent (Invitrogen, USA). About 100 mg sample was finely ground using liquid nitrogen in a pre-chilled autoclaved pestle and mortar. 1 ml TRIzol reagent was added to the sample tissue powder and grinded for 2 min. Cap each tube and lyse the cells by vortexing vigorously for 30 min. Incubate the samples for 10-15 min at room temperature and decant in a 2 mL eppendorf tube. Then add 200 µL of chloroform to each tube and vortex vigorously for 1 min. The samples were incubated for 1 min at room temperature and centrifuged at 13000 rpm for 20 min at 4°C for phase separation. There were three phases one lower phase of brown colour, middle phase contain chloroform-phenol having DNA and upper aqueous phase containing RNA. The upper aqueous phase was pipette out to a fresh tube without disturbing the middle phase and added 600 µL of chilled isopropyl alcohol. Then the samples were mixed well by inverting the tube up and down and incubated in -20°C for 30 min which helped in precipitating RNA. After incubation the samples were centrifuged for 10 min at 12000 g at 4°C where a pellet of RNA formed at the bottom side of the tube. The supernatant was discarded and the pellet was washed with 1.0 mL of 80 % ethanol twice and centrifuged at 12000 g for 1 min at 4°C. The pellets were air dried for 5-10 min avoiding complete drying of pellet. Finally the pellet was dissolved in 50 µL autoclaved double distilled water and incubated for 3 min at 36°C. To reduce the DNA contamination RNase-free DNase I (Promega, USA) was used. Quantity and quality of the RNA was analysed using NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, USA).

3.19 Electrophoresis of RNA

a. Materials and reagents

1. Agarose
2. 5X TBE buffer (pH 8.0)

Table 3.2 Components used for the preparation of TBE buffer

Components	Amount
Tris base	54.0 g
Boric acid	27.5
EDTA 0.5 M (pH 8.0)	20.0 mL
Final Volume	1000.0 mL with distilled water

The buffer was autoclaved and stored at room temperature

3. Gel loading dye (6X):

Table 3.3 Components used for the preparation of 6X Gel loading dye

Components	Amount
Tris-HCL (pH 7.6)	10 mM
Bromophenol blue	0.03%
Xylenecyanol FF	0.03%
Glycerol	60%
EDTA	60.0 mM

4. Ethidium bromide stock solution: 10.0 mg per mL in water
5. Mini gel apparatus and power supply
6. Gel documentation system: Alpha Imager

Agarose gel (0.8 %) was prepared by heat dissolution of 0.40 g of agarose in 50.0 mL of 1X TBE buffer. From the stock solution (10.0 mg/mL), Ethidium bromide was added to a final concentration of 0.5 mg /mL of gel. Before pouring to the gel plate, gel was allowed to cool to almost 50-55°C. Once the gel was ready, it was placed in the electrophoresis tank after removing the comb and tape. The electrophoresis tank was filled with 1X TBE buffer till the complete submergence of the gel. The total RNA samples were mixed with appropriate volume of 6X loading dye before loading. After that, the samples were loaded and run at 5 V/cm, with the help of an electric supply. After 1 h of electrophoresis, the agarose gel was viewed using gel documentation system (Alpha Imager).

3.20 DNase treatment

To remove the DNA contamination from RNA samples, we used Ambion (Applied Biosystems) TURBO DNase. Since we are making cDNA from RNA via reverse transcriptase and attempting to amplify target genes responsible for stress response, if any genomic DNA would present in the sample that could be amplified. Therefore, the result obtained by qRT-PCR may be due to the generation of cDNA or simply the genomic DNA contamination of sample. For avoiding this confusion, RNA samples were treated with DNase which is an enzyme that selectively degrades DNA. Reaction mixture was prepared using 10 µg of RNA, 10 X DNase buffer and TURBO DNase followed by incubation of the content at 37°C for 30 min. DNase inactivation reagent (0.1 volume) was added just after incubation and mixed well. The content was again incubated for 2 min at room temperature followed by centrifugation at 10000 g for 90s. Afterwards, RNA was transferred to fresh tube.

3.21 DNA PCR (negative control)

To check whether there is any contamination of genomic DNA in the RNA sample, PCR was done after DNase treatment. In a sterile 0.5 mL micro centrifuge tube, the following components were added.

Table 3.4: Components used for polymerase chain reaction (PCR)

Components	Volume (µl)	Final concentration
10X PCR buffer, minus Mg	10	1X
10 mM dNTP mixture	2	0.2mM each
50 mM MgCl ₂	3	1.5mM
Ubiquitin primer mix (10 µM each)	5	0.5 µM each
Template mRNA (DNase treated)	1-20	As required
Taq DNA polymerase	0.2-0.5	1.0-2.5 units
DMSO	4	
Nuclease free water	To 100	Not applicable

Tubes were capped and centrifuged briefly to collect the contents. For complete denaturation of the template, the tubes were incubated in a thermal cycler at 94°C for 3 min. 35 cycles of PCR amplification was performed.

Table 3.5: PCR temperature and timing scale

Steps	Temperature (°C)	Duration(seconds)
Denature	94	45
Anneal	55	30
Extend	72	90

The tubes were incubated for an additional 10 min at 72°C and the reaction was maintained at 4°C after recycling. The samples were stored at -20°C until further use. The products were analysed by 2% agarose gel electrophoresis and visualised with SYBR safe attaining.

3.22 cDNA synthesis using two step RT-PCR

The amplification of full length cDNA was done by using Superscript* III First-Strand Synthesis System (Invitrogen™, USA). The starting material can be ranging from 1-5 µg of total RNA. cDNA synthesis was performed in the first step using total RNA primed with oligo (dT), random primers, or a gene-specific primer. In the second step, PCR is performed using gene specific primers of interest. At first in thermocycler, RNA, Oligo (dT) primer and dNTPs are mixed to make a volume of 10 µl and give incubation for 5 min at 65°C which is known as Denaturation. 5 min after, take it out and has to be kept in ice for at least 1 min. In the next step, which is known as annealing step, 10 µl cDNA synthesis mix was added and kept at 50°C for 50 min. cDNA synthesis occurs in that step. The reaction has to be kept at 85°C for 5 min to terminate the reaction. This will ultimately give 20µl of cDNA.

Table 3.6. Components of cDNA synthesis mix

10X RT buffer	2 µl
25 mM MgCl ₂	4 µl
0.1 M DTT	2 µl
RNase OUT™	1 µl
Superscript* III RT	1 µl

- Product were used directly for PCR or stored at -20°C.

3.23 Statistical analyses

The results are expressed as means with standard error (S.E.). The significance difference (at $P < 0.05$) between control and stressed samples were determined by Duncan's multiple range tests at a significance level of 0.05 for all biochemical parameters and was evaluated by analysis of variance (ANOVA). ANOVA and critical difference value were calculated by using SPSS 10.0 (SPSS Inc., Chicago, IL, USA), and Microsoft Excel.

Chapter 4

RESULTS

Melatonin is one of the key cellular mechanisms involved in growth, development and tolerance to biotic and abiotic stress in both plants and animals. Only limited studies have been made to analyse the molecular pathway of melatonin in plants especially in rice, one of the major food crops of the world. Hence, this study used physiological, biochemical and molecular approaches to understand the pathway of melatonin mediated drought tolerance in rice under normal and drought condition.

Drought sensitive Pusa Sugandh 2 was selected for the study. The transgenic of ABA receptor 6 were made by over expressing with the help of CAMV: RD29 promoter and other RNAi line of ABA receptor 6 was made by RNAi technology in the Abiotic stress lab of Dr. Viswanathan C, Head and Principal Scientist at Division of Plant Physiology, ICAR-IARI. These lines show different tolerance capacity against drought. Non transgenic, ABAR6ox and ABAR6-Ri lines are sensitive, tolerant and hyper sensitive respectively to the drought stress. The third event of transgenic was selected for the experiment. The transgenic seeds were grown in lab condition and treated with antibiotics to confirm the transgenic line. After 21 days, seedlings were transplanted to the pot under controlled condition with temperature and relative humidity 30°C and 70% RH respectively. Drought stress was given for 20 days and melatonin application of 200µM was provided as foliar spray to the leaves once drought treatment started through manual sprayer. Melatonin application

at every alternate was provided four times in total and sampling was done at evening time. Samples were collected in liquid nitrogen for different biochemical and molecular analysis.

Transcriptome data of public database through Genevestigator were used to select the ABA dependent and ABA-independent transcription factors involved in drought tolerance and RT-PCR gene expression analysis were used to study the expression pattern in drought stress and exogenous melatonin application. The results of these experiments are presented in this section.

4.1. Plant water status indicators

Drought stress decreases the plant water status. We measured the soil matric potential by using tensiometer device. Similarly, relative water content in leaf was

measured. Soil sample was taken from the each drought treated pot to measure the soil moisture content through gravimetric method. The loss of pot weight was measured in different time interval once treatment was started.

4.1.1 Soil water potential

The tensiometer devices were installed in selected pots which were treated with drought stress. Four readings were taken at different time interval. The soil water potential was found to be -0.08 MPa (**Fig. 4.1**) after 20 days of withhold the irrigation.

4.1.2. Soil moisture content (Gravimetric method)

Soil moisture content was measured by Gravimetric method. The soil moisture content (**Fig. 4.2**) was measured at three days interval and found to be 12.1%, 10.42% and 9.28% in non-transgenic, transgenic ABAR6ox and ABAR6-Ri lines respectively.

4.1.3. Total water loss (Pot weight basis)

Water loss from the pots were measured based on the pot weight difference over the interval of time. It was found that the highest water release (**Fig. 4.3**) was in transgenic ABAR6-Ri lines followed by non-transgenic and transgenic ABAR6ox lines.

4.1.4. Water loss (Pot weight basis)

Water released from pots was measured in time intervals. We observed that in each transgenic and non-transgenic line, the water release (**Fig. 4.4**) increases with melatonin application.

4.1.5. Relative water content (%)

We did not observe any significance difference between non transgenic and transgenic at RWC level in control plants. Moreover, exogenous melatonin application did not show any significant changes on RWC (**Fig. 4.5**) in all the genotypes as compared with their respective mock plants under non-stress condition. However, drought stress significantly reduced the RWC level in all the genotypes as compared with the non-stress condition. The ABAR6-Ri lines showed higher reduction in the RWC level compared to ABAR6ox and NT lines under drought stress, while ABAR6ox line maintained higher level of RWC than both NT and ABAR6-Ri lines under drought stress condition. Exogenous melatonin application

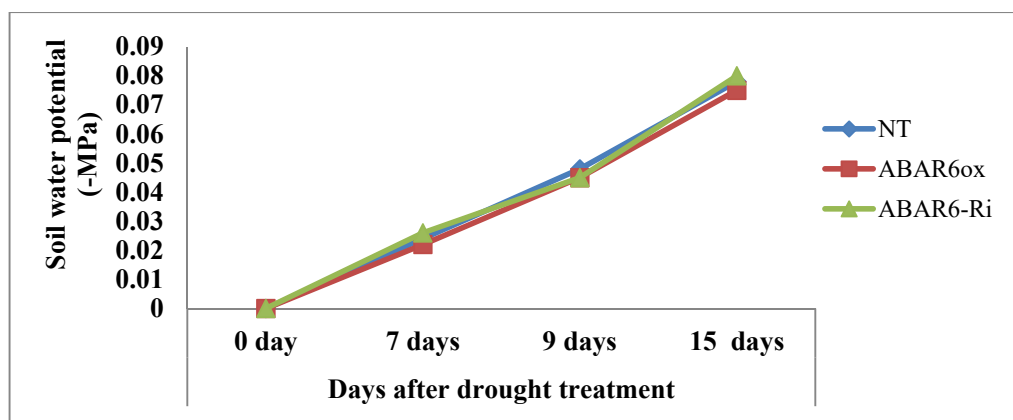


Fig: 4.1. The effect of drought stress on soil water potential (bar) in transgenic and non-transgenic rice genotypes.

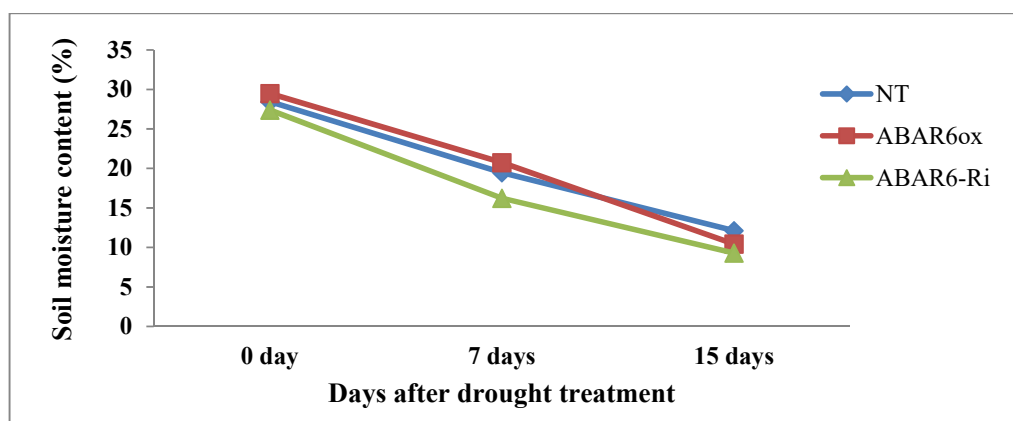


Fig: 4.2. The effect of drought stress on soil moisture content (%) in transgenic and non-transgenic rice genotypes.

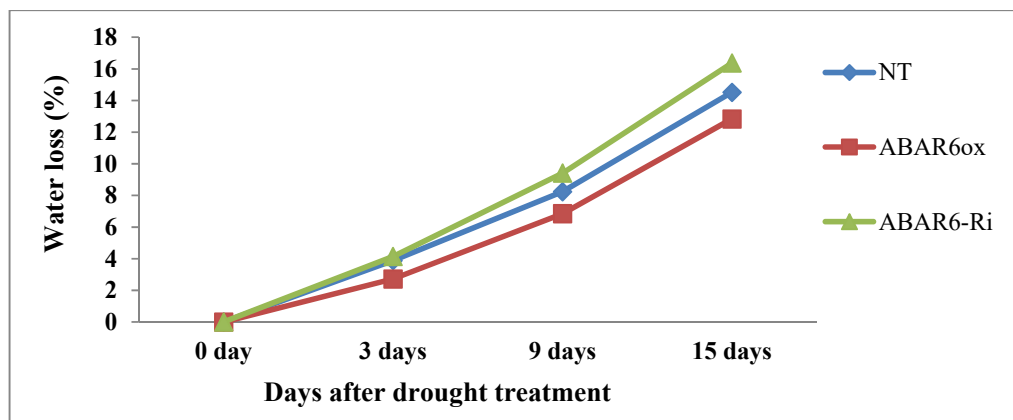


Fig: 4.3. The effect of drought stress on soil water loss (%) from pot in transgenic and non-transgenic rice genotypes.

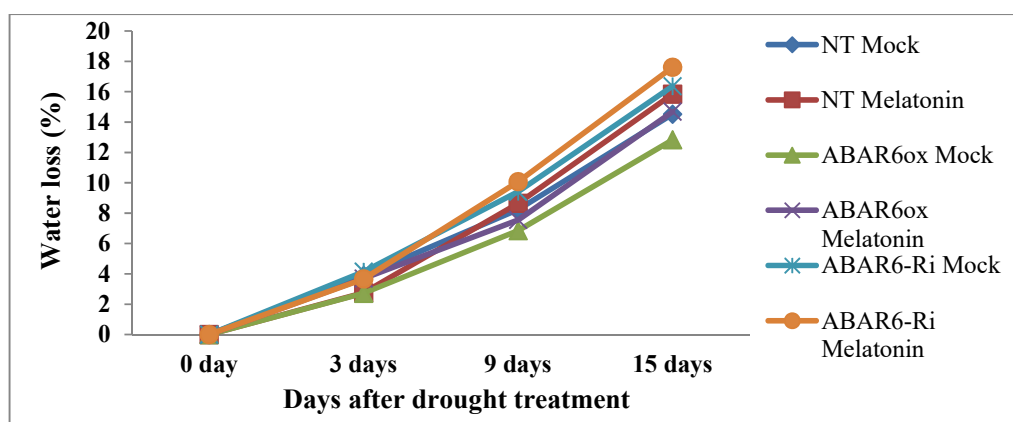


Fig: 4.4. The effect of drought stress on water loss (%) from transgenic and non-transgenic rice genotypes under melatonin application.

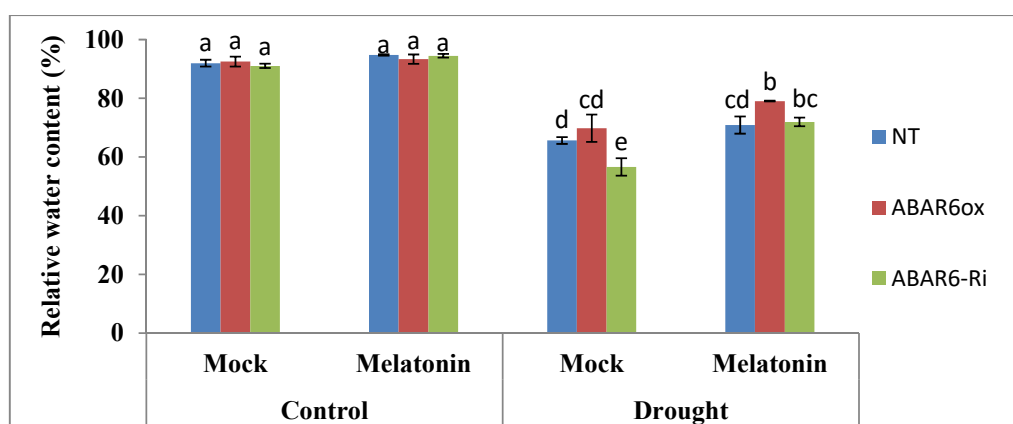


Fig: 4.5. The effect of melatonin application on relative water content (%) in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean ($n=3$). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

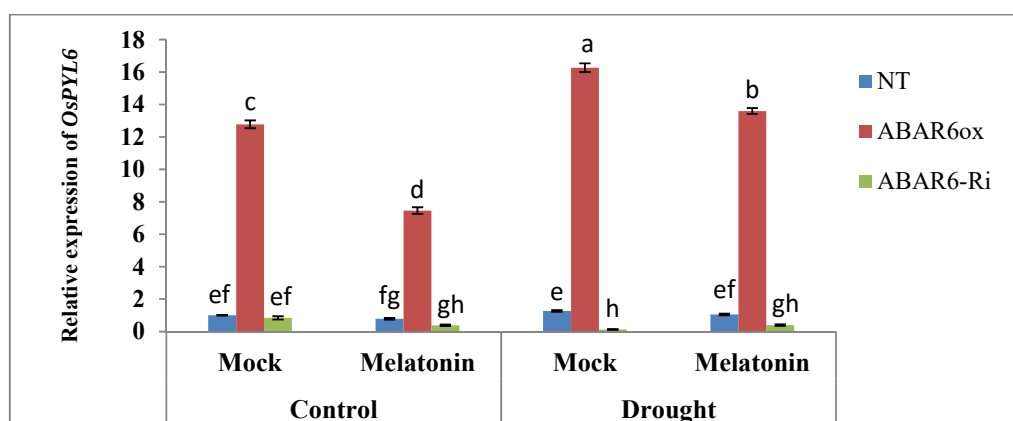


Fig: 4.6. The effect of melatonin application on relative expression of *OsPYL6* in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean ($n=3$). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

moderately increased the RWC level in NT lines, while there was significant increase in the RWC level in both ABAR6ox and ABAR6-ABAR6-Ri lines. ABAR6ox lines maintained higher level of RWC as compared to both NT and ABAR6-Ri lines upon application of exogenous melatonin.

4.2. Transgenic validation through gene expression

The relative expression of *OsPYL6* was studied to verify the transgenic line. The relative expression of *OsPYL6* under control condition was found to be significantly higher (**Fig. 4.6**) in transgenic ABAR6ox line than that of non-transgenic and ABAR6-Ri lines. Both NT and ABAR6-Ri lines maintained same level of expression with no significant difference under non-stress condition. Exogenous melatonin application significantly reduced the expression of *OsPYL6* in ABAR6ox and ABAR6-Ri lines while it was moderate reduction in the NT lines. Drought stress significantly increased the expression in ABAR6ox line while there was significant reduction in ABAR6-Ri lines and slightly increases in NT lines as compared to their respective mock plants in non-stress conditions. Among all, ABAR6ox lines maintained very high expression under stress condition. Exogenous melatonin application significantly reduced the expression in ABAR6ox line, while it was moderate increase in the NT and ABAR6-Ri lines as compared to their respective mock plants in stress condition.

4.3. Hormonal changes in drought stress and melatonin application:

Drought stress changes the hormonal level in plants. ABA is the major stress hormone which elevation is the indicator of the drought stress. Similarly melatonin level also changes with the stress. So through HPLC, the ABA and melatonin content in leaf were measured. *OsNCED3* and *OsNCED5* were selected for the relative expression study of ABA biosynthesis pathway and *OsTDC2*, *OsASMT1*, *OsASMT2*, *OsASMT3* and *OsCOMT* were selected for melatonin biosynthesis. The relative expression of their biosynthesis genes were analysed through RT-PCR. The results of hormone level and their biosynthesis gene expressions in control and stress condition with mock and melatonin treatment are presented in graphical representation below.

4.3.1. ABA content in leaves of rice

Absciseic acid level in the leaves of plants was measured in different lines through HPLC method. Endogenous ABA (**Fig. 4.7**) content under control condition

was found to be significantly higher in ABAR6ox lines as compared to NT lines while there was no significant difference between NT and ABAR6-Ri lines. Exogenous melatonin application enhanced the level of endogenous ABA level significantly higher in ABAR6ox lines as compared with its mock plant under non-stress conditions, while no further significant increase in endogenous ABA was observed in NT and ABAR6-Ri lines. Drought stress significantly increased the ABA content in all the genotypes as compared with non-stress conditions. Both ABAR6ox and ABAR6-Ri lines accumulated significantly higher level of ABA as compared with that of NT under drought. Exogenous melatonin application increased significantly higher level of ABA in all the genotypes as compared to mock plants under drought condition.

4.4. Relative expression of ABA biosynthesis genes by melatonin application

ABA biosynthesis genes were studied through RT-PCR and their expression were analysed in all the lines. ABA biosynthesis genes expression indicates the drought stress in the plants and expressed according to stress and melatonin application. ABA biosynthesis genes *OsNCED3* and *OsNCED5* were analysed.

4.4.1. Relative expression of *OsNCED3*

The relative expression of *OsNCED3* (**Fig. 4.8**) was found to be non-significant in all the genotypes. Exogenous melatonin significantly increased the expression of *OsNCED3* in non-transgenic line while no further significant changes was observed in the transgenic ABAR6ox and AbAR6-Ri lines as compared to their respective mock plants in non-stress condition. Drought stress significantly increased the expression of *OsNCED3* in all the genotypes. ABAR6ox maintained the higher expression than other two non-transgenic and transgenic ABAR6-Ri lines under stress condition. Exogenous melatonin application significantly increased the expression in ABAR60x lines while there were no significant changes in non-transgenic and ABAR6-Ri lines as compared to their mock plants in stress condition.

4.4.2. Relative expression of *OsNCED5*

The relative expression *OsNCED5* (**Fig. 4.9**) under control condition was found to be non-significant among all the genotypes studied. Exogenous melatonin application significantly increased the expression in non-transgenic lines while it was moderately increased in the ABAR6-Ri lines while significant decrease in the

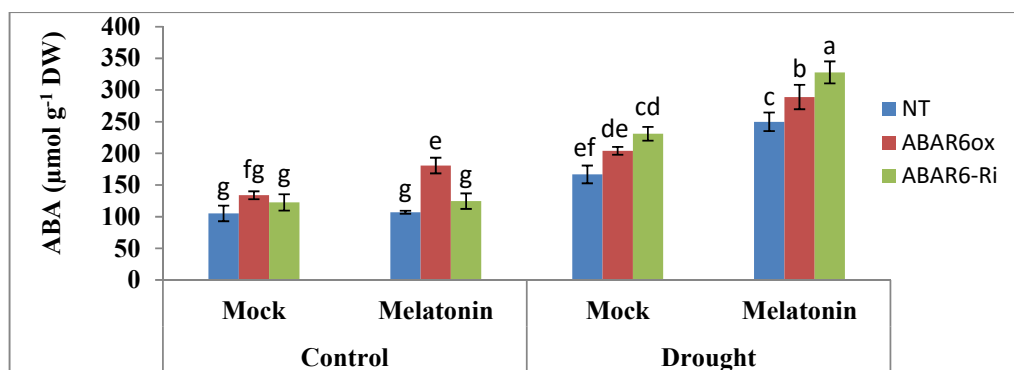


Fig: 4.7. The effect of melatonin application on endogenous abscisic acid ($\mu\text{mol g}^{-1} \text{DW}$) in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

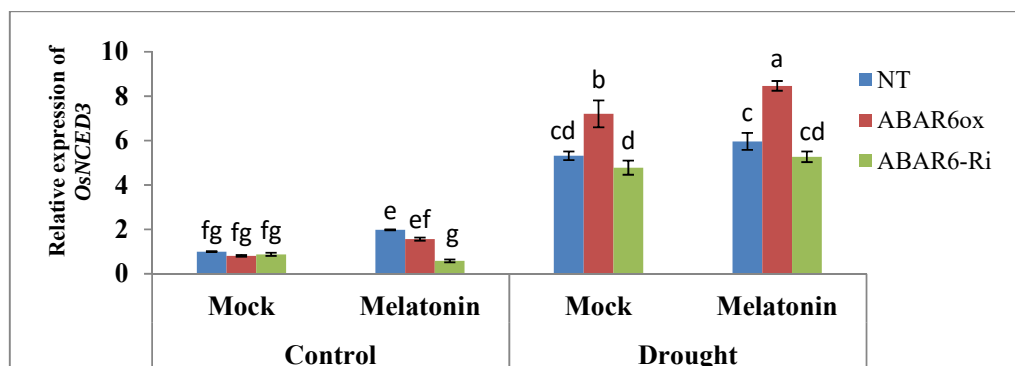


Fig: 4.8. The effect of melatonin application on relative expression of *OsNCED3* in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

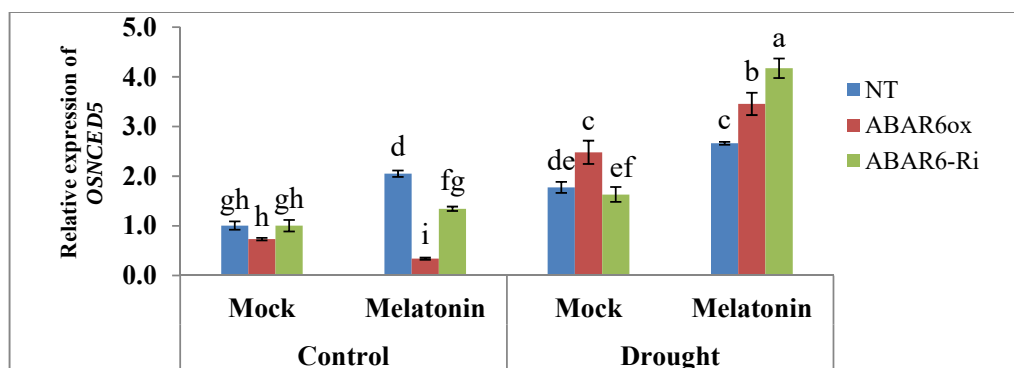


Fig: 4.9. The effect of melatonin application on relative expression of *OsNCED5* in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

ABAR6ox lines as compared to their respective mock plants in non-stress condition. Drought stress significantly increased the expression of *OsNCED5* in all the genotypes as compared to the non-stress condition. ABAR6ox maintained higher expression than the other two non-transgenic and ABAR6-Ri lines in stress condition. Exogenous melatonin application significantly increased the expression in all the genotypes as compared to their respective mock plants in the stress condition. ABAR6-Ri lines maintained higher expression among three genotypes during melatonin application under stress condition.

4.5. Melatonin and its biosynthesis genes under drought stress and exogenous melatonin application

4.5.1. Melatonin content

Melatonin content under control condition was found to be significant between the non-transgenic (NT) and transgenic lines. Exogenous melatonin application enhanced the levels of endogenous melatonin (**Fig. 4.10**) content significantly in NT and ABAR6ox lines as compared with their respective mock plants under non-stress condition, while no further increase in melatonin was observed in ABAR6-Ri line. Drought stress significantly reduced the melatonin content in all genotypes as compared with non-stress conditions. Both ABAR6ox and ABAR6-Ri lines accumulated significantly higher level of melatonin as compared with that of NT under drought. Exogenous melatonin application only moderately increased the endogenous melatonin content in NT and ABAR6-Ri line while that of ABAR6ox increased significantly under stress conditions.

4.5.2. Relative expression of Melatonin Biosynthesis genes

Melatonin biosynthesis genes were studied through RT-PCR and their expression were analysed in all the lines. Melatonin biosynthesis genes expression indicates the drought stress in the plants and expressed according to stress and melatonin application. Melatonin biosynthesis genes *OsCOMT*, *OsTDC2*, *OsASMT1*, *OsASMT2* and *OsASMT3* were analysed.

4.5.2.1. Relative expression of *OsCOMT*

The relative expression of *OsCOMT* (**Fig. 4.11**) under the control condition was found to be non-significant among three lines: Non transgenic, transgenic ABAR6ox and ABAR6-Ri lines. ABAR6-Ri line maintained slightly higher

expression than the NT and ABAR6ox lines under non stress conditions. Exogenous melatonin application increased the relative expression significantly in all the genotypes and ABAR6ox lines maintained higher expression as compared to the NT and ABAR6-Ri lines under non stress conditions. Drought stress significantly increased the expression of *OsCOMT* of all the genotypes as compared with non-stress condition. The relative expression was higher in NT line followed by ABAR6-Ri and ABAR6-ox lines. Exogenous melatonin application increased the expression of *OsCOMT* significantly in transgenic line ABAR6-ox and ABAR6-Ri lines while there were no significant changes in NT lines as compared with their respective mock plants in stress condition.

4.5.2.2. Relative expression of *OsASMT1*

The relative expression of *OsASMT1* (**Fig. 4.12**) under control condition was found to be non-significant among all three genotypes: non transgenic, transgenic ABAR6ox and ABAR6-Ri lines. Exogenous melatonin application increased the expression in NT lines significantly while there was slight increase in ABAR6-Ri lines and significant reduction in ABAR6ox line as compared to their respective mock plants in non-stress condition. Drought stress increased the expression of *OsASMT1* in all the genotypes as compared with their respective mock plants in non-stress condition. ABAR6ox line maintained higher expression among all the genotype under stress condition. Exogenous melatonin application increased the expression significantly in non-transgenic and ABAR6-Ri lines while; it was moderate increase in ABAR6ox line as compared to their respective mock plants in stress condition. ABAR6-Ri line maintained higher expression among all three lines with melatonin application in stress condition.

4.5.2.3. Relative expression of *OsASMT2*

The relative expression of *OsASMT2* (**Fig. 4.13**) under control condition was found to be similar in all the genotypes. Non-transgenic showed slightly higher expression than other two transgenic lines. Exogenous melatonin application did not showed any significant changes in the expression among all the lines. Drought stress increased the expression of *OsASMT2* significantly in ABAR6-Ri lines while it was moderate increase in NT and ABAR6ox lines as compared with their respective mock plants in non-stress condition. Exogenous melatonin application significantly increased the expression in NT and ABAR6ox while it was no significant changes in

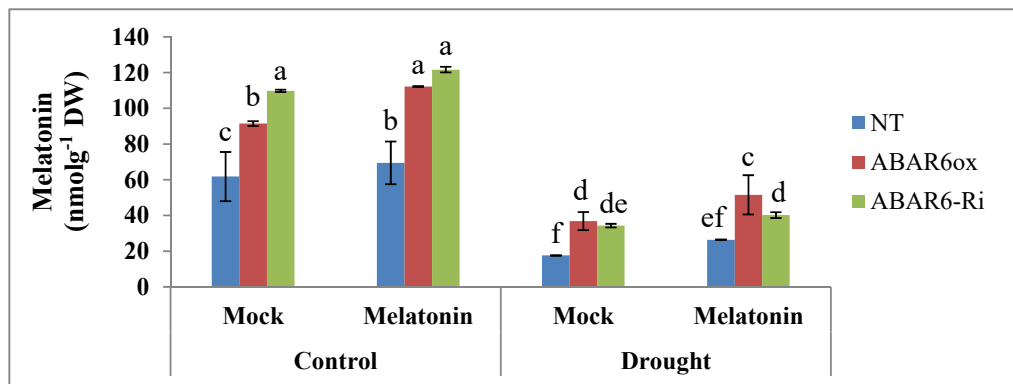


Fig: 4.10. The effect of melatonin application on endogenous melatonin content ($\text{nmol g}^{-1} \text{ DW}$) in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean ($n=3$). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

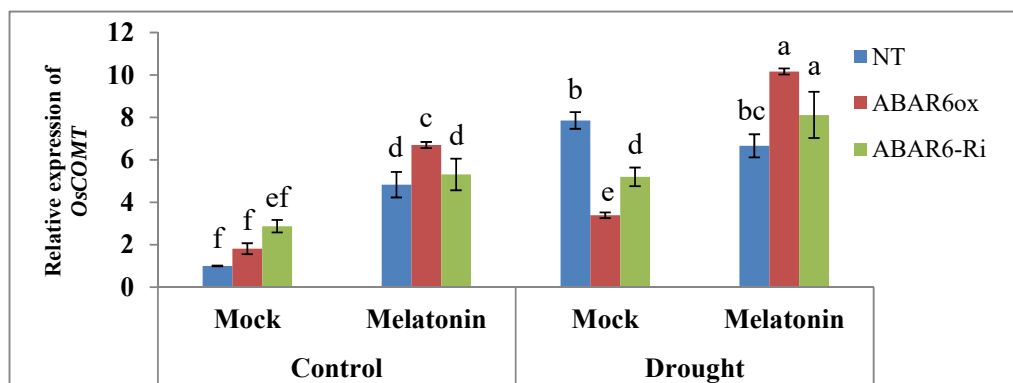


Fig: 4.11. The effect of melatonin application on relative expression of *OsCOMT* in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean ($n=3$). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

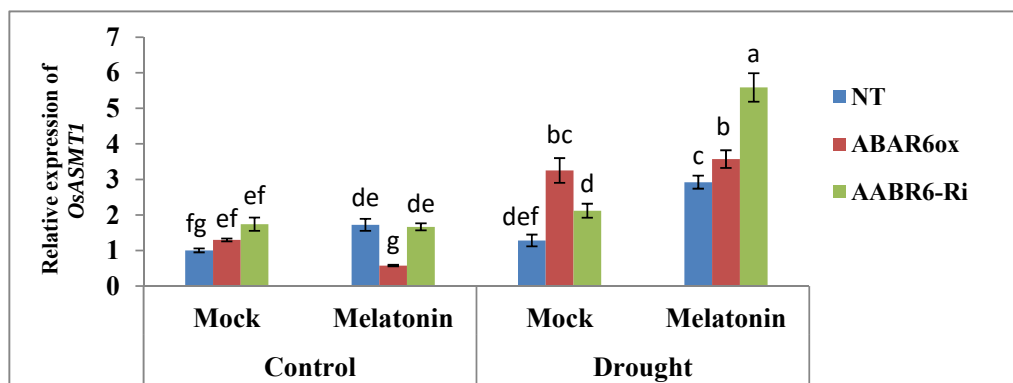


Fig: 4.12. The effect of melatonin application on relative expression of *OsASMT1* in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean ($n=3$). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

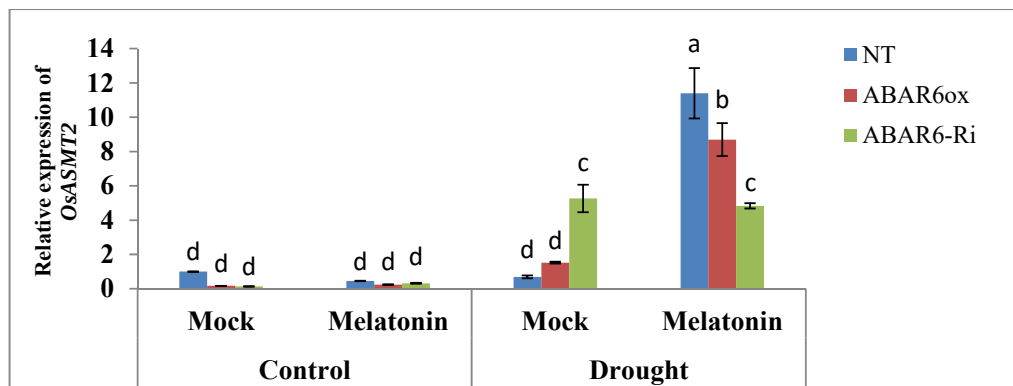


Fig: 4.13. The effect of melatonin application on relative expression of *OsASMT2* in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

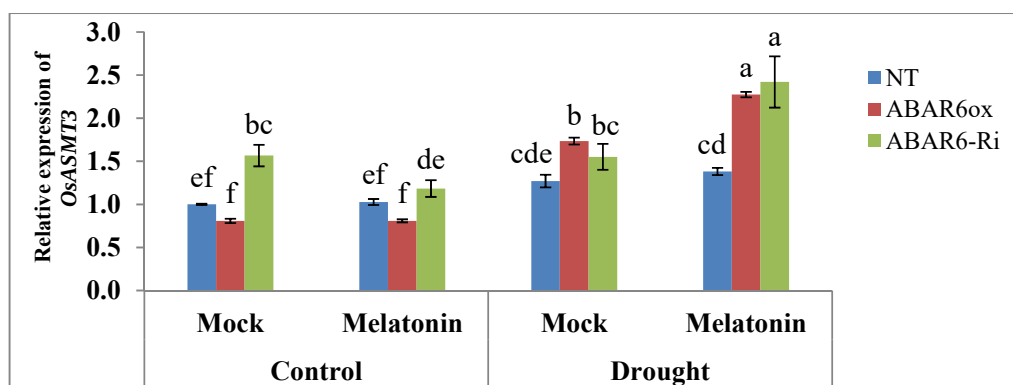


Fig: 4.14. The effect of melatonin application on relative expression of *OsASMT3* in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

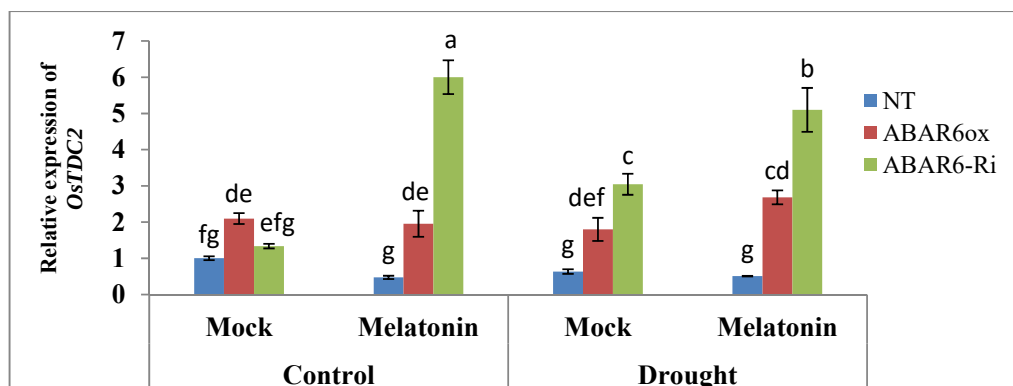


Fig: 4.15. The effect of melatonin application on relative expression of *OsTDC2* in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

expression in ABAR6-Ri lines as compared with their respective mock plants in stress conditions.

4.5.2.4. Relative expression of *OsASMT3*

Relative expression of *OsASMT3* (**Fig. 4.14**) under control condition was found to be significantly higher in ABAR6-Ri line while there was no significant difference between non-transgenic and transgenic ABAR6ox lines. Exogenous melatonin application did not show significant changes in the expression of non-transgenic and ABAR6ox lines while there significant decrease in ABAR6-Ri lines. Drought stress significantly increase the expression of ABAR6ox while, there was no significant changes in the expression of NT and ABAR6-Ri lines as compared with their mock plants in non-stress condition. Exogenous melatonin application significantly increased the expression of *OsASMT3* in transgenic line ABAR6ox and ABAR6-Ri lines while there were no significant changes in the non-transgenic lines as compared with their respective mock plants in stress condition.

4.5.2.5. Relative expression of *OsTDC2*

The relative expression of *OsTDC2* (**Fig. 4.15**) under control condition was found to be significantly higher in transgenic ABAR6ox than that of non-transgenic while, there was non-significant between transgenic ABAR6-Ri and non-transgenic plants. Exogenous melatonin application significantly increased the expression of *OsTDC2* in ABAR6-Ri lines while there were no significant changes in non-transgenic and transgenic ABAR6ox lines as compared with their respective mock plants in non-stress condition. Drought stress significantly increased the expression of *OsTDC2* in ABAR6-Ri lines while there were no significant changes in the non-transgenic and transgenic ABAR6ox lines. Exogenous melatonin application brought no significance changes in the non-transgenic and transgenic ABAR6ox lines while it was significant increase in the ABAR6-Ri lines as compared with their respective mock plants in stress conditions.

4.6. Photosynthetic performance under drought stress and melatonin treatment

Photosynthetic attributes were measured using the LICOR-6400 IRGA machine under control and drought conditions in mock and melatonin treatment. The photosynthetic attributes like Photosynthesis rate, stomatal conductance, transpiration rate and internal carbon dioxide concentration were measured. Fluropen

was used to measure PSII activity. Stomatal aperture size was measured by scanning electron microscope (SEM). The results are listed below in graphical representation way with statistical analysis.

4.6.1. Photosystem II activity

Photosystem II activity (**Fig. 4.16**) under control condition was found to be similar in all the genotypes with non-significant among all the lines. Exogenous melatonin application did not show any significant changes in the PSII activity as compared to their respective mock plants under non stress conditions. Drought stress significantly reduced the PS II activity in all the genotypes as compared with non-stress condition. ABAR6ox and ABAR6-Ri lines maintain slightly better PSII activity as compared to non-transgenic lines. Exogenous melatonin application significantly increased the PSII activity as compared to their respective mock plants under drought condition. This increased PSII activity showed significantly lower level than in the non-stress condition.

4.6.2. Photosynthesis

Photosynthesis (**Fig. 4.17**) under control condition was non-significant among non-transgenic (NT) and transgenic lines. Exogenous melatonin application enhanced the photosynthesis significantly in all the genotypes as compared with their respective mock plants under non stress condition. The enhancement in the photosynthesis was found to be non-significant among all the genotypes under non stress condition. Drought stress significantly reduced the photosynthesis in all the genotypes as compared with non-stress condition. Both ABAR6ox and ABAR6-Ri lines maintained moderately higher photosynthesis than non-transgenic lines. Exogenous melatonin application significantly increased the photosynthesis in non-transgenic and ABAR6ox lines while it was moderate increase in the ABAR6-Ri lines.

4.6.3. Stomatal conductance

Stomatal conductance (**Fig. 4.18**) under control condition was found to be moderately higher in the ABAR6-Ri lines than other non-transgenic and ABAR6ox lines, while they were non-significant different statistically. Exogenous melatonin enhanced the stomatal conductance significantly in all the genotypes. ABAR6-Ri lines maintained slightly higher conductance than the non-transgenic and ABAR6ox

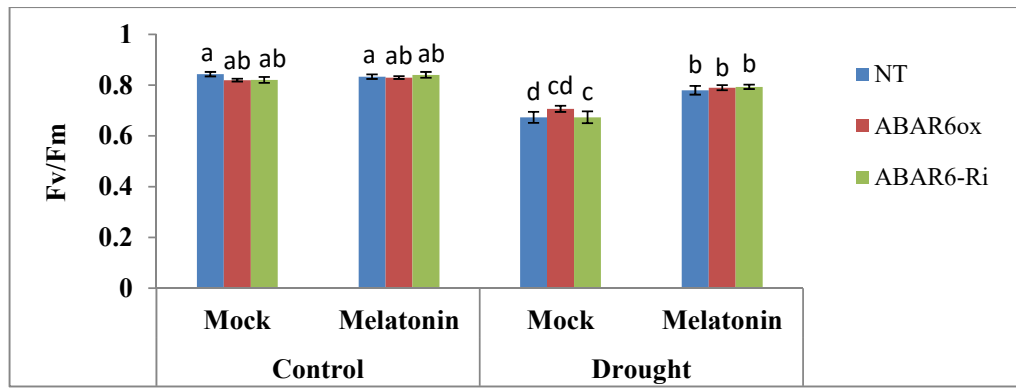


Fig: 4.16. The effect of melatonin application on photosystem II activity in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

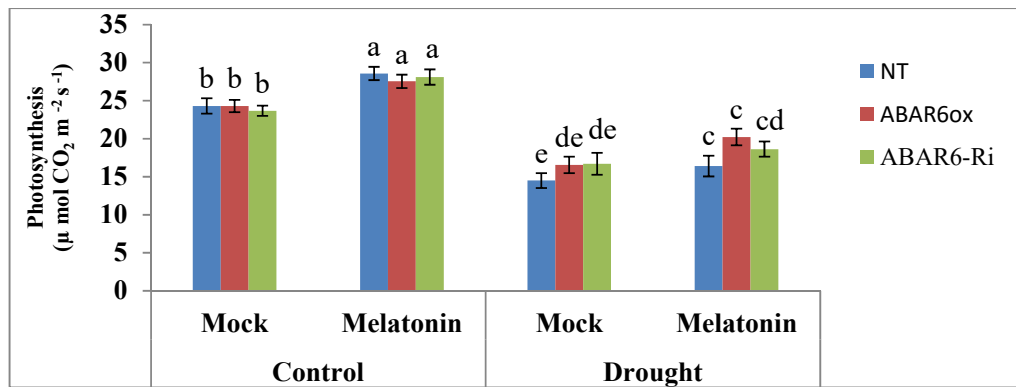


Fig: 4.17. The effect of melatonin application on photosynthesis ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

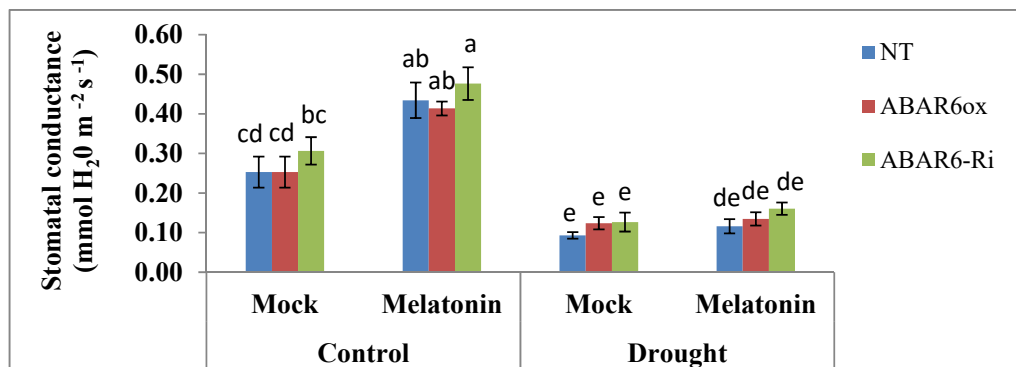


Fig: 4.18. The effect of melatonin application on stomatal conductance ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$) in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

lines. Drought stress significantly reduced the stomatal conductance as compared to the non-stress condition. Exogenous melatonin application moderately increase the stomatal conductance in all the genotypes while they were non-significant different statistically.

4.6.4. Internal CO₂ concentration

Internal carbon dioxide concentration (**Fig. 4.19**) was moderately higher in the ABAR6-Ri lines as compared to the non-transgenic and ABAR6ox lines under control condition, while there was non-significant statistically. Exogenous melatonin application moderately improved the stomatal conductance in all the genotypes without significant difference. Drought stress reduced the stomatal conductance significantly in non-transgenic lines while it was moderate decrease in ABAR6ox and ABAR6-Ri lines as compared with non-stress condition. Exogenous melatonin application moderately increased the internal carbon dioxide concentration while it was no significant changes in transgenic ABAR6ox and ABAR6-Ri lines as compared to their respective mock plants in stress condition.

4.6.5. Stomatal Opening

Stomatal opening (**Fig. 4.20**) under drought stress was found to be significant difference between non transgenic and transgenic lines. ABAR6ox line was found to be lowest stomatal aperture size while non-transgenic and ABAR6-Ri lines were higher aperture size. This showed ABAR6ox is tolerant to drought than other two genotypes. Melatonin application significantly increased the stomatal opening in ABAR6-Ri and ABAR6ox lines while it was no significant change in non-transgenic lines as compared to their respective mock plants in stress condition.

4.6.6. Transpiration

Transpiration rate (**Fig. 4.21**) under control condition was found to be non-significant between non transgenic and transgenic lines. Exogenous melatonin application moderately increased the transpiration rate in NT and ABAR6-Ri lines, while it was significant increase in ABAR6ox line compared to their respective mock plants under non stress conditions. Drought stress significantly reduced the transpiration rate in non-transgenic and ABAR6-Ri lines, while it was moderate reduction in the ABAR6ox line as compared to non-stress condition. ABAR6ox line maintained higher transpiration rate compared to NT and ABAR6-Ri lines under

drought condition. Exogenous melatonin application significantly increased the transpiration rate in NT lines while it was moderate increase in the ABAR6ox and ABAR6-Ri lines under stress condition.

4.7. Photosynthetic pigments under drought stress and melatonin application

Chlorophyll content was measured with DMSO method and all the components like chlorophyll a, chlorophyll b, chlorophyll a/b ratio, total chlorophyll content and total carotenoid content were measured under control and drought condition in mock and melatonin treatment. The results are listed below in graphical representation way with statistical analysis.

4.7.1. Chlorophyll a

Chlorophyll a content (**Fig. 4.22**) under control condition was found to be non-significant among the all genotypes (NT, ABAR6ox and ABAR6-Ri lines). Exogenous melatonin application did not show any significant changes in the chlorophyll a content as compared to their respective mock plants under non stress condition. Drought stress significantly reduced the chlorophyll a level in all the genotypes as compared with non-stress condition. Exogenous melatonin application increased the chlorophyll a level in all the genotypes significantly as compared to their respective mock plants under stress condition. It also restored the level of chlorophyll a level to the level of non-stress conditions.

4.7.2. Chlorophyll b

Chlorophyll b content (**Fig. 4.23**) under control condition was found nearly same without significant difference in all the genotypes (NT, ABAR6ox and ABAR6-Ri). Exogenous melatonin application significantly reduced the chlorophyll b content in NT but there was no significant difference among the ABAR6ox and ABAR6-Ri lines as compared with their respective mock plants under non stress conditions. Drought stress slightly reduced the level of chlorophyll level in all genotypes as compared with non-stress conditions with no significant difference among three lines. Exogenous melatonin application significantly increased the level of chlorophyll b in all the lines as compared to their respective mock plants under stress conditions. The level of chlorophyll b was improved than their respective mock control plants.

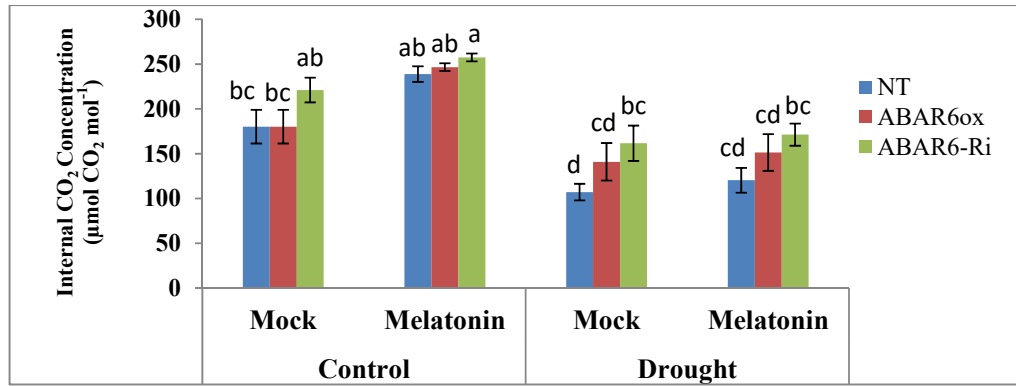


Fig: 4.19. The effect of melatonin application on internal CO₂ (μmol CO₂ mol⁻¹) in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05).

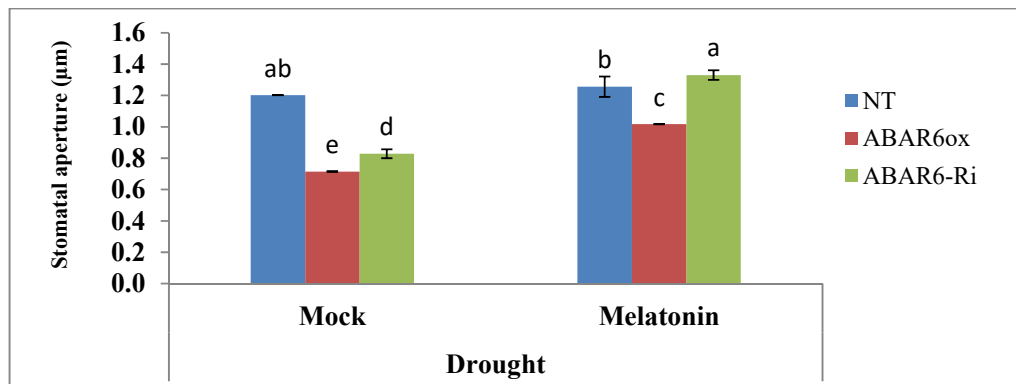


Fig: 4.20. The effect of melatonin application on stomatal opening (aperture size- μm) in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05).

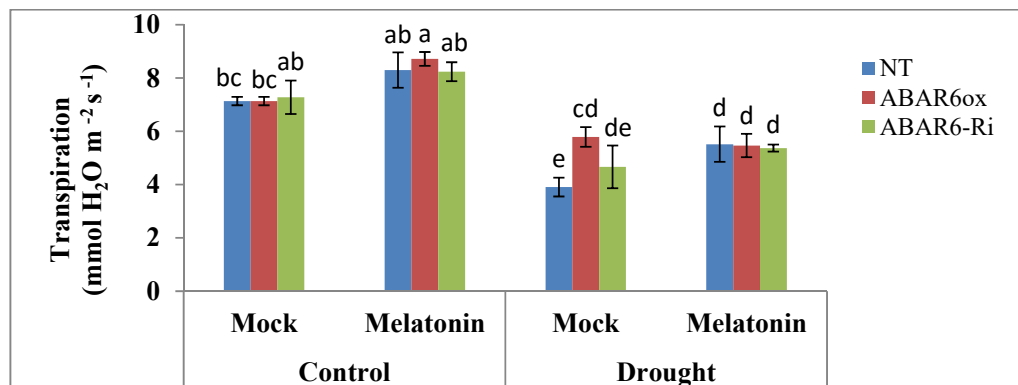


Fig: 4.21. The effect of melatonin application on transpiration (mmol H₂O m⁻² s⁻¹) in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05).

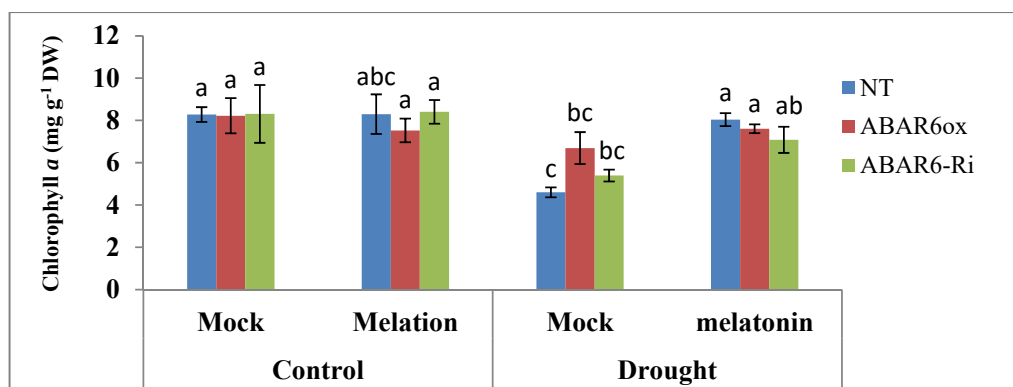


Fig. 4.22. The effect of melatonin application on chlorophyll a (mg g⁻¹ DW) in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05).

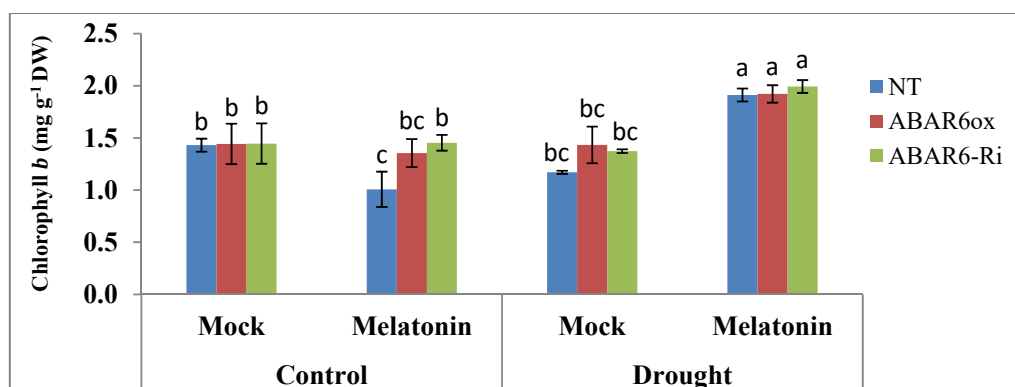


Fig. 4.23. The effect of melatonin application on chlorophyll b (mg g⁻¹ DW) in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05).

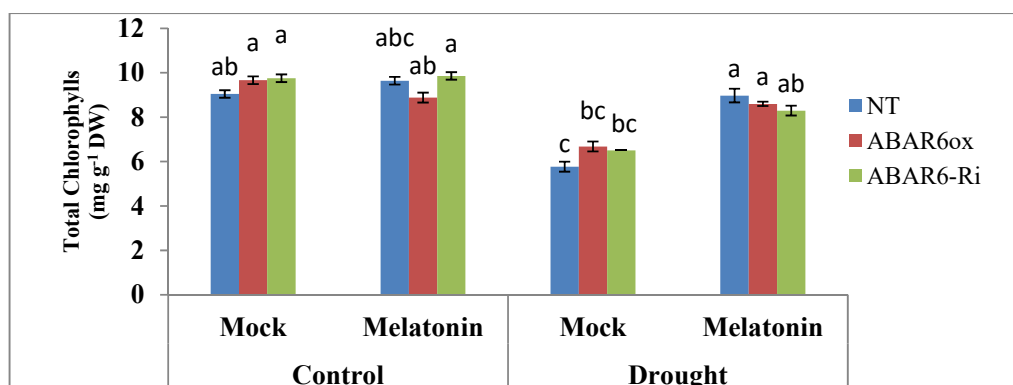


Fig. 4.24. The effect of melatonin application on total chlorophyll (mg g⁻¹ DW) in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05).

4.7.3. Total chlorophyll content

Total chlorophyll content (**Fig. 4.24**) under control condition was found to be similar in all the genotypes with non-significant. Exogenous melatonin application showed non-significant in all the genotypes as compared to their respective mock plants under non stress conditions. Drought stress significantly reduced the chlorophyll level in all the genotypes as compared to the non-stress condition. Exogenous melatonin significantly increased the chlorophyll level in all the genotypes as compared to their mock plants in stress condition and restored the level similar to the non-stress condition.

4.7.4. Total carotenoid content

Carotenoids content (**Fig. 4.25**) under control conditions was found to be equal in all the genotypes with no significant difference. Exogenous melatonin application didn't show any significant changes in the carotenoid content in all plants as compared to their mock plants in non-stress condition. Drought stress significantly reduced the carotenoids content in all the genotypes as compared to their respective mock plants under non stress conditions. ABAR6ox and ABAR6-Ri lines accumulated slightly higher carotenoid level than non-transgenic line under drought condition. Exogenous melatonin application significantly increased the carotenoid level as compared to their respective mock plants under drought stress and restored the carotenoids level similar to that of non-stress plants.

4.8. Proline content under drought stress and melatonin application

Proline content (**Fig. 4.26**) under control condition was found to be no significant difference between non-transgenic and transgenic ABAR6ox while the proline content was significantly higher than other two lines. Melatonin application significantly increased the proline content in the ABAR6ox line while there were no significant changes in non-transgenic and transgenic lines as compared to their mock plants in non-stress conditions. Drought stress significantly increased the proline content in all the genotypes as compared to the non-stress condition. Among three genotypes, ABAR6-Ri line showed lower level of proline while it was higher in non-transgenic line under stress condition. Melatonin application significantly increased the proline content in all the genotypes as compared to stress condition. The proline

level was significantly higher in non-transgenic line and followed by ABAR6ox and ABAR6-Ri lines.

4.9. Reactive oxygen species and antioxidants under drought stress and exogenous melatonin application

ROS are the major indicators of drought stress. We measured the hydrogen peroxide level and lipid peroxidation level in leaf under control and drought stress with mock and melatonin treatment. Similarly the enzymic antioxidants are also important marker in drought stress. Superoxide dismutase, catalase and total peroxidase were measured. The results are listed below in graphical representation with statistical analysis.

4.9.1. Hydrogen peroxide activity

The hydrogen peroxide level (**Fig. 4.27**) under control condition was found to be higher in transgenic ABAR6-Ri lines than other two non-transgenic and transgenic ABAR6ox lines while there was no significant difference between them. Melatonin application significantly increased the hydrogen peroxide level in ABAR6-Ri lines while there was no significant change in the non-transgenic and transgenic ABAR6ox lines in non-stress condition. Drought stress significantly increased the hydrogen peroxide content in all the genotypes as compared to the non-stress condition. ABAR6-Ri accumulated higher hydrogen peroxide level than other two non-transgenic and transgenic ABAR6ox lines. Melatonin application significantly reduced the level of hydrogen peroxide in all the genotypes as compared to their mock plants in stress condition. ABAR6-Ri lines accumulated higher hydrogen peroxide than other two genotypes.

4.9.2. Lipid peroxidation activity

Lipid peroxidation content (**Fig. 4.28**) was measured by the MDA level. MDA level under control condition was found to be non-significant among all the three genotypes. Exogenous melatonin application didn't show any significant changes in the lipid peroxidation activity, while the level of melatonin was equal in the all the genotypes as compared with their respective mock plants in non-stress condition. Drought stress significantly increases the MDA level on non-transgenic and ABAR6-Ri lines while; it was moderate increase in the ABAR6ox lines as compared to non-stress condition. ABAR6ox maintained reduced lipid peroxidation activity as

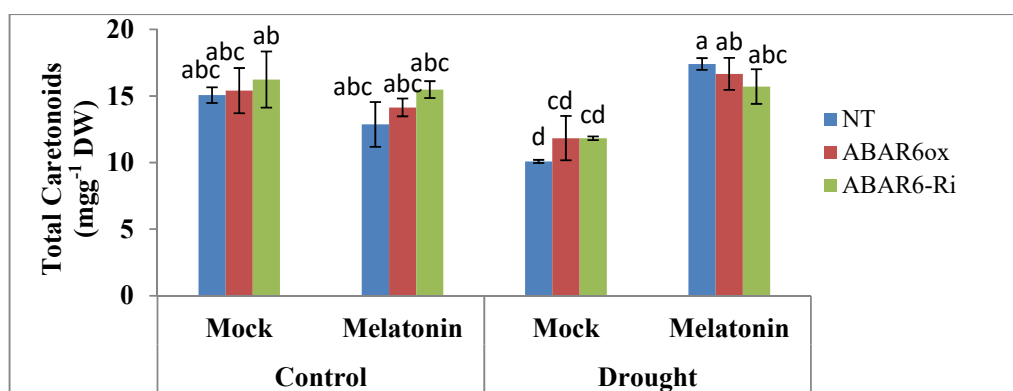


Fig: 4.25. The effect of melatonin application on total carotenoids level (mg g⁻¹ DW) in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05).

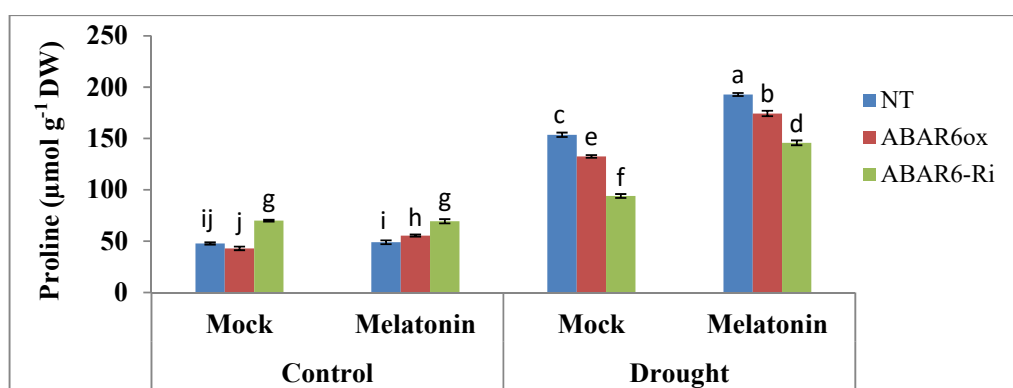


Fig: 4.26. The effect of melatonin application on total carotenoids level (mg g⁻¹ DW) in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05).

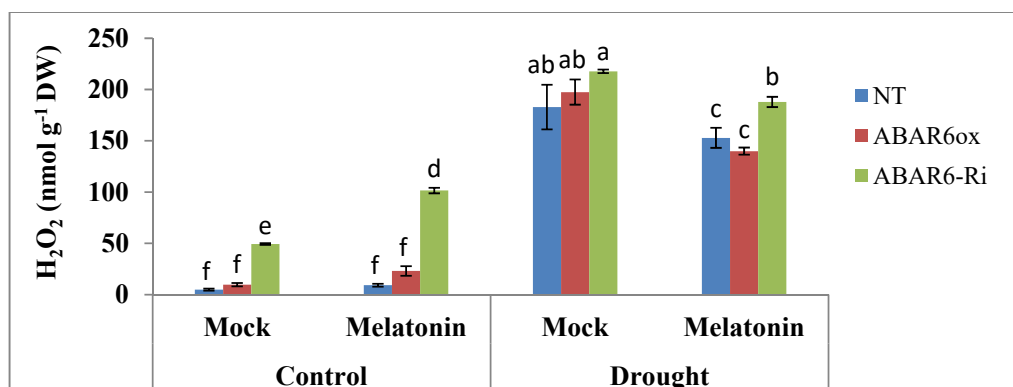


Fig: 4.27. The effects of melatonin application on hydrogen peroxide level (nmol g⁻¹ DW) in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05).

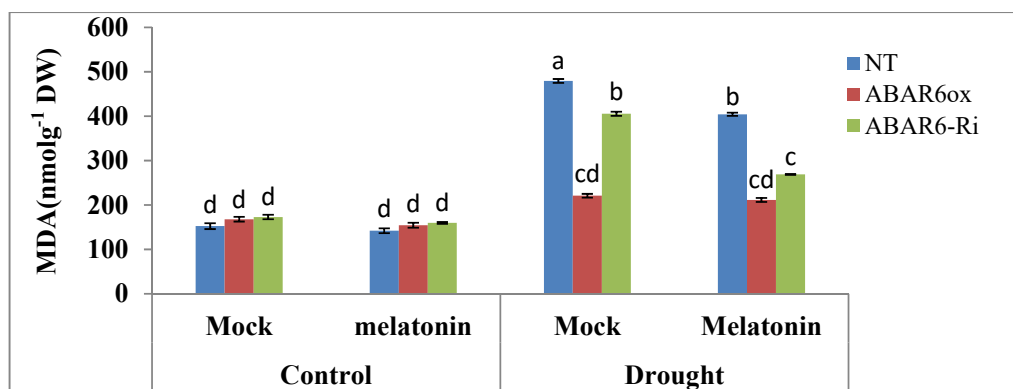


Fig: 4.28. The effects of melatonin application on lipid peroxidation activity (nmol g⁻¹ DW) in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05).

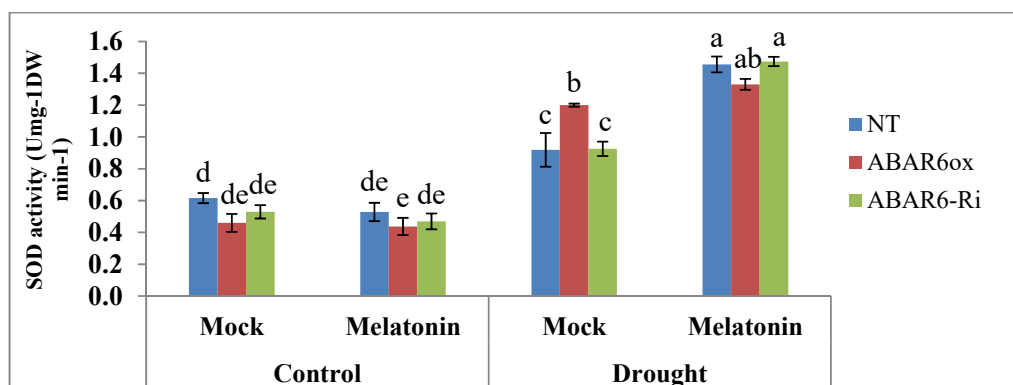


Fig: 4.29. The effects of melatonin application on superoxide dismutase (SOD) activity (Umg⁻¹ min⁻¹ DW) in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05).

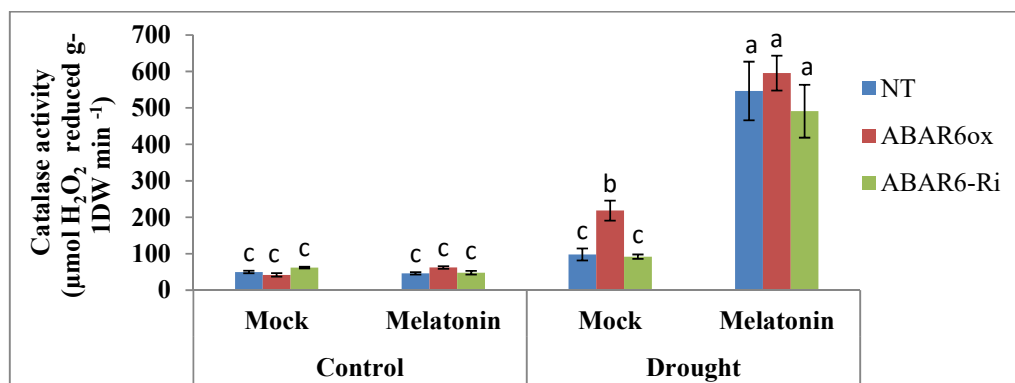


Fig: 4.30. The effects of melatonin application on Catalase activity (μmol H₂O₂ reduced g⁻¹ min⁻¹ DW) in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05).

compared to non-transgenic and ABAR6-Ri lines under drought condition. Exogenous melatonin application significantly reduced the MDA level and lipid peroxidation activity in non-transgenic and ABAR6-Ri lines while; it was not significant changes in the ABAR6ox lines as compared with their respective mock plants in stress condition.

4.9.3. Superoxide dismutase activity

Superoxide dismutase (SOD) activity (**Fig. 4.29**) under control condition was found to be non-significant between transgenic and non-transgenic lines while non-transgenic showed slightly higher activity than the transgenic lines. Exogenous melatonin application didn't show any significant changes in the SOD activity under non stress conditions. Drought stress significantly increased the SOD activity in all the genotypes as compared to their respective mock plants in non-stress conditions. ABAR6ox maintained higher SOD activity than NT and ABAR6-Ri lines under stress condition as compared with non-stress condition. Melatonin application enhanced the SOD activity significantly in NT and ABAR6-Ri lines while it was moderate increased in ABAR6ox lines.

4.9.4. Catalase activity

Catalase activity (**Fig. 4.30**) under control condition was observed non-significant among all the genotypes. Exogenous melatonin application didn't show any significant changes in the catalase activity in all the genotypes as compared to their respective mock plants in non-stress condition. Drought stress significantly increased the catalase activity in the transgenic ABAR6ox line while it was moderate increase in non-transgenic and ABAR6-Ri lines as compared with the non-stress condition. ABAR6ox lines maintained higher catalytic activity than non-transgenic and ABAR6-Ri lines under stress condition. Exogenous melatonin application significantly increased the catalase activity under stress condition as compared with their respective mock plants in stress and non-stress condition. Increase in catalase activity was moderately higher in ABAR6ox line, NT and ABAR6-Ri lines subsequently.

4.9.5. Peroxidase activity:

Peroxidase activity (**Fig. 4.31**) under control condition was found to be non-significant between difference transgenic and non-transgenic lines. Exogenous

melatonin application didn't show any significant changes in the peroxidase activity as compared with their respective mock plants in non-stress condition. Drought stress significantly increases the peroxidase activity in all the genotypes as compared with non-stress condition. ABAR6ox maintained slightly higher peroxidase activity than non-transgenic and ABAR6-Ri lines under drought condition. Exogenous melatonin application enhanced the peroxidase activity significantly in all the genotypes as compared to their respective mock plants in the stress condition.

4.10. Cytokinin biosynthesis gene expression under drought stress and melatonin application

Cytokinin is one of the important components of development biology under different stress condition. . The activity of cytokinin depends upon its biosynthesis gene activity. Based on the previous reports and analysis of transcriptome data of public database through Genevestigator, we selected the cytokinin biosynthesis genes which are differentially expressed in drought stress. The selected genes are *OsIPT7*, *OsIPT9*, *OsLOGL1* and *OsLOGL2* and their relative expression was computed through RT-PCR under control and drought stress with mock and melatonin treatment. The results are listed below in graphical representation with statistical analysis.

4.10.1. Relative expression of *OsIPT7*

The relative expression of *OsIPT7* (**Fig. 4.32**) under control condition was found to be significantly higher expression in transgenic ABAR6ox line than two other non-transgenic and ABAR6-Ri lines while there was non-significant between NT and ABAR6-Ri lines. Exogenous melatonin application did not cause any significant changes in the expression as compared to their respective mock plants in non-stress condition. Drought stress significantly reduced the expression in the ABAR6ox lines while there was no significant change in expression in the NT and ABAR6-Ri lines as compared to their respective mock plants in non-stress condition. . Exogenous melatonin application significantly increased the expression in transgenic ABAR6ox and ABAR6-Ri lines while there was no significant change in the non-transgenic lines as compared to their respective mock plants in the stress condition.

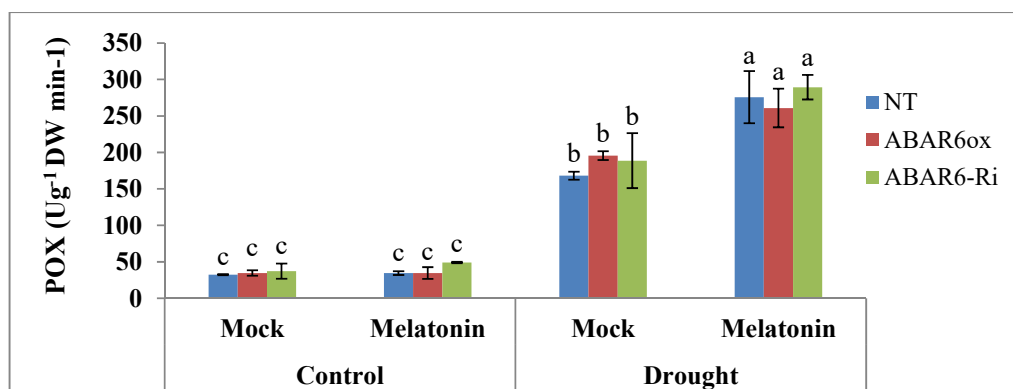


Fig: 4.31. The effects of melatonin application on Catalase activity ($\mu\text{mol H}_2\text{O}_2$ reduced $\text{g}^{-1} \text{min}^{-1} \text{DW}$) in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean ($n=3$). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$)

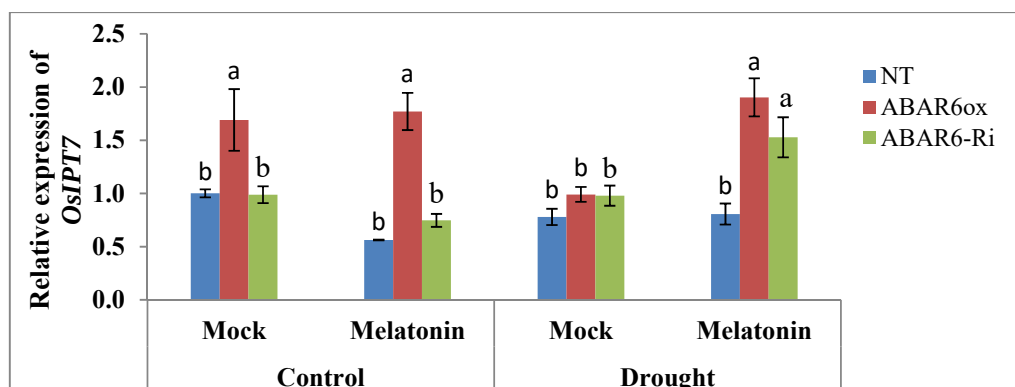


Fig: 4.32. The effects of melatonin application on relative expression of *OsIPT7* in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean ($n=3$). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

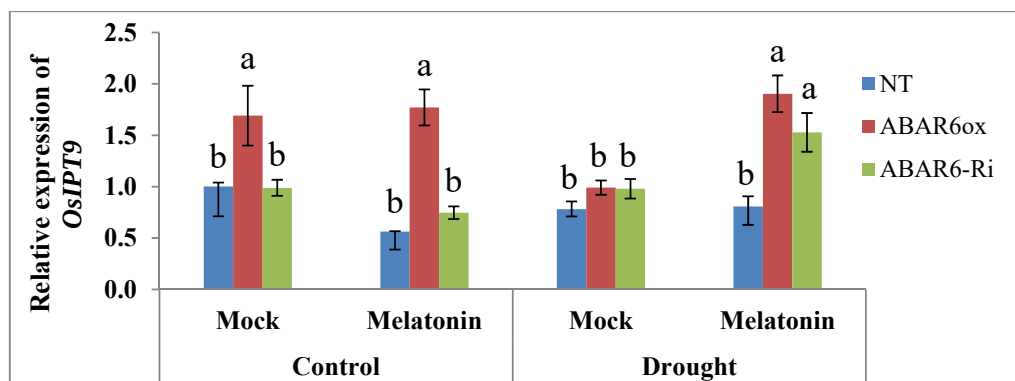


Fig: 4.33. The effects of melatonin application on relative expression of *OsIPT9* in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean ($n=3$). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

4.10.2. Relative expression of *OsIPT9*

The relative expression of *OsIPT9* (Fig. 4.33) under control condition was found to be significantly higher expression in transgenic ABAR6ox line than two other non-transgenic and ABAR6-Ri lines while there was no significant between NT and ABAR6-Ri lines. Exogenous melatonin application did not cause any significant changes in the expression as compared to their respective mock plants in non-stress condition. Drought stress significantly reduced the expression in the ABAR6ox lines while there was no significant change in expression in the NT and ABAR6-Ri lines as compared to their respective mock plants in non-stress condition. Exogenous melatonin application significantly increased the expression in transgenic ABAR6ox and ABAR6-Ri lines while there was no significant change in the non-transgenic lines as compared to their respective mock plants in the stress condition.

4.10.3. Relative expression of *OsLOGL1*

The relative expression of *OsLOGL1* (Fig. 4.34) under control condition was found to be significantly higher expression in transgenic ABAR6ox line than two other non-transgenic and ABAR6-Ri lines while there was non-significant between NT and ABAR6-Ri lines. Exogenous melatonin application did not cause any significant changes in the expression as compared to their respective mock plants in non-stress condition. Drought stress significantly increased the expression in the NT lines while there was no significant change in expression in the ABAR6ox and ABAR6-Ri lines as compared to their respective mock plants in non-stress condition. Exogenous melatonin application significantly increased the expression in transgenic ABAR6-Ri lines while there was no significant change in the non-transgenic lines and significant reduction in the ABAR6ox lines as compared to their respective mock plants in the stress condition.

4.10.4. Relative expression of *OsLOGL2*

The relative expression of *OsLOGL2* (Fig. 4.35) under control condition was found to be significantly higher expression in transgenic ABAR6-RI line than two other non-transgenic and ABAR6ox lines while there was non-significant between NT and ABAR6ox lines. Exogenous melatonin application did not cause any significant changes in the expression of NT and ABAR6ox lines while there was

significant reduction in the ABAR6-Ri lines as compared to their respective mock plants in non-stress condition. Drought stress significantly reduced the expression in the ABAR6-Ri lines while there was significant increase in expression in the NT and ABAR6ox lines as compared to their respective mock plants in non-stress condition. . Exogenous melatonin application significantly increased the expression in transgenic ABAR6ox and ABAR6-Ri lines while there was no significant change in the non-transgenic lines as compared to their respective mock plants in the stress condition.

4.11. Relative expression of ABA dependent transcription factors under drought stress and impact of exogenous melatonin application

There are several genes reported which work through ABA dependent pathway for drought tolerance. Based on the previous reports and analysis of transcriptome data of public database through Genevestigator, we found several ABA dependent transcription factors which are differentially expressed under drought stress. Out of which *OsABF2*, *OsNAC2*, *OsNAC6*, *OsNAC10* and *OsLEA3* genes were selected. The relative expression of these genes was studied under control and drought stress with mock and melatonin treatment through RT-PCR. The results are listed below in graphical representation with statistical analysis.

4.11.1. Relative expression of *OsLEA3*

The relative expression of *OsLEA3* (**Fig. 4.36**) was found to be very low in the control condition in all the genotypes. Melatonin application did not significantly change the relative expression under non stress condition. Drought stress significantly increased the relative expression to higher level as compared to the non-stress condition. ABAR6-Ri accumulated higher relative expression as compared to non-transgenic and transgenic ABAR6ox lines under stress condition. Melatonin application significantly decreased the expression in ABAR6-Ri lines, while significantly increased on ABAR6ox lines with moderate increase in non-transgenic line as compared to their respective mock plants in stress condition.

4.11.2. Relative expression of *OsNAC2*

The relative expression of *OsNAC2* (**Fig. 4.37**) under control condition was found to be significantly higher in non-transgenic line while there was non-significant between ABAR6ox and ABAR6-Ri lines. Exogenous melatonin

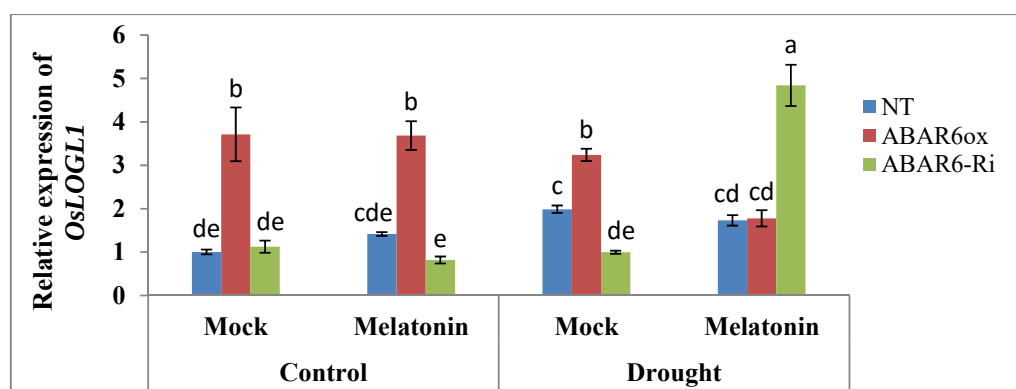


Fig: 4.34. The effects of melatonin application on relative expression of *OsLOGL1* in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

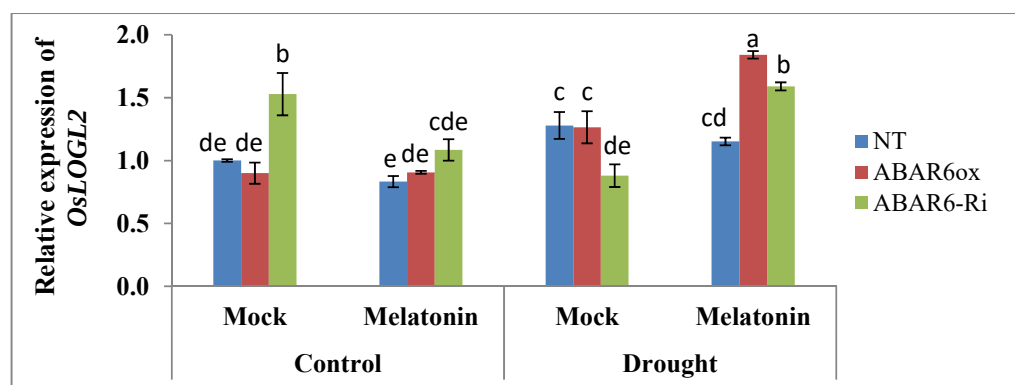


Fig: 4.35. The effects of melatonin application on relative expression of *OsLOGL2* in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

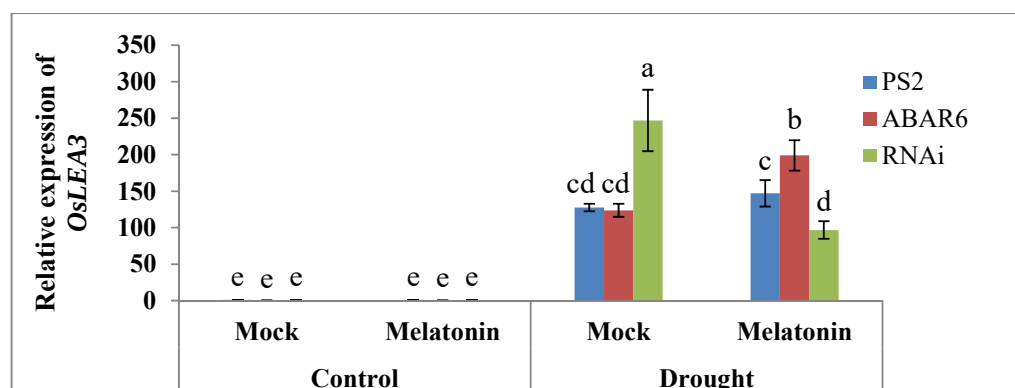


Fig: 4.36. The effects of melatonin application on relative expression of *OsLEA3* in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

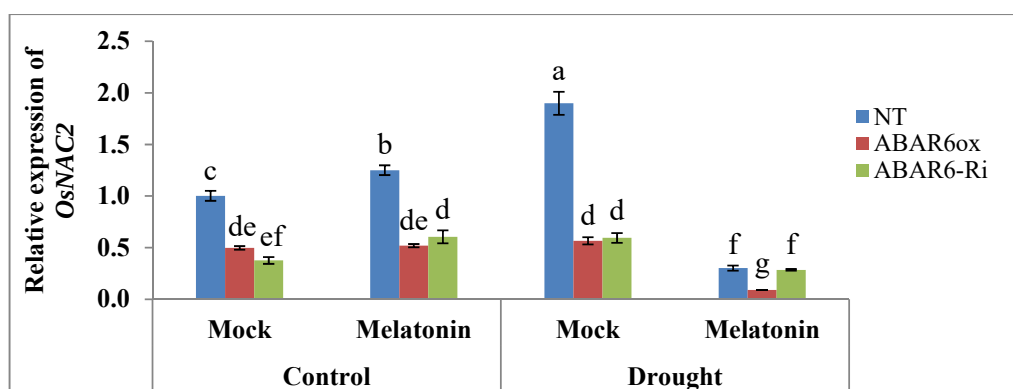


Fig: 4.37. The effects of melatonin application on relative expression of *OsNAC2* in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

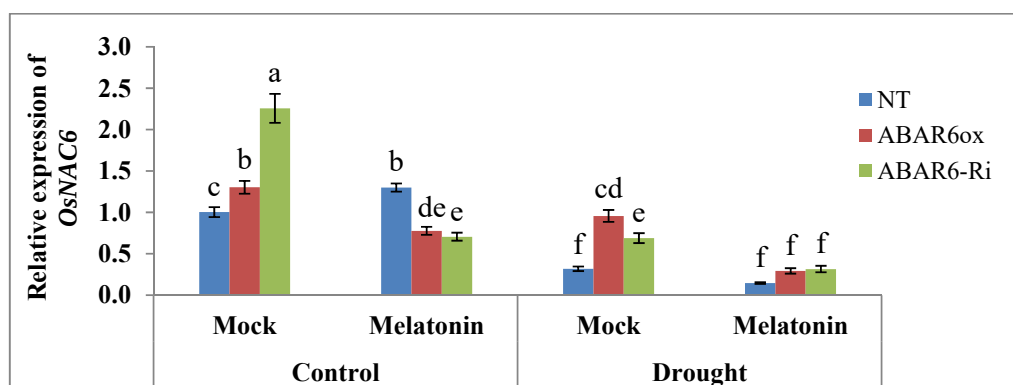


Fig: 4.38. The effects of melatonin application on relative expression of *OsNAC6* in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

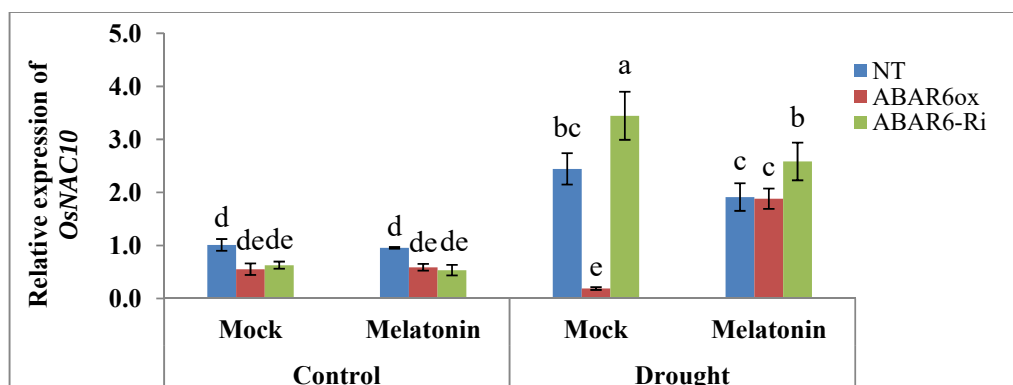


Fig: 4.39. The effects of melatonin application on relative expression of *OsNAC10* in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

application increased the expression in non-transgenic line and ABAR6-Ri line while it was non-significant changes in the ABAR6ox lines as compared to their respective mock plants in non-stress condition. Drought stress significantly increased the expression in non-transgenic line while it was non-significant in transgenic line ABAR6ox and ABAR6-Ri lines as compared to their respective mock plants in non-stress condition. Exogenous melatonin application reduced the expression of *OsNAC2* in all the genotypes significantly with respect to their mock plants in stress condition.

4.11.3. Relative expression of *OsNAC6*

The relative expression of *OsNAC6* (Fig. 4.38) under control condition was found to be significant among the transgenic and non-transgenic genotypes. ABAR6-Ri line maintained higher relative expression followed by ABAR6ox and non-transgenic line in non-stress condition. Exogenous melatonin application significantly decreased the relative expression of *OsNAC6* while it was significantly increased in the non-transgenic line as compared to their respective mock plants in non-stress condition. Drought stress significantly reduced the relative expression of *OsNAC6* in all the genotypes as compared to the non-stress condition. Exogenous melatonin significantly reduced the expression of *OsNAC6* both ABAR6ox and ABAR6-Ri lines while there was moderate decrease on the non-transgenic line as compared to their mock plants in the stress condition.

4.11.4. Relative expression of *OsNAC10*

The relative expression of *OsNAC10* (Fig. 4.39) under the control condition was found to be non-significant among all the three genotypes. Exogenous melatonin application did not show any significant difference among all the genotypes as compared to their respective mock plants in the non-stress condition. Drought stress significantly increased the expression in the non-transgenic and ABAR6-Ri lines while no significant changes in the ABAR6ox lines as compared to the non-stress condition. Exogenous melatonin application significantly reduced the relative expression in ABAR6-Ri line, moderately decrease in the non-transgenic line while significantly increase in the ABAR6ox lines as compared to their mock plants in stress condition.

4.11.5. Relative expression of *OsABF2*

The relative expression of *OsABF2* (**Fig. 4.40**) under control condition was found to be no significant difference between transgenic and non-transgenic line. Exogenous melatonin application significantly increased the relative expression of *OsABF2* in ABAR6ox line while there was no significant difference in NT and ABAR6-Ri lines as compared to their respective mock plants in no stress condition. Drought stress significantly increased the relative expression in all the genotypes as compared to non-stress condition. ABAR6ox significantly maintained higher expression than other NT and ABAR6-Ri lines under stress condition. Exogenous melatonin application significantly reduced the expression of *OsABF2* in all the genotypes as compared with their respective mock plants in stress condition.

4.12. Relative expression of ABA independent transcription factors under drought stress and impact of exogenous melatonin application

There are several genes reported which work through ABA independent pathway. Based on the previous reports and analysis of transcriptome data of public database through Genevestigator, we found the following DREBs transcription factors which are differentially expressed under drought stress and also work via ABA independent pathway. *OsDREB1A*, *OsDREB1B*, *OsDREB1F*, *OsDREB2A* and *OsDREB2B* were selected for study. The relative expression of these genes was studied under control and drought stress with mock and melatonin treatment through RT-PCR. The results are listed below in graphical representation with statistical analysis.

4.12.1. Relative expression of *OsDREB1A*

The relative expression of *OsDREB1A* (**Fig. 4.41**) under the control condition was found to be non-significant among transgenic genotypes and significantly higher in the non-transgenic genotypes. Exogenous melatonin application did not show any significant difference between transgenic ABAR6ox and ABAR6-Ri lines while significant reduce in the non-transgenic line as compared to their respective mock plants in the non-stress condition. Drought stress significantly increased the expression in transgenic ABAR6ox and ABAR6-Ri lines while it was significantly reduced in non-transgenic lines as compared to the non-stress condition. Exogenous melatonin application significantly reduced the relative expression in non-transgenic

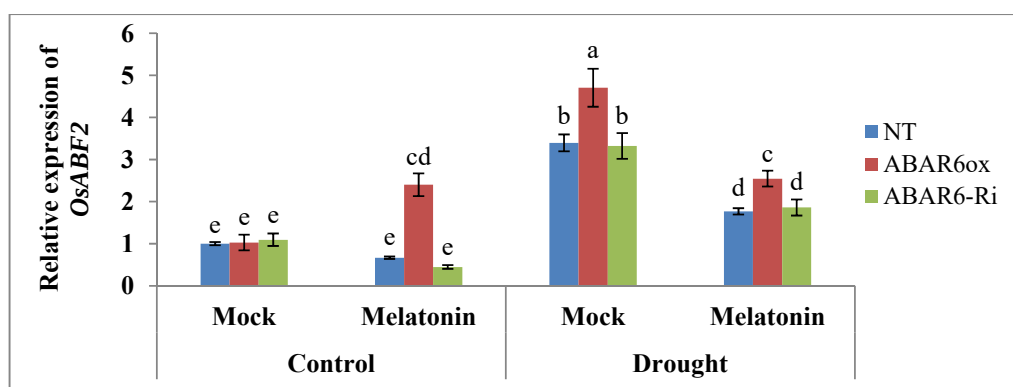


Fig. 4.40. The effects of melatonin application on relative expression of *OsABF2* in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

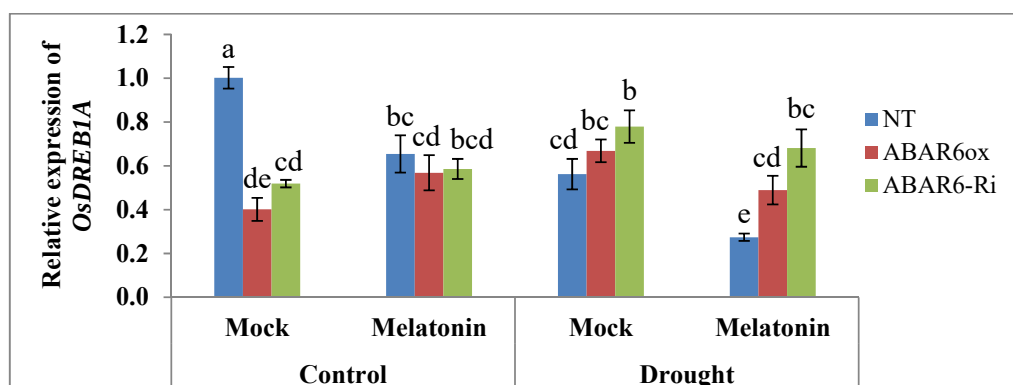


Fig. 4.41. The effects of melatonin application on relative expression of *OsDREB1A* in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

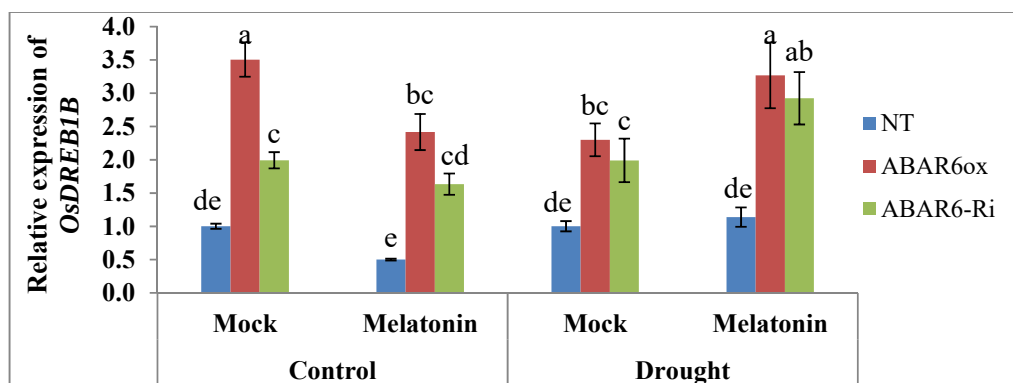


Fig. 4.42. The effects of melatonin application on relative expression of *OsDREB1B* in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

line moderately decrease in the transgenic lines as compared to their mock plants in stress condition.

4.12.2. Relative expression of *OsDREB1B*

The relative expression of *OsDREB1B* (Fig. 4.42) under control condition was found to be significant among all the genotypes. ABAR6ox line maintained higher relative expression followed by ABAR6-Ri lines than non-transgenic lines. Exogenous melatonin application significantly reduced the expression in the ABAR6ox line while it was moderate reduction in the NT and ABAR6-Ri lines. Drought stress significantly reduced the relative expression in the ABAR6ox line while it was no significant change in the NT and ABAR6-Ri lines as compared to their respective mock plants in non-stress condition. Exogenous melatonin application significantly increased the expression of *OsDREB1B* in ABAR6ox and ABAR6-Ri lines while there no significant changes in the NT line as compared to their respective mock plants in the stress condition.

4.12.3. Relative expression of *OsDREB1F*

The relative expression of *OsDREB1F* (Fig. 4.43) under control condition was found to be significantly lower in the transgenic line ABAR6ox and ABAR6-Ri as compared to the non-transgenic line. Exogenous melatonin application significantly reduced the relative expression in non-transgenic line while it was no significant difference in the transgenic line ABAR6ox and ABAR6-Ri as compared to their respective mock plants in the non-stress condition. Drought stress significantly increased the relative expression of *OsDREB1F* in the ABAR6-Ri lines while it was no significant difference in NT and ABAR6ox lines. Exogenous melatonin application significantly increased the relative expression in tall the genotypes as compared to their mock plants in stress condition.

4.12.4. Relative expression of *DREB2A*

The relative expression of *OsDREB2A* (Fig. 4.44) under control condition was found to significantly higher in ABAR6ox while no significant difference in ABAR6-Ri to non-transgenic line. Exogenous melatonin application significantly increased the relative expression in ABAR6ox line while there was no significant change in the non-transgenic and transgenic ABAR6-Ri lines as compared to their respective mock plants in the non-stress condition. Drought stress significantly

increased the relative expression of *OsDREB2A* in ABAR6-Ri lines while there was no significant difference in NT and ABAR6ox lines as compared to their respective mock plants in the non-stress condition. Exogenous melatonin application significantly increased the relative expression in the ABAR6ox and ABAR6-Ri lines while there was no significant change in the relative expression in NT line as compared to their respective mock plants in the stress condition.

4.12.5. Relative expression of *OsDREB2B*

The relative expression of *OsDREB2B* (**Fig. 4.45**) under control condition was found to be significantly higher in ABAR6ox line while there was non-significant between non-transgenic and transgenic ABAR6-Ri lines. Exogenous melatonin application did not show any significant change in relative expression of *OsDREB2B* as compared to their respective mock plants in non-stress condition. Drought stress significantly reduced the relative expression of ABAR6ox while there was no significant change in the NT and ABAR6-Ri lines as compared to their mock plants in non-stress condition. Exogenous melatonin application showed significant increase in expression in the NT and ABAR6-Ri while moderate increase in the ABAR6ox lines as compared to their respective mock plants in the stress condition.

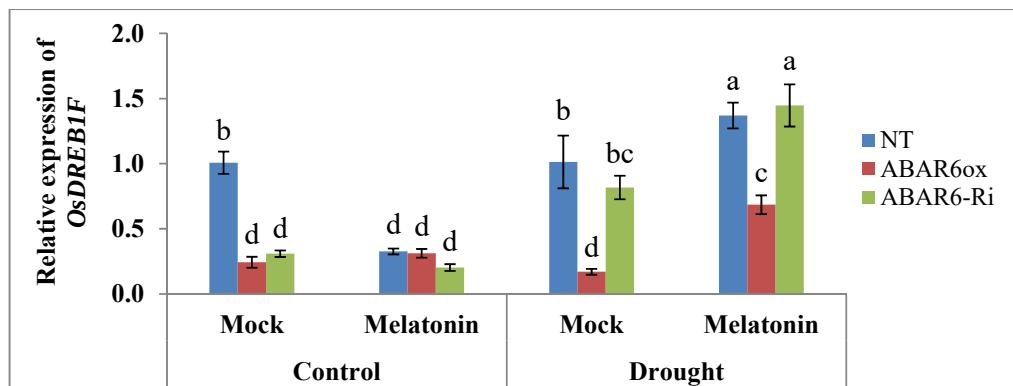


Fig: 4.43. The effects of melatonin application on relative expression of *OsDREB1F* in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

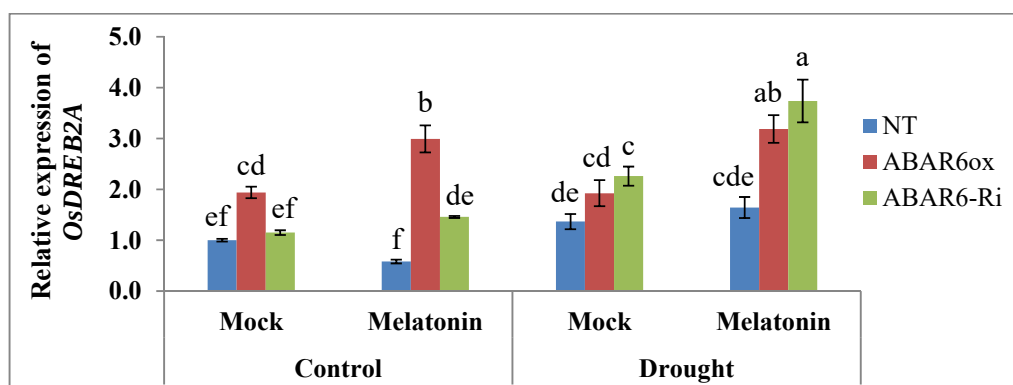


Fig: 4.44. The effects of melatonin application on relative expression of *OsDREB2A* in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

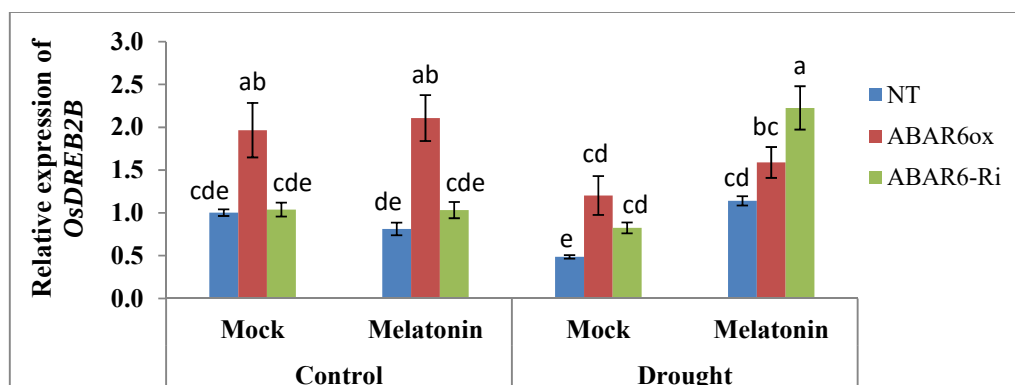


Fig: 4.45. The effects of melatonin application on relative expression of *OsDREB2B* in transgenic and non-transgenic rice genotypes under control and drought condition. Vertical bars indicate $\pm$ standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$).

5.1. Melatonin improves the leaf water status:

Water is the most important component of life. In plants, water supports for stabilizing the turgidity of cell for structure and transportation of growth nutrients and other several organic compounds throughout the plant system. Water is in the living protoplasm which serves as raw materials for various biochemical process and reaction like photosynthesis and through transpiration, buffering the plant against wide temperature fluctuations. Reduced leaf water status is the primary effect of drought stress in rice.

This study showed that melatonin application improved the relative water content (RWC) (**Fig. 4.5.**) in both transgenic and non-transgenic lines under drought stress. It was observed that ABAR6ox line maintained higher and ABAR6-Ri line maintained lower RWC in drought condition. Melatonin application rescued the drought sensitive ABAR6-Ri line by significantly increasing RWC. The enhanced RWC suggests that melatonin can improve tolerance level in drought. Previous studies have shown that melatonin application under drought stress significantly improve leaf water status by decreasing water potential (Ye et al., 2016), higher turgor pressure (Meng et al., 2014; Cui et al., 2017) and higher RWC (Turk et al., 2014). Thus improved leaf water status of rice by melatonin application under drought stress plays an important role in drought tolerance.

5.2. Melatonin improves photosynthesis and better function of photosynthetic pigments

Drought stress affects the photosynthesis, the main metabolic process which determines crop growth and production. Carbon dioxide diffusional limitations is the major component limiting photosynthesis due to early stomatal closure, reduced activity of photosynthetic enzymes, the biochemical components related to triose-phosphate formation and decreased photochemical efficiency of PSII. Drought stress reduces the photosynthetic rate of rice (Ji et al., 2012, Lauteri et al., 2014; Yang et al., 2014). Drought stress also impairs activity of PSII in rice flag leaf (Pieters and Souki, 2005). Similarly drought reduces the stomatal conductance and transpiration.

This study showed that melatonin reduced the adverse effect of drought stress on photosynthesis in rice. Melatonin application (200 μ M) significantly increased the photosynthesis rate (**Fig. 4.17.**) in NT and ABAR6ox genotypes while there was moderate increase in ABAR6-Ri lines compared to drought stress. Melatonin increased the photosynthesis in all genotypes under non stress condition. Similarly, it was observed that melatonin moderately improved transpiration (**Fig. 4.21.**) and stomatal conductance (**Fig. 4.18.**) while there was no change in internal carbon dioxide concentration in mesophyll cell (**Fig. 4.19.**) under drought stress. Enhanced photosynthesis rate, transpiration and stomatal conductance suggest that melatonin application improve the plant functioning in drought stress. Previous studies have shown that melatonin application maintained significantly photosynthesis and stomatal conductance under water deficit in wheat (Cui et al., 2017), maize (Fleta-Soriano et al., 2017), apple (Li et al., 2014; Wang et al., 2013), rapeseed (Li et al., 2015) and alfalfa (Antoniou et al., 2017). Thus improved photosynthetic performance of rice by melatonin application under drought stress plays an important role in drought stress tolerance in rice.

In control condition, ABAR6 receptor showed neutral effect in non-transgenic and ABAR6-Ri genotypes with respect to photosynthesis rate, transpiration, stomatal conductance and internal CO₂ concentration. By melatonin application, the effect of ABAR6 receptor was not changed under non stress condition. Under drought stress, ABAR6 receptor showed neutral effect in non-transgenic and ABAR6-Ri genotypes with respect to photosynthesis, stomatal conductance, and internal CO₂ concentration in mesophyll cells while it was depletive effect in non-transgenic genotype and slightly reduced in ABAR6-Ri genotype with respect to transpiration. Melatonin application neutralizes depletive effect of ABAR6 receptor in non-transgenic line while it was slightly additive effect in ABAR6-Ri genotypes in all photosynthetic parameters selected under drought stress. This showed that Melatonin can rescue the depletive effect of ABAR6 receptor in rice under drought condition.

PSII activity is important for providing reducing power NADPH and ATP. When PSII activity crosses the limit of balance tradeoff, there is excess reduction of photosynthetic electron transport chain which triggers the ROS formation. Therefore, there must be balance between photochemical activity and the photo assimilate's

demand. Drought stress severely impairs PSII activity in rice plants (Pieters and Souki, 2005).

This study showed that melatonin application enhanced the PSII activity (**Fig. 4.16.**) under drought stress in all the genotypes. Enhanced PSII activity suggests the role of melatonin in photo protection under stress condition. Previous studies have reported that melatonin application maintained significantly higher PSII activity under drought stress in maize (Ye et al., 2016; Fleta Soriano et al., 2017), wheat (Cui et al., 2017), apple (Li et al., 2014; Wang et al., 2012), and rye grass (Zhang et al., 2017). Thus, melatonin treated plants maintained high photosynthetic rate under drought stress enabling a much greater supply assimilated to growing tissue through protection of photo induced damage in rice.

Drought causes reduced photosynthetic pigments which ultimately reduced the light harvest and loss of reducing energy. Chlorophyll absorbs light and transfers light energy to the reaction center of the photosystem. Both chlorophyll a and b are prone to soil drying. However, other pigment such as carotenoids have additional roles in chloroplast photosystem structure, light harvesting and photo protection, and partially help the plants to withstand adversaries of drought. Drought reduced the chlorophyll content rice (Pirdashti et al., 2009; Cha-um et al., 2010; Sikuku et al., 2012; Maisura et al., 2014).

This study showed that melatonin application (200 μ M) in rice leaf reduced the adverse effect of drought stress in photosynthetic pigments. Melatonin application significantly increased the chlorophyll a (**Fig. 4.22.**), total chlorophyll (**Fig. 4.25.**) and total carotenoid content (**Fig. 4.26.**) in NT and ABAR6ox genotypes while moderate increase in ABAR6-Ri lines under drought stress, while chlorophyll b (**Fig. 4.23.**) was significantly increased in all the genotypes. Enhanced level of photosynthetic pigments suggests that melatonin plays key role in increasing photosynthetic performance under drought stress. Previous studies also have shown that melatonin application significantly increased the chlorophyll content in ryegrass (Zhang et al., 2017), wheat (Li et al., 2018), watermelon (Nawaz et al., 2018), Kiwi fruit (Liang et al., 2018) and carotenoids content (Arnao and Hernández-Ruiz, 2014) under stress condition. Melatonin application delayed the leaf senescence by delaying the chlorophyll degradation and suppressed the transcript levels of pheophorbide a oxygenase (PAO), chlorophyll degradation gene, and the senescence-

associated gene 12 (SAG12) (Wang et al., 2012; Zhang et al., 2013; Liang et al., 2018). In Arabidopsis, melatonin application down regulated the chlorophyllase and pheophorbide a oxygenase, two chlorophyll-degrading enzymes (Weeda et al., 2014). Thus melatonin treated plants maintained higher level of photosynthetic pigments under drought stress and promote tolerance to some extent.

5.3.Melatonin improves the endogenous Melatonin level

Light, temperature, various environmental stresses like drought, salinity etc. increase endogenous melatonin levels in plants (Arnao and Hernández-Ruiz, 2009, 2013). Our reported that exogenous melatonin (250 μ M) under drought stress increased the endogenous melatonin content (**Fig. 4.10.**) in both transgenic and non-transgenic lines. The relative expression of *OsCOMT* (**Fig. 4.11.**) was significantly induced in transgenic ABAR6ox and ABAR6-Ri lines while *OsASMT1* (**Fig. 4.12.**) was significantly higher in non-transgenic and transgenic ABAR6-Ri lines under stress condition. *OsASMT3* (**Fig. 4.13.**) and *OsTDC2* (**Fig. 14.**) were highly up regulated in the transgenic ABAR6ox and ABAR6-Ri lines while *OsASMT2* was significantly increased in non-transgenic and ABAR60x lines by melatonin application under stress condition. This showed that ABAR6-Ri line maintained higher expression of melatonin biosynthesis genes. These results states that increase in melatonin content was positively correlated to the increase in melatonin biosynthesis genes. Previous studies also showed that exogenous melatonin application increased the endogenous melatonin content (Zhang et al., 2017; Wang et al., 2017; Ke et al., 2018; Ahammed et al., 2018). It was also observed that melatonin induced the melatonin biosynthesis genes (Park et al., 2013; Zheng et al., 2017, Zhao et al., 2018,). It is also reported that the melatonin biosynthesis gene, TDC overexpression in rice showed the delaying leaf senescence and RNAi suppression of TDC lowers serotonin levels and promoted the senescence process (Byeon et al., 2014). Thus, increase in melatonin content and its biosynthesis gene expression promotes the drought tolerance in rice by adjusting leaf water status and increasing antioxidative pathway.

5.4.Melatonin improves the endogenous ABA level

Absciscic acid (ABA) is a stress tolerance hormone. ABA accumulation in response to water stress has been well observed in rice (Wang *et al.*, 2007; Ye et al., 2011; Kumar *et al.*, 2013). ABA closes the stomata and induces the antioxidant

enzymes thus imparting the tolerance to drought (Latif et al., 2014; Li et al., 2014) and improving protein transport, carbon metabolism and expression of resistance proteins (Zhou et al., 2014).

Our results showed that exogenous melatonin application significantly increased the ABA level (**Fig. 4.7.**) in both transgenic and non-transgenic lines under drought stress condition. ABA biosynthesis gene *OsNCED5* (**Fig. 4.9.**) was significantly increased in both the genotypes viz, non-transgenic and transgenic while *OsNCED3* (**Fig. 4.8**) was significantly expressed in ABAR6ox as compared to other genotypes under drought condition. It is somehow contrary to the previous report that exogenous melatonin application significantly increased the tolerance of both drought-tolerant *Malus prunifolia* and drought-sensitive *M. hupehensis* plants through down-regulation of *MdNCED3*, and up-regulates its catabolic genes, *MdCYP707A1* and *MdCYP707A2*, thereby reducing ABA contents in drought-stressed plants (Li et al., 2014). Similarly it was reported that Melatonin treated seed of cucumber showed decrease of ABA in germination by up regulating ABA catabolic genes like *CYP707A1* and *CYP707A2* and down regulating ABA biosynthesis genes like *NCED2* (Zhang et al., 2014). Countering the above evidences, it was reported that foliar and rhizospheric treatment of melatonin in barley under combined drought and cold stress increases endogenous ABA level in leaves (Li et al., 2016) while Fu et al., (2017) reported that exogenous treatment of melatonin under cold stress in *Elymus nutans* increases the ABA level. Thus with these results and evidences, it is concluded that melatonin upregulated the ABA biosynthesis gene and increased the ABA content which is important aspects of inducing drought tolerance.

5.5.Melatonin improves Osmotic adjustment

Osmotic adjustment is accumulation of solutes due to decrease in osmotic potential in different stress (Steponkus et al., 1982; Turner, 1986; Munns, 1988). Osmotic adjustment assists in turgor maintenance and cell volume during drought stress and has been great importance in drought tolerance of crops (Turner and Jones, 1980; Yang et al., 1983; Morgan, 1984). Proline is one of the important osmolytes which helps to better perform under drought conditions (Kemble and Mac-Pherson, 1954; Vajrabhaya et al, 2001; Verbruggen and Hermans, 2008,). Proline also acts a

metal chelator (Hayat et al, 2012). In plants, Proline content increased under drought stress, more than other amino acids.

This study showed that melatonin application significantly increased the proline content (Fig. 4.27.) in both transgenic (ABAR6ox and ABAR6-Ri) and non-transgenic line. Drought sensitive ABAR6-Ri accumulated lower level of proline as compared to other two lines under drought stress while it was improved significantly by melatonin application. There was no significant difference in proline content in non-stress condition. Increased level of proline suggests osmotic adjustment as major phenomenon of melatonin mediated drought tolerance. Previous studies also showed that melatonin significantly increased the proline content under different stress condition (Turk et al., 2014; Ye et al., 2016; Zhang et al., 2017; Liang et al., 2018). Thus increased proline content by melatonin application during drought stress enables better osmotic management and osmo-protection for better drought tolerance in rice.

5.6. Melatonin reduces the reactive oxygen species and lipid peroxidation activity by increasing the antioxidative enzyme activities.

The ROS generation is an important drought responsive factor in plants (Miller et al., 2010). ROS such as singlet oxygen ($^1\text{O}_2$), hydrogen peroxide (H_2O_2), and superoxide anion (O_2^-) are toxic byproducts of stress metabolism and also act as important signal transduction molecules depending on cellular amount (Miller et al., 2010). ROS acts as important stress signalling molecules at different concentration from low to moderate which triggers the stress responsive adaptations. ROS at high level causes oxidative damage of lipids, proteins and DNA leading to retarded growth and eventual cell death (Hou et al., 2009). During drought stress, CO_2 fixation limitation will reduce the regeneration of NADP^+ , thus leading to excessive reduction of electrons in photosynthetic reactions. Similarly, it is also reported that increased photorespiration causes more than 70 % of total H_2O_2 produced during drought stress (Noctor et al., 2002). This study showed that melatonin significantly decreased H_2O_2 content (**Fig. 4.28.**) in both transgenic (ABAR6ox and ABAR6-Ri) and non-transgenic lines. Drought sensitive ABAR6-Ri line accumulated higher hydrogen H_2O_2 level compared to non-transgenic and transgenic ABAR6ox line which was significantly improved by melatonin application. In non-stress condition, ABAR6-Ri lines accumulated higher level of H_2O_2 than other two. Reduced H_2O_2 suggests that

melatonin triggers the antioxidant activity in plants. Previous studies also have shown that melatonin application during drought stress reduces oxidative stress by reducing the level of ROS (Li et al., 2014; Wang et al., 2017; Antoniou et al., 2017; Cui et al., 2018). Thus, melatonin plays an important role in drought tolerance in rice by reducing the H_2O_2 level in stress condition.

Lipid peroxidation is a continuous reaction induced by free radicals which leads to cell membrane damage by altering the unsaturated fatty acids. Poly unsaturated fatty acids are susceptible to peroxidation which damages the membranes and disrupt the cellular function under drought stress. This study showed that melatonin application reduced the MDA level in both (**Fig. 4.29**) transgenic and non-transgenic lines. Lower MDA level indicates lower peroxidation activity. Drought tolerance ABAR6ox lines maintained lower lipid peroxidation activity as compared to other two lines, while there was lower MDA level in non-stress condition in all genotypes. Reduced lipid peroxidation level indicates the membrane protection by melatonin application. Previous studies showed that melatonin ameliorates the drought stress induce membrane damage by reducing the lipid peroxidation activity (Antoniou et al., 2017). Similarly it was also reported that melatonin improved the membrane damage in heat stress (Deng et al., 2017) and cold stress (Bajwa et al., 2014; Li et al., 2018) and salt stress (Zheng et al., 2017; Chen et al., 2017). Thus melatonin promotes the membrane protection in drought stress by reducing the lipid peroxidation activity and ROS level.

An efficient antioxidative system (non enzymic and enzymic antioxidants) is needed for scavenging or detoxification of excess ROS (Noctor and Foyer, 1998). In enzymatic systems, super oxide dismutase (SOD) catalyses the dismutation of O_2^- to H_2O_2 . Catalase (CAT) and ascorbate peroxidase (POX) can break down H_2O_2 . However, drought stress membrane damage can interrupt the function of scavenging enzymes (Jagtap and Bhargava, 1995; Dat et al., 1998). Thus, high antioxidant capacity is important factor for drought stress tolerance.

This study showed that melatonin application in rice leaf under drought stress significantly increased the SOD (**Fig. 4.30.**), CAT (**Fig. 4.31**) and POX (**Fig. 4.32.**) activity. ABAR6ox moderately maintained higher activity of enzymic antioxidants than other non-transgenic and ABAR6-Ri lines under drought condition. This increase in detoxification of ROS through increasing the activity of enzymic

antioxidants promises the tolerance of plants to drought stress. Previous studies also have shown that melatonin systemically improved drought stress-induced damage by increasing the activity of SOD, CAT and POX (Cu et al., 2017; Antoniou et al., 2017; Liang et al., 2018; Li et al., 2018). Similarly melatonin elevated ASA-GSH cycle enzyme activity such as ascorbate peroxidase (APX), mono dehydro ascorbate reductase (MDHAR) and ascorbic acid level under severe stress (Wang et al., 2012; Meng et al., 2014; Liu et al., 2015; Shi et al., 2015; Cui et al., 2017; Liang et al., 2018). Thus enhancement of antioxidant enzymes can be one of the key strategies of melatonin for reducing or preventing oxidative damage and improving the drought resistance of plants.

5.7. Melatonin improves the cytokinin biosynthesis gene expression

Cytokinin, an important plant hormone regulates a wide array of processes crucial to plant which regulates the different crucial plant development process (Werner and Schmulling, 2009; Kieber and Schaller, 2014). Cytokinin plays an key role in regulating cell divisions and the maintenance of stem cells in apical meristems of both shoots (Gordon et al., 2009; Zhao et al., 2010; Su et al., 2011) and roots (Dello Ioio et al., 2008; Zhang et al., 2013).

This study showed that exogenous melatonin application significantly increased the relative expression of *OsIPT7* (**Fig. 4.33.**) and *OsIPT9* (**Fig. 4.34.**) in transgenic line ABAR6ox and ABAR6-Ri lines under drought conditions. Similarly exogenous melatonin application increased the expression of *OsLOGL1* (**Fig. 4.35.**) and *OsLOGL2* (**Fig. 4.36.**) in transgenic rice ABAR6-Ri lines significantly while there was moderate increase in other two non-transgenic and transgenic ABAR6ox lines under stress condition. This result was positively correlated with maintenance of chlorophyll content photosynthetic performance and anti-oxidants activity under stress condition. Previous study revealed that cytokinin and melatonin had additive effects to suppress drought-induced leaf senescence in creeping bent grass seedlings by up-regulating genes for melatonin and cytokinin responses and signaling and down regulating the genes for degradation of chlorophyll and senescence suppression (Ma et al., 2018). Similarly it was reported that melatonin induces heat tolerance by reducing the senescence through up regulating cytokinin biosynthesis genes and down regulating the cytokinin degrading genes (Zhang et al., 2017). Thus melatonin

application increased the cytokinin level in plants by upregulating the cytokinin biosynthesis genes under drought stress and promotes the drought tolerance in rice.

5.8.ABA receptor over expression induces drought tolerance

ABA is stress hormone which regulates the plant development and tolerance to abiotic stress like drought. The function of PYLS in stress response mediated by ABA is important for understanding and improving the stress tolerance in rice. This study showed that exogenous melatonin application decreased the *OsPLYL6* (**Fig. 4.6.**) moderately without any significant changes in both transgenic and non-transgenic line under stress condition. This showed that melatonin may reduce the ABA signaling under drought stress while adopting alternative ABA independent pathway for drought tolerance. Previous study showed that transgenic line over expressing PYL9 (Zhao et al., 2016), PYL5 (Kim et al., 2014) and PYL3 (Lenka et al., 2018) increased the drought tolerance by reducing the transpiration water loss and reducing the growth under stress condition.

5.9. Melatonin down regulates the ABA signaling pathway

Absciscic acid is important endogenous messenger in plants during drought stress. Understanding the signaling mechanism of ABA is critical for improving plant performance under drought stress. Many ABA-dependent drought-related genes contain a conserved ABA-responsive element (ABRE) with ACGT cores in their promoter regions (Uno et al., 2000). These genes are regulated by ABRE binding proteins-AREB or ABRE binding factors-ABFs (Uno et al., 2000; Fujita et al., 2009), ABI5 (ABA insensitive 5), MYB (myeloblastosis), MYC (myelocytomatosis), NAC (NAM, ATAF, CUC), and ERF (ethylene response factor).

NAC transcription factors appear to be involved in ABA transcriptional network. They are involved in various abiotic and biotic stress regulations. Several reports have shown that they promote drought tolerance in rice. This study showed that exogenous melatonin application significantly decreased the relative expression of *OsNAC2* (**Fig. 4.38.**) and *OsNAC6* (**Fig. 4.39.**) in both transgenic and non-transgenic lines while *OsNAC10* (**Fig. 4.40.**) was significantly decreased in ABAR6-Ri line, moderately decrease in non-transgenic line and significantly increased in ABAR6ox line under drought condition. Previous transcriptomic study revealed that

melatonin application down regulated NAC transcription factors under stress condition (Zhang et al., 2014, Liang et al., 2015). Thus, down regulation of NAC transcription factors by melatonin application and same time achieving drought tolerance shows melatonin works through ABA independent pathway.

AREBs/ABFs belong to the bZIP (basic leucine zipper) transcription factor subfamily. ABFs confer tolerance to various environmental stresses like chilling, freezing, high temperature and drought stress. This study showed that exogenous melatonin application significantly reduced the expression of *OsABF2* under drought stress in both transgenic and non-transgenic line. ABAR6ox maintained significantly higher expression of *OsABF2* (Fig. 4.41.) than other two genotypes under drought condition. Similarly *OsLEA3* (Fig. 4.37.) was significantly down regulated in ABAR6ox and ABAR6-Ri lines under drought stress by exogenous melatonin application. Reduced expression was positively correlated to drought tolerance under melatonin treatment. Previous study also showed that AREB1/ABF2, AREB2/ABF4, and ABF3 induced by high salt, dehydration or ABA treatment in vegetative tissues (Riechmann et al., 2000; Yoshida et al., 2010). It was also reported that overexpression of ABF3 improved plant performance to tolerance to chilling, freezing, high temperature, and drought stress (Kim et al., 2004). Constitutively expression of ABP9 (ABRE binding protein 9) in Arabidopsis improved photosynthetic performance under drought stress and heat shocks (Zhang et al., 2008). Comparative transcriptomic analyses revealed that exogenous melatonin application in bermuda grass down regulated the ABA signaling genes like RCAR3, LEA protein and transcription factors like AREB3, RABs, etc (Shi et al., 2014) while upregulated the PP2C related genes and other ABA independent transcription factors. Thus, melatonin promotes the drought tolerance by inducing the ABA independent gene network and down regulating the ABA dependent signaling gene network.

5.10. Melatonin up regulates the ABA independent dehydration response element binding factors

Shinozaki; (2000) - reported that many drought induced genes don't respond to ABA treatment, suggesting existence of ABA-independent pathways in response to drought stress. ABA-independent genes contain a conserved drought responsible element (DRE) and C-repeat (CRT) cis-acting elements in which combination with DRE-binding protein (DREB) or C-repeat-binding factor (CBF) transcription factors.

The dehydration responsive element (DRE) has a 9 bp conserved core sequence (5'-TACCGACAT-3') (Yamaguchi-Shinozaki and Shinozaki, 1993) which is essential for the induction of *rd29A* gene expression by osmotic stress, water deficit and high salinity, low temperature without involving ABA (Yamaguchi-Shinozaki and Shinozaki, 1994; Saleh et al., 2005). Induction of DREB2s, DRE-binding proteins by drought and high-salinity stress shows their role in stress-responsive gene expression (Liu et al., 1998; Nakashima et al., 2000; Sakuma et al., 2002). Transgenic *Arabidopsis* plants expressing DREB1B/CBF1 or DREB1A/CBF3 showed strong tolerance to freezing, drought, and high salinity stresses. DREB1A/CBF3 transgenics showed accumulation of osmoprotectants, such as proline and various sugars (Gilmour et al., 2000). These results suggest that DREBs/CBFs target multiple genes (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999; Dubouz et al., 2003; Ito et al., 2006).

This study showed that exogenous melatonin application significantly increased the *OsDREB2A* (**Fig. 4.45.**) and *OsDREB2B* (**Fig. 4.46.**) expression in transgenic ABAR6-Ri lines while it was moderate increase in non-transgenic and transgenic ABAR6ox lines under drought stress. Similarly exogenous melatonin application increased the relative expression of *OsDREB1B* (**Fig. 4.43.**) in transgenic ABAR6ox and ABAR6-Ri lines while *OsDREB1F* (**Fig. 4.44.**) was found to be increased in both non transgenic and transgenic lines. *OsDREB1A* (**Fig. 4.42.**) was found to be non-significant changes in transgenic line while reduced in non-transgenic lines. These increased in *OsDREBs* are positively correlated with the increase in proline and photosynthetic performance. Previous study also showed that exogenous melatonin induced the transcripts of CBF/DREB1s which are involved in both abiotic and biotic stresses in *Arabidopsis* (Shi et al., 2015). The transcriptome study revealed that exogenous melatonin induced the expression of DREB transcription factors (Zhang et al., 2014). Melatonin up regulated expression of DREB2A transcription factor for enhancing the drought tolerance by decreasing chlorophyll degradation (Ma et al., 2018). Thus, *OsDREBs* mediated signaling pathway may be partially involved in melatonin mediated drought tolerance in rice.

Chapter 6

SUMMARY AND CONCLUSIONS

Rice is the major staple food crop of the world. To sustain the food security, rice production must be doubled by 2050. This appears to be a major challenge as the current genetic gain in growth rate of rice yield is only 1% per year. Drought stress and other environmental stress further exacerbate this challenge which imposes major limitation for rice productivity and yield stability. Therefore there is need of adoption of next generation breeding tools such as gene/QTL mapping, Gene.QTL-networking pyramiding, transgenics and genome editing to develop high yielding rice cultivars with climate resilience. This warrants the detailed understanding of gene expression programs involved in stress tolerance in rice. Although rice genome has been sequenced in 2005 (IRGSP, 2005), only very few rice genes have been characterized for stress tolerance. Hence, more efforts are required for the deciphering the function of rice genes for stress tolerance and climate resilience rice.

Melatonin is one of the key antioxidant molecule reported in plants for detoxification of ROS under stress condition like drought and also inducing the higher activity of antioxidants. Melatonin cross talks with other plant hormones for stress tolerance. Melatonin promotes the drought tolerance in several cereal crops and horticultural crops. It has mitigates several other stresses like heat, salt, cold and light. Although melatonin promotes drought tolerance in rice, but its molecular pathway and cross talk with other plant hormones like ABA and cytokinin under drought stress is poorly known.

Considering the significance of rice in food and environmental security of the nation, and huge research gap in understanding the molecular pathway of melatonin in drought tolerance in rice, this thesis work on “*Deciphering the molecular pathway of melatonin mediated drought tolerance in rice*” was carried out with following objectives:

1. Physiological and biochemical elucidation of melatonin-mediated drought tolerance mechanism in rice by using non transgenic and transgenic line of ABA receptor PYL6.

2. Molecular expression of melatonin mediated ABA-dependent and ABA-independent genes promoting drought tolerance in transgenic rice.

This study used transgenic (ABA receptor *PYL6* over expressed line-*ABAR6-ox* and RNAi line of ABA receptor *PYL6-ABAR6-Ri*) and non-transgenic (NT) rice to study the drought tolerance in rice through exogenous melatonin application. Here, *ABAR6ox* acts as drought tolerant and *ABAR6-Ri* acts as drought sensitive rice while non-transgenic rice acts as control between them. Transgenic lines were made from Pusa Sugandh 2 variety and these transgenic were made in Dr. Chinnusamy V. lab, Division of Plant Physiology, ICAR-IARI. The rice plants at maximum tillering stage were given drought treatment by withholding irrigation for 20 days and melatonin application was give after one week of drought treatment. Physiological and biochemical traits of rice leaf for drought tolerance with molecular pathway were analyzed under drought stress by melatonin application. The salient findings are summarized below.

- Exogenous melatonin application improved the RWC in both transgenic and non-transgenic lines under drought stress. It was observed that *ABAR6ox* line maintained higher and *ABAR6-Ri* line maintained lower RWC in drought condition. Melatonin application rescued the drought sensitive *ABAR6-Ri* line by significantly increasing their RWC.
- Exogenous melatonin application increased the endogenous melatonin content in both transgenic and non-transgenic lines under drought stress. The relative expression of melatonin biosynthesis genes such as *OsTDC2*, *OsCOMT*, *OsASMT1*, *OsASMT2* and *OsASMT3* up regulated in both non transgenic and transgenic lines. *ABAR6-Ri* line maintained higher expression of melatonin biosynthesis genes. These results states that increase in melatonin content was positively correlated to the increase in melatonin biosynthesis genes.
- Exogenous melatonin application significantly increased the ABA level in both transgenic and non-transgenic lines under drought stress condition. ABA biosynthesis gene *OsNCED5* was significantly increased in both the genotypes non transgenic and transgenic while *OsNCED3* was significantly expressed in *ABAR6ox* as compared to other genotypes under drought condition.

- Exogenous melatonin application significantly increased the chlorophyll a, chlorophyll b, total chlorophyll and total carotenoid content in both non transgenic and transgenic lines. Melatonin also significantly increased the photosynthesis rate, transpiration and stomatal conductance in all the rice lines under non stress condition. It was also observed that melatonin application enhanced the PSII activity under drought stress in all the lines studied. This study showed that melatonin application in rice leaf reduced the adverse effect of drought stress in photosynthetic pigments and photosynthetic performance.
- Exogenous melatonin significantly decreased H₂O₂ and MDA level. Lower MDA level indicates lower peroxidation activity. These results suggest that melatonin has important role in ROS detoxification in plants under drought stress.
- It was also reported that melatonin increased the proline content under drought stress. This correlates with the higher water status in plants by inducing the osmotic adjustment.
- Exogenous melatonin application decreased the relative expression of *OsNAC2*, *OsNAC6*, *OsABF2* and *OsLEA3* in both transgenic and non-transgenic lines while *OsNAC10* was significantly decreased in ABAR6-Ri line, moderately decrease in non-transgenic line and significantly increased in ABAR6ox line under drought condition. Down regulation of ABA responsive transcription factors by melatonin application and at the same time achieving drought tolerance shows melatonin works through ABA independent pathway.
- Exogenous melatonin application significantly increased the relative expression of cytokinin biosynthesis genes *OsIPT7*, *OsIPT9*, *OsLOGL1* and *OsLOGL2* in transgenic line under drought conditions. This result was positively correlated with maintenance of chlorophyll content photosynthetic performance and anti-oxidants activity under stress condition. Similarly, melatonin application increased relative expression of *OsDREB1B*, *OsDREB1B*, *OsDREB2A* and *OsDREB2B* which is positively correlated with the elevated level of osmo-protectants like free proline, soluble sugars

(sucrose and raffinose), and metabolites (galactinol and myo-inositol), thus improving the drought stress tolerance and leaf water status in plants

In conclusion, this study showed that melatonin is the key regulator of drought tolerance in plants. Exogenous melatonin application induced the ABA-independent drought promoting genes like DREB transcription factors and cytokinin biosynthesis pathway. This is positively correlated with the improved photosynthetic pigments and photosynthetic performance under drought stress by melatonin application. Proline level increased under drought stress which correlates the DREB transcription factors and melatonin mediated drought tolerance by inducing the osmotic adjustment. It was observed that melatonin induced the expression of ABA biosynthesis genes and down regulates the ABA dependent signaling which highlights the possible role of melatonin cross talk with ABA and its receptor under drought conditions. This showed that melatonin works via ABA independent pathway for drought tolerance in plants. Functional analysis and transgenic approaches will further illuminate the molecular pathway of melatonin mediated drought tolerance.

“Deciphering the molecular pathway of melatonin mediated drought tolerance in rice”

ABSTRACT

The study entitled **“Deciphering the molecular pathway of melatonin mediated drought tolerance in rice”** was carried out to check whether melatonin promotes drought tolerance via ABA dependent or ABA-independent pathway at maximum tillering stage under drought condition in rice. Pusa Sugandh 2, drought sensitive rice variety was selected for study. The transgenic of ABA receptor 6 developed at Abiotic Stress lab of Dr. Viswanathan C., Division of Plant Physiology, ICAR-IARI, by over expressing with the help of CAMV: RD29 promoter and other RNAi line of ABA receptor 6 by RNAi technology were utilized for this study. ABAR6ox acts as drought tolerant and ABAR6-Ri acts as drought sensitive rice while non-transgenic rice acts as control between them. The transgenic seeds were grown in lab condition and treated with antibiotics to confirm the transgenic line. After 21 days, seedlings were transplanted to the pot under controlled condition with temperature and relative humidity. Drought stress was given for 20 days and melatonin application of 200 μ M was given in the leaves once drought treatment started through manual sprayer. Melatonin application at every alternate was provided four times in total and sampling was done at evening time.

To understand the melatonin's role in alleviating negative effects of drought stress in rice, we studied different physiological and biochemical traits. It was observed that exogenous melatonin application during drought stress significantly improved plant water status by increasing the RWC and proline content. It also improved the photosynthetic pigments like chlorophyll and carotenoids, photosynthetic rate, stomatal conductance, transpiration rate and stomatal opening which are limitations for crop growth under drought stress. The antioxidants like catalase, superoxide dismutase and peroxidase were significantly increased and lipid peroxidation and hydrogen peroxide content were reduced by melatonin application. Moreover, there was increase in abscisic acid (ABA) and melatonin content in the leaf tissue under drought stress. These results showed that melatonin is involved in stress tolerance under drought stress and is potential molecule for decreasing the stress level in plants. The qRT-PCR expression analysis showed that exogenous melatonin application induced the melatonin biosynthesis genes (*OsASMT1*, *OsASMT2*, *OsASMT3* and *OsTDC3*) and ABA biosynthesis genes (*OsNCED3*,

OsNCED5) under drought stress. Melatonin application rescued adverse effects of drought stress on drought sensitive ABAR6-Ri lines.

Based on the previous reports and analysis of transcriptome data of public database through Genevestigator, we identified the ABA responsive and ABA independent genes involved in drought tolerance. We found several ABA dependent transcription factors which are differentially expressed under drought stress. Among them, *OsABF2*, *OsNAC2*, *OsNAC6*, *OsNAC10* and *OsLEA3* genes were selected for the study. Similarly, we found that most of the C-repeat-binding factors (CBFs)/Drought response element Binding factors (DREBs) -transcription factors work via ABA independent pathway under drought stress. *OsDREB1A*, *OsDREB1B*, *OsDREB1F*, *OsDREB2A* and *OsDREB2B* were selected for study. The relative expression of these genes was studied under control and drought stress with mock and melatonin treatment through qRT-PCR gene expression analysis. It was observed that exogenous melatonin application decreased the relative expression of *OsNAC2*, *OsNAC6*, *OsABF2* and *OsLEA3* in both transgenic and non-transgenic lines while *OsNAC10* was significantly decreased in ABAR6-Ri line, moderately decrease in non-transgenic line and significantly increased in ABAR6ox line under drought condition. Down regulation of ABA responsive transcription factors by melatonin application and at the same time achieving drought tolerance shows melatonin works through ABA independent pathway. Similarly it was observed that exogenous melatonin application significantly increased relative expression of *OsDREB1B*, *OsDREB1B*, *OsDREB2A*, and *OsDREB2B* which is positively correlated with the elevated level of osmoprotectants like free proline, soluble sugars(sucrose and raffinose), and metabolites (galactinol and myo-inositol), thus improving the drought stress tolerance and leaf water status in plants. It was also observed that the relative expression of cytokinin biosynthesis genes *OsIPT7*, *OsIPT9*, *OsLOGL1* and *OsLOGL2* in transgenic line under drought conditions were increased This result was positively correlated with maintenance of chlorophyll content photosynthetic performance and anti-oxidants activity under stress condition. These results suggest that melatonin promote drought tolerance through ABA independent pathway by upregulating CBF/DREB2s transcription factors and further downstream stress responsive genes. Together, the results demonstrate that *OsDREB*-mediated signaling pathway may be involved in melatonin-mediated drought stress response in rice at least partially.

Keywords:Rice, Drought, Melatonin, ABA,Cytokinin, DREB, Antioxidants, ROS

“धान में मेलाटोनिन मध्यस्थ सूखे की सहनशीलता को आणविक मार्ग द्वारा समझने”

सारांश

"धान में मेलाटोनिन मध्यस्थ सूखे की सहनशीलता को आणविक मार्ग द्वारा समझने" के अध्ययन में यह जांचा गया कि क्या मेलाटोनिन सूखे की स्थिति के तहत धान के अधिकतम टिलरिंग चरण में, एबीए आश्रित या एबीए-स्वतंत्र मार्ग के माध्यम से सूखा सहनशीलता को बढ़ावा देता है या नहीं। धान की सूखा संवेदनशील प्रजाति, पुसा सुगंध 2 के ट्रांसजेनिक किस्म पर अध्ययन किया गया। एबीए रिसेप्टर 6 का ट्रांसजेनिक को सीएएमवी: आरडी 29 प्रमोटर की मदद से अति अभिव्यक्ति किया गया और एबीए रिसेप्टर 6 की आरएनएआई लाइन को आरएनएआई प्रौद्योगिकी द्वारा बनाया गया। इन दोनों ट्रांसजेनिक प्रजाति को, आईसीएआर-आईएआरआई के पादप कार्यकी संभाग के डॉ विश्वनाथन सी, प्रभागाध्यक्ष और प्रधान वैज्ञानिक के अजैव तनाव प्रयोगशाला में बनाया गया। एबीएआरसिक्सओक्स सूखा सहनशील, एबीएआरसिक्सआरआई सूखा संवेदनशील तथा गैर-ट्रांसजेनिक धान, उनके बीच नियंत्रण के रूप में लिया गया था। ट्रांसजेनिक बीजों को प्रयोगशाला की स्थिति में उगाया गया और ट्रांसजेनिक लाइन की पुष्टि करने के लिए एंटीबायोटिक्स के साथ उपचारित किया गया। 21 दिनों के बाद, रोपण को तापमान (30°C) और सापेक्ष आर्द्रता (70 %) के साथ नियंत्रित स्थिति के तहत गमलो में रोपित किया गया। 20 दिनों के लिए सूखा तनाव दिया गया और मैनुअल स्प्रेयर के माध्यम से सूखा उपचार शुरू होने के बाद पत्तियों में 200 μM मेलाटोनिन का छिड़काव किया गया। प्रत्येक वैकल्पिक दिन पर मेलाटोनिन आवेदन कुल मिलाकर चार बार प्रदान किया गया और शाम को नमूनाकरण किया गया।

धान में सूखा तनाव के नकारात्मक प्रभाव को मेलाटोनिन के माध्यम से कम करने की भूमिका का अध्ययन, के लिए विभिन्न कार्यकी और जैव रासायनिक लक्षणों का अध्ययन किया गया। यह देखा गया कि सूखा तनाव के दौरान बाहरी मेलाटोनिन आवेदन ने आरडब्ल्यूसी और प्रोलिन मात्रा को बढ़ाकर पौधों की पानी की स्थिति में काफी सुधार किया है। इसने क्लोरोफिल और कैरोटेनोइड, प्रकाश संश्लेषण दर, स्टोमेटा प्रवाहकत्व, प्रत्यारोपण दर और स्टोमेटा के खुलने जैसे प्रकाश संश्लेषक रंगों में भी सुधार किया जो कि सूखा तनाव के तहत फसल की वृद्धि के लिए सीमाएं हैं। मेलाटोनिन के आवेदन से कैटलस, सुपरऑक्साइड डिमूटेज और पेरॉक्साइडस जैसे एंटीऑक्सिडेंट्स में काफी वृद्धि हुई थी और लिपिड पेरॉक्साइडेशन और हाइड्रोजन पेरॉक्साइड में कमी हुई। इसके अलावा, सूखे तनाव के तहत पत्ती के ऊतक में एब्सिसिक एसिड (एबीए) और मेलाटोनिन की मात्रा में वृद्धि हुई। इन परिणामों से पता चला है कि सूखा तनाव के तहत मेलाटोनिन तनाव सहनशीलता में शामिल है और पौधों में तनाव स्तर को कम करने के लिए संभावित अणु है। क्यूआरटी-पीसीआर अभिव्यक्ति विश्लेषण से पता चला है कि बाहरी मेलाटोनिन आवेदन ने सूखा तनाव के तहत मेलाटोनिन जैव संश्लेषण जीन (ओएसएसएमटी 1, ओएसएसएमटी 2, ओएसएसएमटी 3 और ओएसटीडीसी 3) और एबीए बायोसिंथेसिस जीन (ओएसएससीईडी 3, ओएसएससीईडी 5) प्रेरित किया है। मेलाटोनिन आवेदन सूखा

संवेदनशील एबीएआरसिक्सआरआई लाइनों पर सूखा तनाव के प्रतिकूल प्रभावों से बचाया।

जेनेवेस्टिगेटर के माध्यम से सार्वजनिक डेटाबेस के ट्रान्सक्रिप्टोम डेटा के विश्लेषण और पिछली रिपोर्ट्स के आधार पर, हमने सूखा सहनशीलता में शामिल एबीए उत्तरदायी और एबीए स्वतंत्र जीन की पहचान की। हमने कई एबीए आश्रित ट्रान्सक्रिप्शन कारकों को पाया जो सूखा तनाव के तहत अलग-अलग व्यक्त किए जाते हैं। उनमें से, अध्ययन के लिए ओएसएबीएफ 2, ओएसएनएसी 2, ओएसएनएसी 6, ओएसएनएसी 10 और ओएसएलए 3 जीन का चयन किया गया था। इसी प्रकार, हमने पाया कि अधिकांश सी-रेपीटिंग बॉन्डिंग फैक्टरस (सीबीएफस) / ड्रॉउट रिस्पांस इलीमेन्ट बॉन्डिंग फैक्टरस (डीआरईबीस)-ट्रान्सक्रिप्शन फैक्टर, सूखा तनाव के तहत एबीए स्वतंत्र मार्ग के माध्यम से काम करते हैं। अध्ययन के लिए ओएसडीआरबी 1 ए, ओएसडीआरबी 1 बी, ओएसडीआरबी 1 एफ, ओएसडीआरबी 2 ए और ओएसडीआरबी 2 बी का चयन किया गया था। इन जीनों की सापेक्ष अभिव्यक्ति का अध्ययन क्यूआरटी-पीसीआर जीन अभिव्यक्ति विश्लेषण के माध्यम से नियंत्रण और सूखे तनाव के साथ मॉक और मेलाटोनिन उपचार के साथ किया गया था। यह देखा गया कि सूखे की स्थिति के तहत बाहरी मेलाटोनिन अनुप्रयोग से ट्रान्सजेनिक और गैर-ट्रान्सजेनिक लाइनों में ओएसएनएसी 2, ओएसएबीएसी 2, ओएसएबीएफ 2, ओएसएलए 3 की सापेक्ष अभिव्यक्ति को कम किया है, जबकि ओएसएनएसी 10, एबीएआरसिक्सआरआई लाइन में काफी कमी आई है, जो गैर-ट्रान्सजेनिक लाइन में मामूली कमी आई है और एबीएआरसिक्सओक्स में काफी वृद्धि हुई है। मेलाटोनिन अनुप्रयोग द्वारा एबीए ट्रान्सक्रिप्शन फैक्टर में कमी हुई और सूखे सहिष्णुता को प्राप्त करने के बाद यह पता चलता है कि मेलाटोनिन, एबीए स्वतंत्र मार्ग के माध्यम से काम करता है। इसी प्रकार यह देखा गया था कि बाहरी मेलाटोनिन अनुप्रयोग से ओएसडीआरबी 1 बी, ओएसडीआरबी 1 एफ, ओएसडीआरबी 2 ए, और ओएसडीआरबी 2 बी की सापेक्ष अभिव्यक्ति में काफी वृद्धि की है जो प्रोलीन, घुलनशील शर्करा (सुक्रोज और रैफिनोज, और मेटाबोलाइट्स (गैलेक्टिनोल और मायोइनोजिटोल) जैसे ओस्मोप्रोटेक्टेंट्स के ऊंचे स्तर से सकारात्मक रूप से सहसंबंधित है। इस प्रकार सूखे तनाव सहनशीलता और पौधों में पत्ती के पानी की स्थिति में सुधार देखा गया। यह भी देखा गया कि सूखे की स्थिति के तहत ट्रान्सजेनिक लाइन में साइटोकिनिन बायोसिंथेसिस जीन ओएसआईपीटी 7, ओएसआईपीटी 9, ओएसएलजीएल 1 और ओएसएलजीएल 2 की सापेक्ष अभिव्यक्ति बढ़ी जिसके परिणामस्वरूप तनाव की स्थिति के तहत क्लोरोफिल मात्रा, प्रकाश संश्लेषण प्रदर्शन और एंटी-ऑक्सीडेंट गतिविधि रखरखाव के साथ सकारात्मक रूप से सहसंबंधित था। इन परिणामों से पता चलता है कि मेलाटोनिन सीबीएफ/डीआरईबीएस ट्रान्सक्रिप्शन कारकों और आगे की डाउन स्ट्रीम प्रतिक्रियाशील जीन को भी अपग्रेड करके एबीए स्वतंत्र मार्ग के माध्यम से सूखा सहनशीलता को बढ़ावा देता है। साथ में, परिणाम यह भी दर्शाते हैं कि ओएसडीआरईबी-मध्यस्थ संकेत पथ धान में आंशिक रूप से मेलाटोनिन-मध्यस्थ सूखे तनाव प्रतिक्रिया में शामिल हो सकता है।

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APPENDIX

A. ANOVA table

	Relative water content (%)					PSII			
Source of	DF	SS	MS	F. cal	Sig.	SS	MS	F. cal	Sig.
treatment	11	5047.01	458.819	36.293	< 0.0001	0.104	0.009	16.551	< 0.0001
error	24	303.412	12.642			0.014	0.001		
Total	35	5350.42				0.118			
	Transpiration					Photosynthesis			
Source of	DF	SS	MS	F. cal	Sig.	SS	MS	F. cal	Sig.
treatment	11	79.370	7.215	10.952	< 0.0001	779.878	70.898	24.458	< 0.0001
error	24	15.812	0.659			69.572	2.899		
Total	35	95.183				849.450			
	Stomatal Conductance					Chlorophyll a			
Source of	DF	SS	MS	F. cal	Sig.	SS	MS	F. cal	Sig.
treatment	11	0.556	0.051	14.665	< 0.0001	56.983	5.180	3.833	0.003
error	24	0.083	0.003			32.436	1.351		
Total	35	0.639				89.419			
	Chlorophyll b					Total chlorophyll			
Source of	DF	SS	MS	F. cal	Sig.	SS	MS	F. cal	Sig.
treatment	11	2.991	0.272	6.127	0.000	70.623	6.420	3.578	0.004
error	24	1.065	0.044			43.071	1.795		
Total	35	4.056				113.694			
	Total carotenoids					ABA content			
Source of	DF	SS	MS	F. cal	Sig.	SS	MS	F. cal	Sig.
treatment	11	168.244	15.295	3.502	0.005	188621.2	17147.4	34.909	< 0.0001
error	24	104.830	4.368			11788.9	491.204		
Total	35	273.075				200410.1			
	leaf melatonin content					Catalase			
Source of	DF	SS	MS	F. cal	Sig.	SS	MS	F. cal	Sig.
treatment	11	41818.1	3801.6	62.261	< 0.0001	1546794.4	140617.7	37.072	< 0.0001
error	24	1465.4	61.060			91033.410	3793.1		
Total	35	43283.5				1637827.8			
	SOD					Peroxidase			
Source of	DF	SS	MS	F. cal	Sig.	SS	MS	F. cal	Sig.
treatment	11	5.545	0.504	61.930	< 0.0001	0.375	0.034	103.427	< 0.0001
error	24	0.195	0.008			0.008	0.000		
Total	35	5.740				0.382			
	internal CO ₂								
Source of	DF	SS	MS	F. cal					
treatment	62318.5	5665.3	4.953	0.001					
error	27452.2	1143.8							
Total	89770.7								