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Research article

Melatonin application reduces fluoride uptake and toxicity in rice seedlings by altering abscisic acid, gibberellin, auxin and antioxidant homeostasis

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ABSTRACT

The manuscript presents an elaborate report on the ameliorative effects of exogenous melatonin in soil-grown seedlings of the rice variety, IR-64 subjected to prolonged fluoride stress. Exogenous melatonin stimulated the physiological growth of the stressed seedlings by triggering high accumulation of gibberellic acid (GA) and melatonin via up regulation of the biosynthetic genes like *GA3ox*, *TDC*, *SNAT* and *ASMT*. The endogenous abscisic acid (ABA) content increased via induction of *NCED3* and suppression of *ABA8ox1*. However, the ABA-dependent genes like *TRAB1*, *WRKY71* and *OSBZ8* were down regulated in presence of high endogenous GA and melatonin. High melatonin level led to low indole-3-acetic acid accumulation in the treated seedlings during fluoride stress. Melatonin significantly decreased fluoride bioaccumulation by suppressing its uptake via CLC1 and CLC2, and also restored *P–H$^+$/ATPase* expression. The damage indices like chlorosis (accompanied by low *RuBisCo*), malondialdehyde, electrolyte leakage, methylglyoxal (detoxified by glyoxalase II) and protein carbonylation were greatly reduced. Increased proline synthesis, activation of the ascorbate-glutathione cycle and enhanced activity of glutathione peroxidase, catalase and guaiacol peroxidase led to low ROS accumulation and localization in the melatonin-treated plants exposed to stress. Overall, melatonin treatment alleviated fluoride-mediated injuries by restricting fluoride uptake, refining the defence machinery and altering the phytohormone homeostasis.

1. Introduction

The toxic element, fluorine is the 13th most abundant element found in the earth's crust. Ingestion of fluoride (F^-) beyond 1.5 mg L^{-1} is restricted by the World Health Organization (WHO), since it causes serious biohazard, characterized by irreversible fluorosis and neurological symptoms (Banerjee and Roychoudhury, 2019a). Rice is the staple food crop of South East Asia. Rice irrigation in India and Bangladesh (two of the largest producers) is usually carried out using groundwater from deep-bored pipelines. Uncontrolled anthropogenic activity and subsided groundwater level lead to large quantities of F^- being extracted from the mineral bed along with groundwater (Hong et al., 2016). As a result, stretches of cultivable lands across West Bengal and Bihar in India, along with Bangladesh and Pakistan are currently experiencing acute endemic fluorosis (Meenakshi and Maheshwari, 2006).

In our previous study, we showed that the mega-yielding indica rice cultivar, IR-64 grown under hydroponic conditions and exposed to F^- concentration of 25 mg L^{-1} for 20 days exhibited high bioaccumulation of F^- (Banerjee et al., 2019a), indicating at a potential biohazard for the consumers. The passive uptake of F^- ions in rice tissues through the chloride channels, viz., CLC1 and CLC2 has been suggested (Banerjee et al., 2019a; Nakamura et al., 2006). The F^- stress increased the expression of *plasma membrane-H$^+$/ATPase* (*P–H$^+$/ATPase*) due to altered ionic homeostasis (Banerjee et al., 2019a).

The F^- acts as an 'accumulative poison' and triggers uncontrolled production of reactive oxygen species (ROS) (Gupta et al., 2009). Such oxidative stress stimulates chlorophyll (Chl) degradation and inhibition of the photosynthetic efficiency by suppressing the CO_2-fixing enzyme, ribulose-1, 5-bispohosphate carboxylase/oxygenase (RuBisCo) (Banerjee and Roychoudhury, 2018). Excess ROS promotes lipid peroxidation and malondialdehyde (MDA) production, stimulates electrolyte leakage (EL) (Che-Othman et al., 2017) and irreversible carbonylation of proteins (Basu et al., 2012). Plant necrosis is triggered by the accumulation of methylglyoxal (MG), the cytotoxic by-product of glycolysis (Banerjee et al., 2019b). Activation of the glyoxalase (Gly) synthesising genes enables efficient detoxification of MG under suboptimal conditions (Ghosh et al., 2014). In order to tackle oxidative stress, plants recruit elaborate antioxidant machinery comprising of osmolytes like proline (Pro) and the components of ascorbate-

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glutathione (AsA-GSH) cycle (Gill and Tuteja, 2010). A high ratio of reduced glutathione (GSH): oxidized glutathione (GSSG) determines the cellular ability of the system to tolerate stress (Foyer and Noctor, 2011). The F^- stress strongly inhibited the enzymes of the AsA-GSH cycle, viz., monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR) and glutathione peroxidase (GPX) in IR-64 seedlings (Banerjee and Roychoudhury, 2019a, 2019b). The antioxidant enzymes like superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and guaiacol peroxidase (GPOX) also regulate oxidative stress in rice (Banerjee and Roychoudhury, 2018).

Melatonin (Mel) is an auxinic antioxidant biomolecule with appreciable ameliorative roles against abiotic stresses like salinity, drought and extreme temperature (Li et al., 2019a). GSH is considered to be one of the most potential antioxidants within the plant cell since it actively maintains the reducing environment and the cellular redox state. Mel has been regarded to be a more potent antioxidant and protective agent compared to even GSH (Poeggeler et al., 2002). The intrinsic nature of Mel to act as a nodal antioxidant upon exogenous application to plants has been recently shown in several plant species and under different types of sub-optimal conditions (Su et al., 2019; Qiu et al., 2019; Huang et al., 2019; Siddiqui et al., 2019). Abscisic acid (ABA) is the universal stress phytohormone, gibberellic acid (GA) is the growth promoter in plants and indole-3-acetic acid (IAA) is the most abundant auxin. The functions of Mel in regulating the homeostasis of these phytohormones during F^- stress in rice are uncharacterized. Mel biosynthesis is regulated by tryptophan decarboxylase (TDC), serotonin-N-acetyltransferase (SNAT) and acetylserotonin-O-methyltransferase (ASMT) (Li et al., 2012). The rate limiting enzyme, 9-cis-epoxycarotenoid dioxygenase 3 (NCED3), and the catabolic enzyme ABA-8-oxidase 1 (ABA8ox1) primarily determines ABA homeostasis (Roychoudhury et al., 2013). The ABA-mediated downstream signaling is activated during stress by the ABA-dependent transcription factors encoded by *Oryza sativa basic leucine zipper 8* (*OSBZ8*) [the master regulatory *trans*-acting factor], *TRAB1* and *WRKY71* (Roychoudhury et al., 2013). Gibberellin-3-oxidase (GA3ox) is the rate limiting enzyme for synthesising GAs in plants (Israelsson et al., 2004).

The aim of this study was to examine the efficacy of exogenous application of Mel in regulating rice physiology during fluoride stress. Exogenous treatment of protective chemical agents to the susceptible plants for generating tolerance is an economic and effective strategy as compared to traditional breeding programs which yield limited success and is time consuming (Paul et al., 2017). The manuscript exhaustively elucidates the roles of Mel in (i) influencing the ABA-GA-IAA homeostasis and ABA-mediated signaling; (ii) regulating F^- uptake and bioaccumulation; and (iii) activating the antioxidant machinery to abrogate systemic injuries.

2. Materials and methods

2.1. Plant materials, growth conditions and stress treatment

Freshly harvested seeds of *Oryza sativa* L. cv. IR-64 were collected from Chinsurah Rice Research Station (West Bengal, India). The seeds were washed with distilled water and sowed in containers filled with soil (containing 1.4% nitrogen, 1.4% phosphorus, 1.4% potash, 4% calcium, 0.7% magnesium, 1% sulphur, 60% moisture, 15% cellulose, 10% lignin, carbon: nitrogen ratio as 25: 1 and near neutral pH). Four sets of samples were maintained:

Set 1: Seeds grown in double distilled water for total 20 days, i.e., Control (Cont)
Set 2: Seeds grown in 25 mg L^{-1} NaF for total 20 days (NaF)
Set 3: Seeds grown in 20 μM Mel for total 20 days (Mel)
Set 4: Seeds grown in 25 mg L^{-1} NaF along with 20 μM Mel for total 20 days (Mel + NaF)

The containers were kept in dark at room temperature (25 °C) till germination. After germination, the pots were maintained under normal sunlight and photoperiod, and the seedlings were harvested after 20 days. Prior to the selection of the actual Mel concentration, we used 10, 20 and 30 μM Mel to test their efficacy in overcoming F^- toxicity in rice seedlings grown under hydroponic conditions. It was found that all the selected concentrations of Mel improved seedling growth under F^- stress. However, only at 20 μM Mel concentration, both the control and stressed seedlings showed significant phenotypic improvement compared to the other two concentrations of Mel (supplementary material). Our observation was in line with Liang et al. (2015), who showed that exogenous application of 20 μM Mel alleviated salt-induced injuries and delayed senescence in rice. Hence, 20 μM Mel was selected as the optimum Mel concentration for mitigating F^--induced injuries in the soil-grown seedlings.

2.2. Estimation of seedling biomass, measurement of root and shoot length, relative water content (RWC) and electrolyte leakage (EL)

Fresh weight (FW), dry weight (DW), and root and shoot length were measured from 50 seedlings from each set according to Banerjee and Roychoudhury (2019b). RWC was calculated as [{(FW-DW)/(turgid weight-DW)} × 100] following Barr and Weatherley (1962). The electrolyte leakage was measured using a conductivity meter (Digital Instruments Corporation, India) as described by Campos et al. (2003).

2.3. Estimation of F^- bioaccumulation and Mel, GA, IAA and ABA contents in rice tissues

0.2 g each of shoot and root was homogenised in TISAB buffer and mineralized after which the F^- content was measured using a F^--sensitive electrode (Cole-Palmer, USA) (Banerjee et al., 2019a). Mel was extracted from the rice tissues following the protocol described by Padumanonda et al. (2014), stored in 80% methanol-water solution and subsequently quantified using enzyme-linked immunosorbent assay (ELISA) at 405 nm (Padumanonda et al., 2014). GA was quantified colorimetrically following the technique described by Graham and Thomas (1961). IAA was extracted in NaH_2PO_4–NaOH buffer and quantified by first derivation synchronous fluorescence spectroscopy as described by Liu and Wan (2013). For ABA quantification, the tissues were homogenised in 20 mM potassium phosphate buffer (pH 7.4) and the hormone content was determined through competitive ELISA using ABA immunoassay kit as previously standardized (Banerjee and Roychoudhury, 2019b).

2.4. Estimation of chlorophyll (Chl), methylglyoxal (MG), protein carbonylation, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) content and ROS localization

Chl was extracted in 80% (v/v) chilled acetone according to Arnon (1949). MG content was measured on the basis of utilization of N-acetyl-L-cysteine at 288 nm following Banerjee et al. (2019b). Protein carbonyl content was measured using dinitrophenyl hydrazine following Banerjee et al. (2019a). MDA content was calculated according to Banerjee and Roychoudhury (2019b) using 155 mM^{-1} cm^{-1} as the molar extinction coefficient. H_2O_2 was spectrophotometrically measured at 390 nm and the content was estimated from a standard curve (Velikova et al., 2000). ROS localization was performed by incubating freshly harvested leaves in dichlorodihydrofluorescein-diacetate-acetyl-ester and subsequent imaging using an epi-fluorescence microscope (Olympus) following Kristiansen et al. (2009).

2.5. Estimation of Pro, AsA and GSH redox state

Free Pro content was assayed spectrophotometrically using freshly

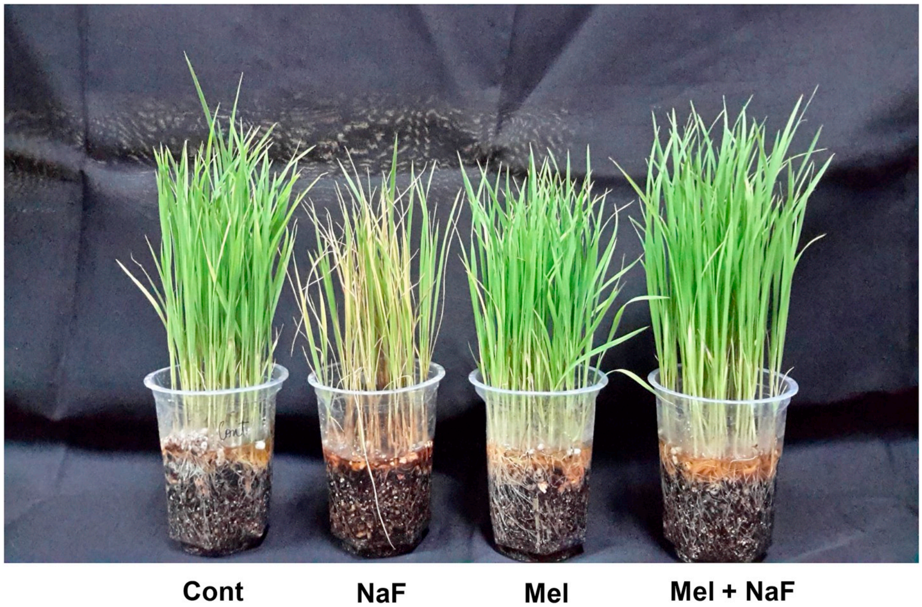

Fig. 1. Visual assessment of growth performance of IR-64 seedlings grown with water (Cont), 25 mg L^{-1} NaF (NaF), 20 μM Mel (Mel), and 25 mg L^{-1} NaF plus 20 μM Mel (Mel + NaF).

Table 1
Assessment of basic physiological parameters in IR-64 seedlings grown with water (Cont), 25 mg L^{-1} NaF (NaF), 20 μM Mel (Mel) and 25 mg L^{-1} NaF plus 20 μM Mel (Mel + NaF). The data are the mean values (n = 3) ± standard error (SE). The SE ($p \leq 0.05$) in each case is represented as '*' (for comparison within treatments). The symbols represent significance at $p \leq 0.05$.

Parameters	Cont	NaF	Mel	Mel + NaF
Shoot length (cm)	10.0 ± 1.2	7.9 ± 2.1*	9.6 ± 1.4*	10.5 ± 0.8
Root length (cm)	5.1 ± 0.9	2.8 ± 1.1*	4.9 ± 0.8*	5.4 ± 1.0*
Fresh weight (mg)	81.1 ± 3.4	58.7 ± 4.1*	80.2 ± 3.2*	98.7 ± 2.3*
Dry weight (mg)	12.9 ± 1.8	8.3 ± 0.7*	12.1 ± 1.1*	13.7 ± 2.1
Relative water content	97.8 ± 3.2	72.0 ± 2.1*	94.7 ± 2.9*	98.1 ± 4.5
Electrolyte leakage (%)	17.9 ± 3.1	37.8 ± 3.8*	19.0 ± 2.3*	18.3 ± 2.2

prepared acid ninhydrin following Banerjee and Roychoudhury (2019b). AsA content was spectrophotometrically determined according to Banerjee and Roychoudhury (2019b). GSH and total glutathione was quantified using Ellman's reagent at 412 nm according to Bonifacio et al. (2011) and Moron et al. (1979) respectively. Glutathione redox state was represented as: [(GSH/total glutathione) × 100].

2.6. Analysis of the activity of antioxidative enzymes like SOD (EC 1.15.1.1), CAT (EC 1.11.1.6), GPOX (EC 1.11.1.7), APX (EC 1.11.1.11), MDHAR (EC 1.6.5.4), DHAR (EC 1.8.5.1), GR (EC 1.8.1.7) and GPX (1.11.1.9)

The SOD activity was determined by incubating the extract in a solution containing nitroblue tetrazolium salt, TEMED and riboflavin. 1

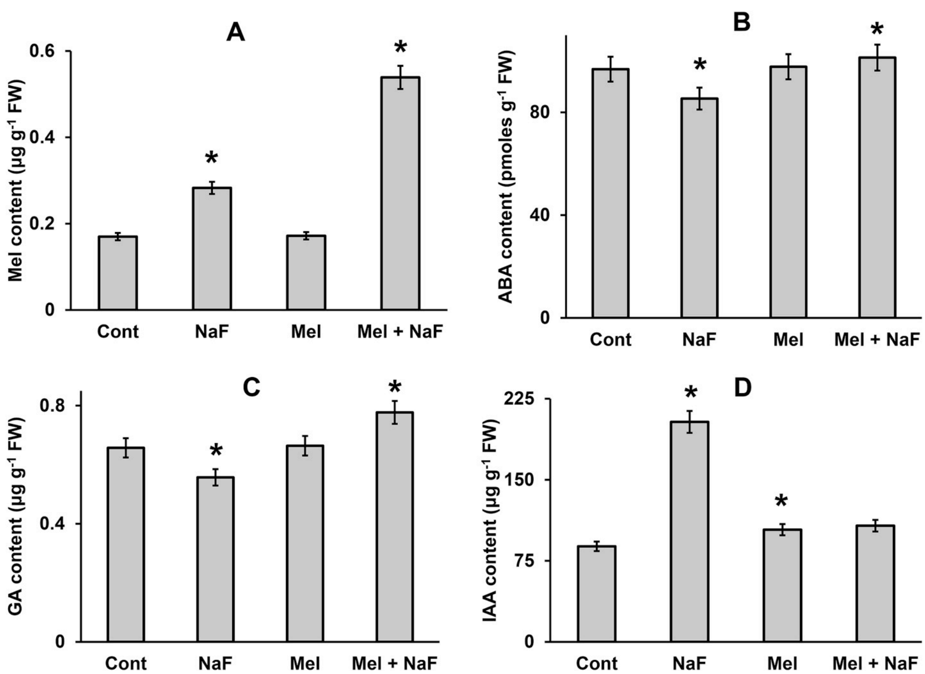

Fig. 2. Endogenous content of Mel (A), ABA (B), GA (C) and IAA (D) in IR-64 seedlings grown with water (Cont), 25 mg L^{-1} NaF (NaF), 20 μM Mel (Mel) and 25 mg L^{-1} NaF plus 20 μM Mel (Mel + NaF). The data are the mean values (n = 3) ± standard error (SE). The SE ($p \leq 0.05$) in each case is represented by the vertical bar in each graph. '*' designated on top of the bars represent significance at $p \leq 0.05$.

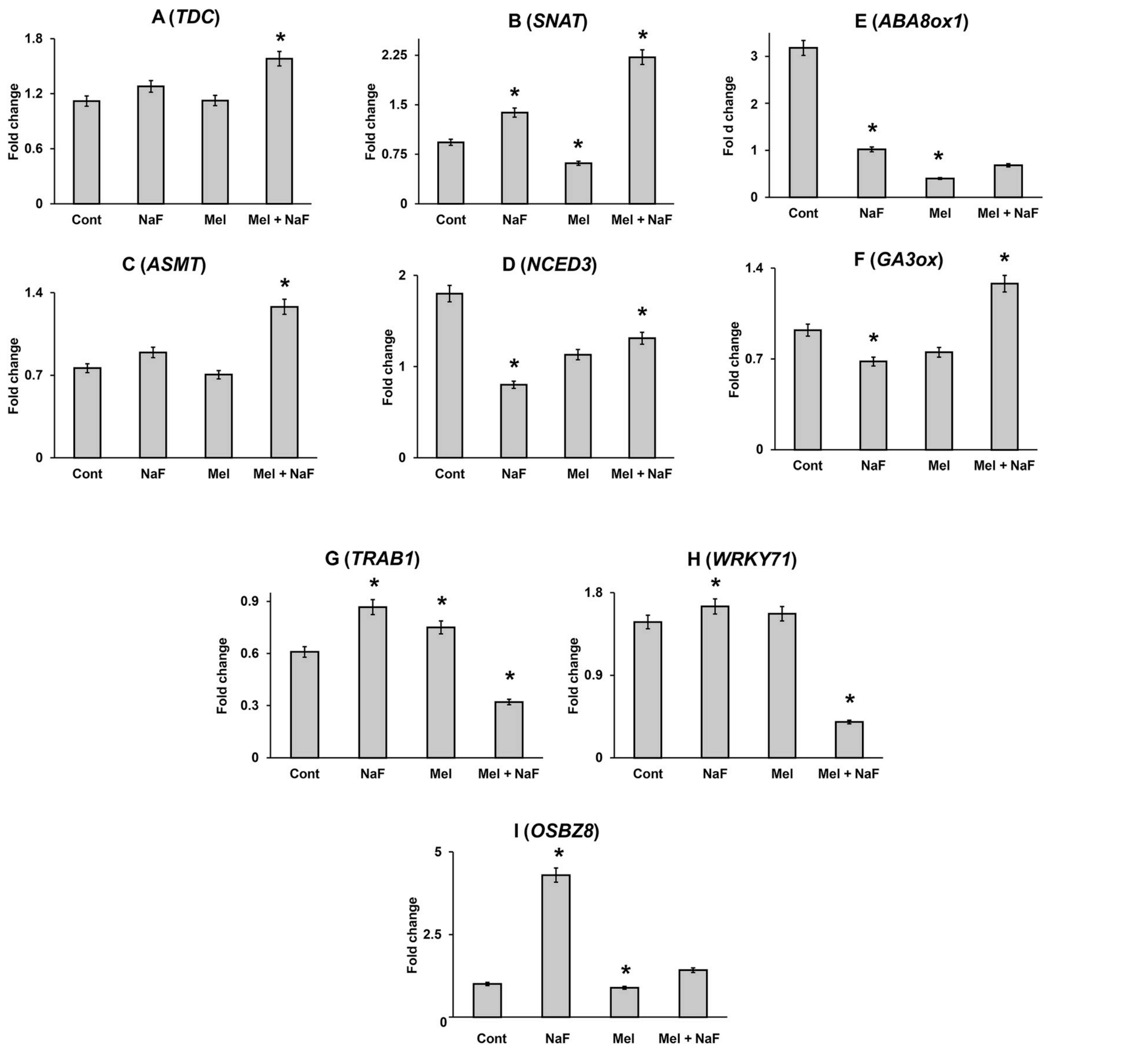

Fig. 3. Transcript level of *TDC* (A), *SNAT* (B), *ASMT* (C), *NCED3* (D), *ABA8ox1* (E), *GA3ox* (F), *TRAB1* (G), *WRKY71* (H) and *OSBZ8* (I) in IR-64 seedlings grown with water (Cont), 25 mg L^{-1} NaF (NaF), 20 µM Mel (Mel) and 25 mg L^{-1} NaF plus 20 µM Mel (Mel + NaF). The data are the mean values ($n = 3$) ± standard error (SE). The SE ($p \leq 0.05$) in each case is represented by the vertical bar in each graph. '*' designated on top of the bars represent significance at $p \leq 0.05$.

U of SOD activity was represented as the amount of enzyme which inhibited 50% of the initial rate of reaction in the absence of enzyme (Alonso et al., 2001). The CAT activity was calculated by assaying the utilization of H_2O_2 at 240 nm using the molar extinction coefficient of 40 $mM^{-1} cm^{-1}$ (Velikova et al., 2000). The GPOX activity was determined according to the extinction coefficient 26.6 $mM^{-1} cm^{-1}$ (Srinivas et al., 1999). The APX activity was calculated based on the oxidation of ascorbate using extinction coefficient of 2.8 $mM^{-1} cm^{-1}$ (Nakano and Asada, 1981). The MDHAR, DHAR and GR activity was estimated following Colville and Smirnoff (2008). The decrease in NADP absorbance was used to assay the GPX activity (Awasthi et al., 1975).

2.7. Expression analysis of genes

Total RNA, isolated from freshly harvested seedlings using RNAisoplus (Takara, Japan), was checked using nanodrop spectrophotometer and treated with DNase I (1U μL^{-1}). Maxima first strand cDNA synthesis kit (Thermo Scientific, USA) was used to reverse transcribe the mRNA. Comparative RT-PCR was performed to monitor gene expression using standard reagents and gene specific primers as previously described by Paul and Roychoudhury (2018).

2.8. Protein estimation and statistical analysis

The total protein content for all enzyme assays was estimated according to Bradford (1976). The experiments were performed in completely randomized design using three replicates (n = 3) with each replication containing an average of 50 seeds. The statistical significance was calculated using one way analysis of variance (ANOVA) at $p \leq 0.05$ in XLSTAT 2018.

3. Results and discussion

3.1. Mel improves physiological growth by altering hormonal homeostasis

The growth of the stressed seedlings (assessed visually) improved upon Mel supplementation (Fig. 1). Exogenous Mel increased the shoot and root length, along with biomass (Table 1) of the stressed seedlings, compared to those in the NaF-treated plants where growth was

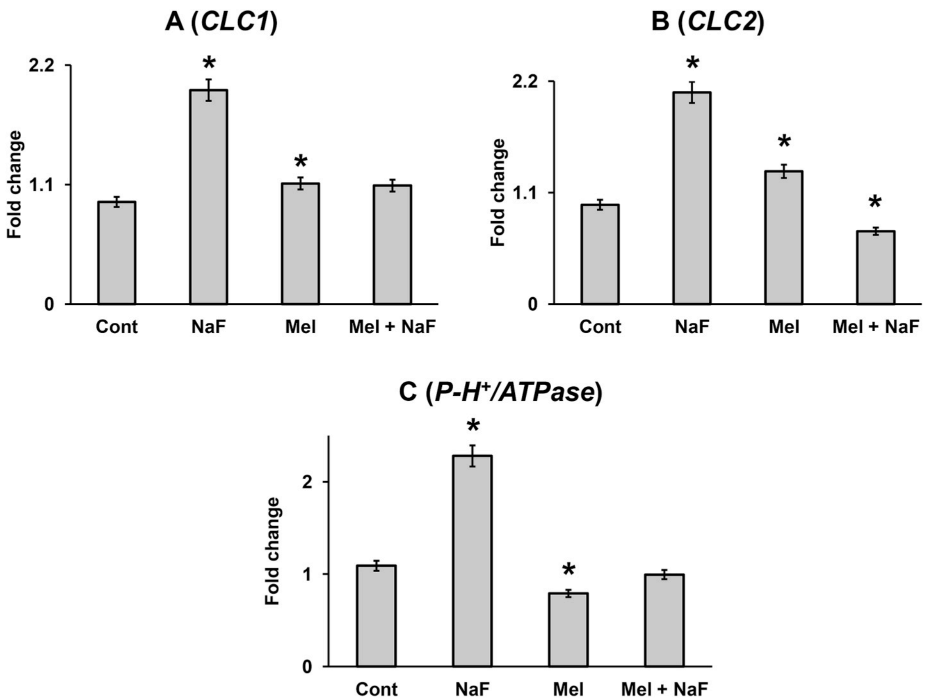

Fig. 4. Transcript level of *CLC1* (A), *CLC2* (B) and *P–H⁺/ATPase* (C) in IR-64 seedlings grown with water (Cont), 25 mg L^{-1} NaF (NaF), 20 µM Mel (Mel) and 25 mg L^{-1} NaF plus 20 µM Mel (Mel + NaF). The data are the mean values ($n = 3$) ± standard error (SE). The SE ($p \leq 0.05$) in each case is represented by the vertical bar in each graph. '*' designated on top of the bars represent significance at $p \leq 0.05$.

Table 2
Assessment of the parameters associated with oxidative injuries in IR-64 seedlings grown with water (Cont), 25 mg L^{-1} NaF (NaF), 20 µM Mel (Mel) and 25 mg L^{-1} NaF plus 20 µM Mel (Mel + NaF). The data are the mean values ($n = 3$) ± standard error (SE). The SE ($p \leq 0.05$) in each case is represented as '*' (for comparison within treatments). The symbols represent significance at $p \leq 0.05$.

Parameters	Cont	NaF	Mel	Mel + NaF
Fluoride accumulation in shoot (mg g^{-1} FW)	0.04 ± 0.02	3.10 ± 0.09*	0.03 ± 0.03*	0.90 ± 0.08*
Fluoride accumulation in root (mg g^{-1} FW)	0.02 ± 0.01	0.81 ± 0.10*	0.02 ± 0.01*	0.45 ± 0.02*
Chlorophyll (µg g^{-1} FW)	40.3 ± 2.1	8.5 ± 1.1*	37.1 ± 3.7*	53.4 ± 4.2*
Malondialdehyde (µM g^{-1} FW)	1.10 ± 0.05	2.10 ± 0.10*	1.20 ± 0.05*	0.9 ± 0.1
H_2O_2 (nmol g^{-1} FW)	6.1 ± 0.9	8.5 ± 0.2*	6.9 ± 1.1*	6.5 ± 0.8
Methylglyoxal (µg g^{-1} FW)	231.7 ± 10.5	471.0 ± 13.5*	268.6 ± 11.9*	44.8 ± 7.8*
Protein carbonylation (mol carbonyl mol^{-1} BSA)	1.50 ± 0.04	2.70 ± 0.10*	1.40 ± 0.05*	1.80 ± 0.07

significantly retarded. The stressed sets treated with Mel showed an increase in RWC by 1.4-fold and suppression of EL by 2.1-fold compared to the F^--stressed seedlings (Table 1). Such Mel-mediated surge in physiological growth has been reported in *Cucumis sativus* and *Malus hupehensis* seedlings which exhibited normal development even under salt stress (Nawaz et al., 2016; Li et al., 2012).

The F^- stress triggered the endogenous accumulation of Mel by inducing the expression of the Mel-biosynthetic genes, viz., *TDC*, *SNAT* and *ASMT*, compared to control. However, Mel application during stress elevated the endogenous Mel level by about 2.0-fold compared to the stressed seedlings by further up regulating the associated anabolic genes (Figs. 2A and 3A, B, C). ABA content decreased during F^- stress due to suppression of the rate limiting enzyme, viz., *NCED3* (Figs. 2B and 3D). This was in line with our previous observation for F^--stressed IR-64 (Banerjee and Roychoudhury, 2019b). Zhang et al. (2014) reported that application of Mel decreased ABA accumulation in salt-stressed cucumber seedlings. Reduced ABA content was also found in the Mel-treated perennial ryegrass exposed to heat stress (Zhang et al., 2017). However, Fu et al. (2017) and Li et al. (2016) reported increased ABA accumulation in Mel-treated Chinese ryegrass and barley exposed to cold stress. We found that exogenous Mel stimulated endogenous ABA accumulation during F^- stress by activating *NCED3* and suppressing the ABA-catabolic gene, viz., *ABA8ox1* with respect to those in the NaF-treated sets (Figs. 2B and 3D, E). However, the ABA content in the Mel- and NaF-treated seedlings was close to that in the control plants. Thus, Mel variably affects ABA synthesis during different types of abiotic stresses. GA synthesis was inhibited during F^- stress due to down regulation of *GA3ox* compared to that in control seedlings. Exogenous Mel significantly triggered *GA3ox* expression and GA content during F^- stress (Figs. 2C and 3F) which eventually accounted for the stimulated seedling growth and shoot elongation even under sub-optimal conditions. It is already known that Mel stimulates GA production and acts synergistically in plants to enhance seedling growth (Zhang et al., 2014). Interestingly, IAA content increased by about 2.5-fold in the NaF-treated seedlings compared to control plants. However, upon Mel application, the IAA content was restored to a level close to that in the control plants (Fig. 2D). This might be due to the fact that both Mel and IAA use tryptamine as the common precursor (Banerjee and Roychoudhury, 2017). Hence, IAA content subsided upon Mel treatment since the stressed seedlings possibly channelized more tryptamine towards the production of Mel, the more potent antioxidant compared to IAA. Arnao and Hernandez-Ruiz (2018) reported that Mel closely mimics the activity of auxins. Thus, the growth-promoting effects of IAA were also executed by Mel in the Mel- and F^--treated seedlings.

The expression of ABA-dependent genes, viz., *TRAB1*, *WRKY71* and *OSBZ8* significantly increased during F^- stress, but were greatly suppressed in the Mel- and NaF-treated plants (Fig. 3G, H, I). The elevated expression of these genes in spite of reduced ABA content has been accredited to the slow degradation and conservation of the hormone due to down regulated *ABA8ox1* (Fig. 3E) (Banerjee and Roychoudhury, 2019b). However, the suppression of the *TRAB1*, *WRKY71* and *OSBZ8* genes in spite of higher ABA and down regulated

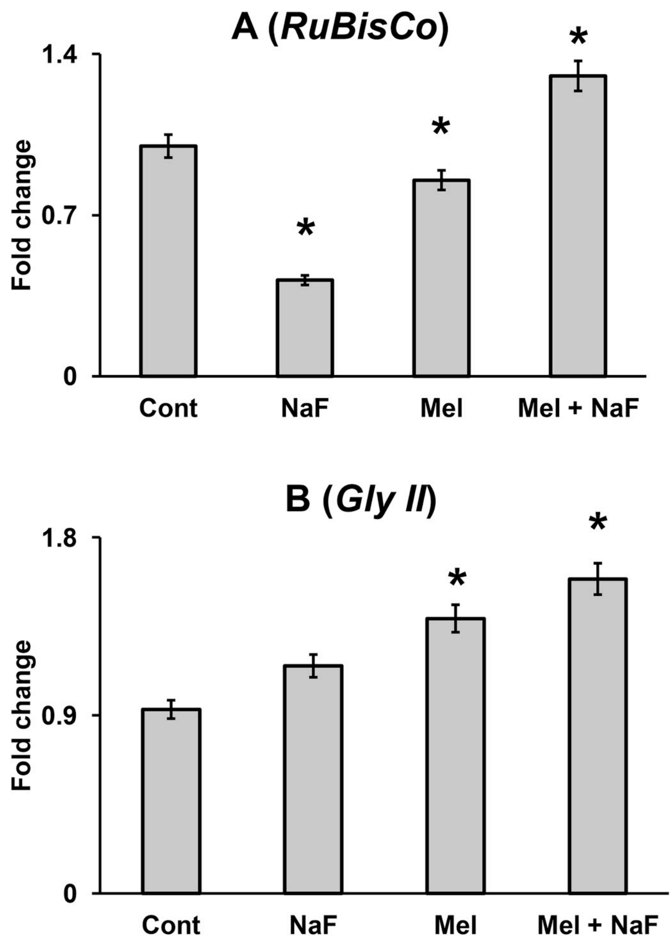

Fig. 5. Transcript level of *RuBisCo* (A) and *Gly II* (B) in IR-64 seedlings grown with water (Cont), 25 mg L^{-1} NaF (NaF), 20 μM Mel (Mel) and 25 mg L^{-1} NaF plus 20 μM Mel (Mel + NaF). The data are the mean values (n = 3) ± standard error (SE). The SE ($p \leq 0.05$) in each case is represented by the vertical bar in each graph. '*' designated on top of the bars represent significance at $p \leq 0.05$.

ABA8ox1 in the Mel- and F^--treated seedlings might be due to the Mel- and GA-mediated antagonistic regulation of the ABA-dependent pathway, as has been previously reported (Debnath et al., 2019). Activation of these ABA-dependent genes during F^- stress might be due to low GA accumulation (Fig. 2C). However, upon Mel treatment, the significant increase in GA and Mel content in the stressed seedlings imposed a negative regulation on such ABA-dependent genes in spite of higher endogenous ABA level than the NaF-treated plants. The antagonistic regulation between GA and ABA has been reported across several plant species and in various developmental stages (Liu and Hou, 2018). As a result, expression of the rate limiting gene *NCED3* was maintained at a much lower level in the Mel- and NaF-treated seedlings than that in the control plants.

3.2. Mel reduces F^- uptake and bioaccumulation

Exogenous Mel significantly suppressed the expression of both *CLC1* and *CLC2* during F^- stress (Fig. 4A and B) which significantly reduced F^- uptake and accumulation by 3.4-fold in the shoot and 1.8-fold in the root compared to that in the NaF-treated plants (Table 2). Yadu et al. (2018) reported reduced F^- bioaccumulation in *Cajanus cajan* seedlings treated with Mel and NaF for five days. Mel treatment also restored the *P–H^+/ATPase* expression (which was strongly induced during NaF stress) close to that in the control (Fig. 4C), thus showing the maintenance of normal proton homeostasis even under stressed situations.

3.3. Mel ameliorates F^--induced injuries

Prolonged F^- stress triggers chlorosis and generation of ROS (Banerjee et al., 2019a). The Chl content was reduced by 4.7-fold during F^- stress due to strong inhibition of *RuBisCo* (Table 2, Fig. 5A). Exogenous Mel induced *RuBisCo* expression by 3.1-fold and increased the Chl content by 6.2-fold during stress compared to that in the NaF-treated seedlings (Table 2, Fig. 5A). The Mel-mediated activation of the stalled photosynthetic machinery has been reported in *Ulva* sp., *Chara australis* and *Cynodon dactylon* during salt stress (Li et al., 2019a). Alternatively, the efficient revival of the photosynthetic machinery in the Mel- and NaF-treated seedlings might also be due to the Mel-dependent down regulation of the *chloride channels* (*CLC1* and *CLC2*) and reduction in the F^- uptake, ultimately resulting in reduced F^--toxicity.

The F^- accumulation imposes oxidative stress on the cells and triggers uncontrolled production of ROS, which leads to lipid peroxidation in membranes and generation of by-products like MDA (Banerjee and Roychoudhury, 2018). NaF treatment stimulated H_2O_2 and MDA accumulation in rice seedlings (Table 2). Fluorescence imaging revealed severe accumulation of ROS in the leaves of the F^--stressed seedlings (Fig. 6). However, Mel treatment decreased ROS localization (Fig. 6) and reduced H_2O_2 and MDA production by 1.3-fold and 2.3-fold respectively in the stressed plants compared to the NaF-treated seedlings (Table 2). Maintenance of significantly higher *Gly II* transcript level enabled the Mel- and F^--treated seedlings to reduce the cytotoxic MG accumulation significantly by 10.5-fold compared to that of the stressed plants (Fig. 5B, Table 2). In line with our observation, Li et al. (2019b) reported efficient MG detoxification in Mel-treated, heat-stressed maize. Gly II is regarded as the chief MG detoxifying enzyme in rice during salt stress (Ghosh et al., 2014). Debska et al. (2012) showed that unregulated production of ROS carbonylates arginine, lysine, threonine and Pro residues in proteins. Due to efficient ROS scavenging by Mel, the protein carbonylation was reduced by 1.5-fold in the Mel and NaF-treated plants compared to the F^- stressed seedlings (Table 2). There is no report on the effects of Mel on protein carbonylation in plants.

3.4. Mel activates the antioxidant machinery during stress

Exogenous Mel stimulated higher Pro accumulation in the stressed plants compared to that in the NaF-treated seedlings (Table 3). The compatible solute, Pro is also involved in mediating post-stress recovery and preserving stress memory (Roychoudhury et al., 2015). Sarropoulou et al. (2012) reported increased Pro accumulation in the melatonin-treated cherry rootstocks. Mel application activated the inhibited AsA-GSH cycle during F^- stress. Such Mel-mediated regulation of this crucial antioxidant cycle has been observed in apple leaves (Wang et al., 2012). Exogenous Mel channelized AsA towards the AsA-GSH cycle during stress and hence accumulated lower level of this antioxidant compared to that in the NaF-treated plants (Table 3). The stressed seedlings treated with Mel exhibited 2.7-, 1.7- and 3.4-fold increases in MDHAR, DHAR and GR activity respectively leading to the maintenance of 2.3-fold higher GSH redox state compared to the NaF-treated plants (Table 3). The expression of the respective genes was also higher in the Mel- and NaF-treated seedlings compared to the F^--stressed plants (Fig. 7A, B, C). The higher level of GSH enabled the Mel- and F^--treated seedlings to maintain higher *GPX* expression and enzyme activity compared to the F^--stressed plants (Fig. 7D, Table 3). GSH is the chief reducing equivalent within the plant cell and is also associated with MG detoxification (Hasanuzzaman et al., 2017). The high GSH/GSSG ratio helped the Mel-treated seedlings to maintain cellular osmoticum and low MG content even under prolonged F^- stress.

The SOD activity and *SOD* gene expression increased significantly during F^- stress. Interestingly, we observed that application of Mel during stress restored the SOD activity and gene expression close to that

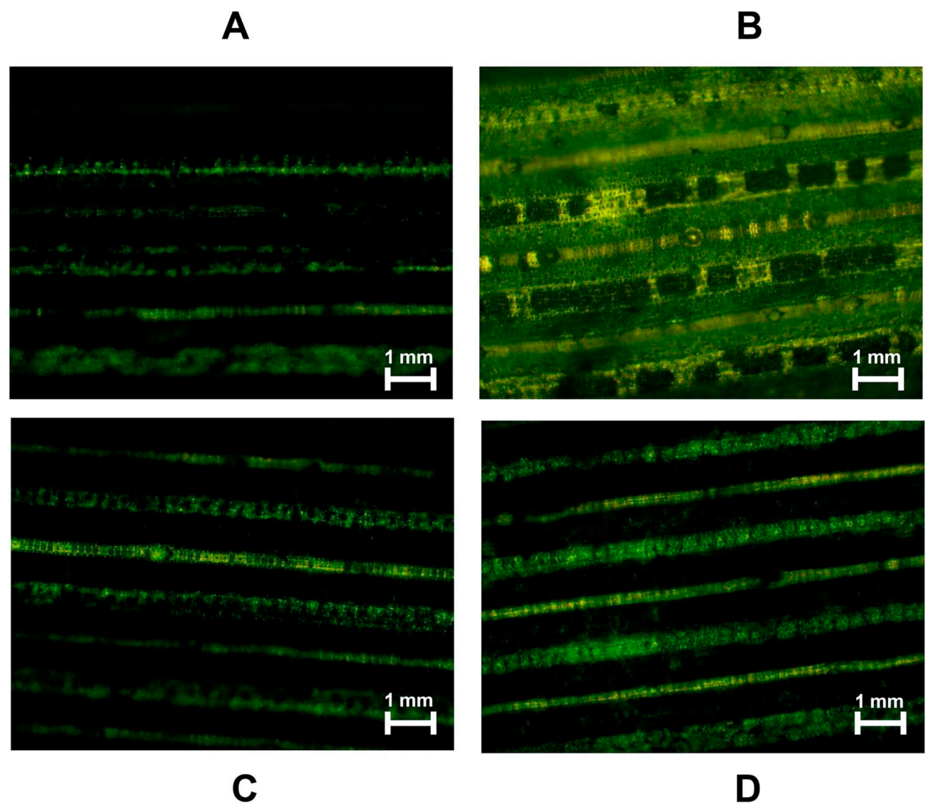

Fig. 6. Fluorescence imaging of ROS localization in the leaves of IR-64 seedlings grown with water (A), 25 mg L^{-1} NaF (B), 20 μM Mel (C) and 25 mg L^{-1} NaF plus 20 μM Mel (D). Scale bar within the figures represents 1 mm length.

Table 3
Assessment of the endogenous content of osmolyte and antioxidants in IR-64 seedlings grown with double distilled water (Cont), 25 mg L^{-1} NaF (NaF), 20 μM Mel (Mel) and 25 mg L^{-1} NaF plus 20 μM Mel (Mel + NaF). The data are the mean values ($n = 3$) ± standard error (SE). The SE ($p \le 0.05$) in each case is represented as '*' (for comparison within treatments). The symbols represent significance at $p \le 0.05$.

	Parameters	Cont	NaF	Mel	Mel + NaF
Osmolyte	Proline (μg g^{-1} FW)	11.1 ± 2.1	17.1 ± 2.3*	8.6 ± 1.0 *	19.9 ± 1.2*
Non-enzymatic antioxidants	Ascorbic acid (mg g^{-1} FW)	2.0 ± 1.1	4.5 ± 1.6*	2.9 ± 1.7*	2.5 ± 0.9
	Glutathione redox state (%)	49.9 ± 2.7	38.1 ± 4.3*	43.0 ± 2.9*	89.5 ± 4.1
Enzymatic antioxidants	MDHAR activity (nmol min^{-1} g^{-1} FW)	0.51 ± 0.03	0.25 ± 0.02*	0.47 ± 0.07*	0.68 ± 0.05*
	DHAR activity (μmol ascorbate produced min^{-1} g^{-1} FW)	0.79 ± 0.04	0.41 ± 0.02*	0.54 ± 0.02*	0.69 ± 0.04*
	GR activity (μmol min^{-1} g^{-1} FW)	17.8 ± 2.5	7.2 ± 1.4*	20.7 ± 3.1*	24.5 ± 2.1*
	GPX activity (μmol min^{-1} g^{-1} FW)	47.8 ± 2.6	31.8 ± 1.7*	50.8 ± 3.1*	59.2 ± 2.6*
	SOD activity (U g^{-1} FW)	0.21 ± 0.02	0.40 ± 0.06*	0.12 ± 0.05*	0.15 ± 0.07
	APX activity (μmol min^{-1} g^{-1} FW)	1.1 ± 0.3	3.0 ± 0.5*	2.3 ± 0.2*	2.3 ± 0.4
	CAT activity (μmol min^{-1} g^{-1} FW)	0.15 ± 0.02	0.32 ± 0.09*	0.17 ± 0.03*	0.48 ± 0.08*
	GPOX activity (μmol min^{-1} g^{-1} FW)	28.5 ± 3.2	38.1 ± 2.8*	30.9 ± 4.3	171.2 ± 5.2*

in the control seedlings (Table 3, Fig. 7E). Similar trend was observed in case of *APX* expression (Fig. 7F). The APX activity was also reduced in the Mel-and F^--treated seedlings compared to the NaF-treated plants, but APX activity in the former was 2.0-fold higher than the control (Table 3). Exogenous Mel led to the efficient scavenging of H_2O_2 during F^- stress by increasing the expression and activity of CAT compared to that in the NaF-treated plants (Fig. 7G, Table 3). The GPOX activity was triggered by 4.5-fold in the stressed seedlings upon Mel treatment compared to that in the NaF-treated plants (Table 3). Lower *APX* expression in the Mel- and F^--treated plants was possibly due to reduced AsA sink, since the metabolite was utilized in the AsA-GSH cycle for GSH synthesis. The possible explanation for low SOD activity in Mel-treated seedlings under stress might be due to efficient ROS scavenging by the antioxidants and most importantly by Mel itself, as a result of which induced *SOD* expression and SOD activity was not of much necessity in presence of Mel, thereby reducing the metabolic burden. Mel has been found to scavenge superoxide radicals and is regarded to be five times more potent than GSH in detoxifying ROS (Poeggeler et al 1996, 2002).

4. Conclusion

Fluoride toxicity negatively affected the growth of IR-64 seedlings. Exogenous Mel significantly improved the growth and physiology of F^--stressed rice seedlings by stimulating the endogenous production of Mel and GA. In spite of higher ABA accumulation, Mel negatively regulated the expression of the major ABA-dependent, osmotic stress-responsive genes. Therefore, this study clearly verified the inter-regulation of the chief plant growth regulators under the influence of Mel. It seemed that the Mel-treated plants countered fluoride stress more through Mel- and GA-dependent signaling rather than using the ABA-dependent pathway. Due to the existence of a common precursor tryptamine, the high endogenous Mel level was accompanied with low IAA accumulation. The Mel-mediated hormonal regulation down

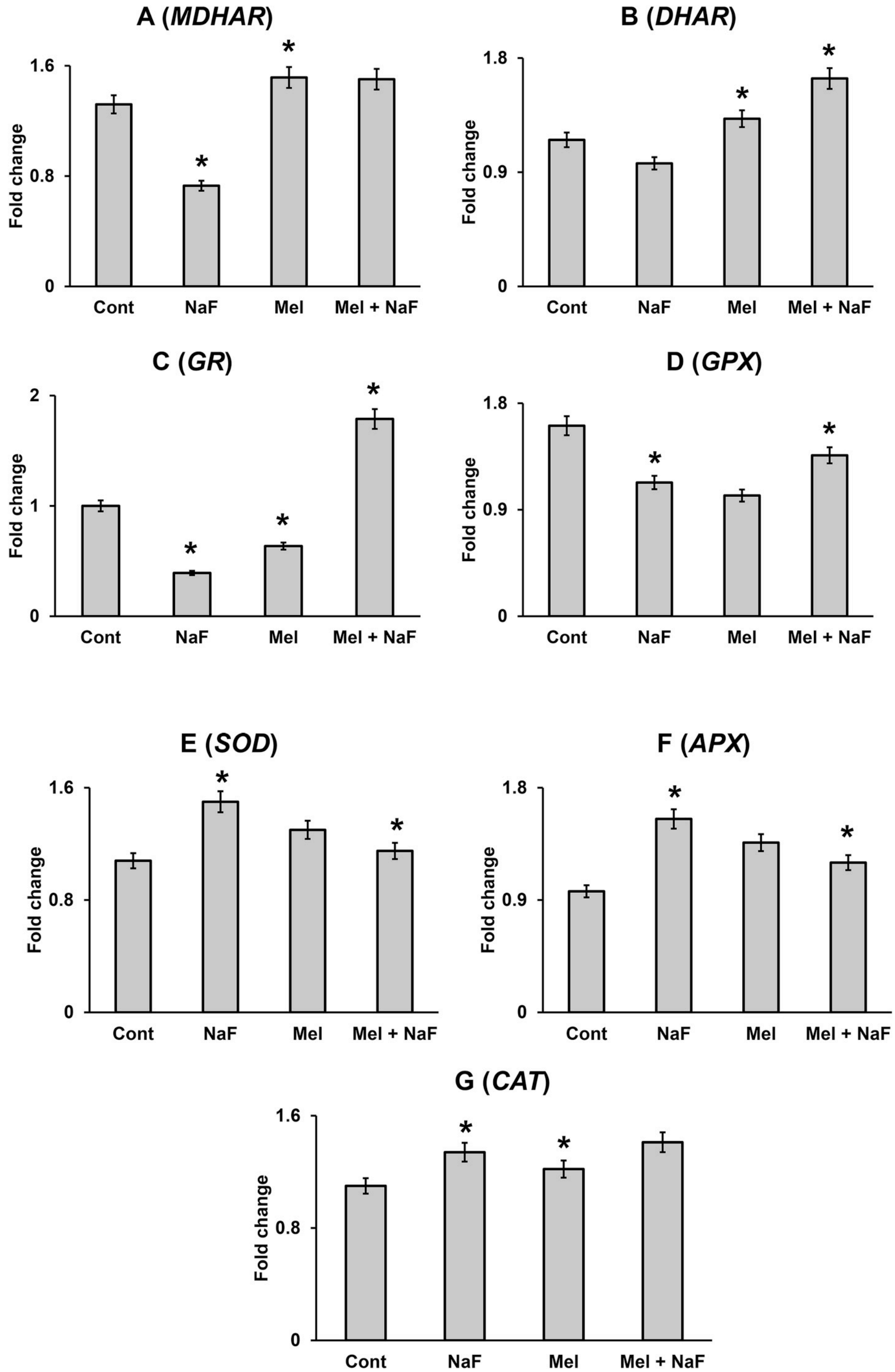

Fig. 7. Transcript level of *MDHAR* (A), *DHAR* (B), *GR* (C), *GPX* (D), *SOD* (E), *APX* (F) and *CAT* (G) in IR-64 seedlings grown with water (Cont), 25 mg L^{-1} NaF (NaF), 20 μM Mel (Mel) and 25 mg L^{-1} NaF plus 20 μM Mel (Mel + NaF). The data are the mean values ($n = 3$) ± standard error (SE). The SE ($p \le 0.05$) in each case is represented by the vertical bar in each graph. '*' designated on top of the bars represent significance at $p \le 0.05$.

regulated the genes encoding chloride channels in the stressed seedlings and also restored the cellular proton homeostasis. Due to low cytosolic F^- level, the plants experienced lower extent of physiological injuries. The major physiological and antioxidant enzymes, together with their corresponding genes were not inhibited. Instead, exogenous Mel activated the overall defence machinery to scavenge and detoxify the excess ROS and MG that were produced under F^- exposure. The study clearly holds environmental significance, since it illustrates the potentiality of Mel as an effective antioxidant, useful in ameliorating the F^--induced injuries in rice plants grown in soil. Apart from elucidating the yet uncharacterized molecular and hormonal interactions operative during Mel and NaF treatments, the results also highlight the basic mechanism of Mel as a protective chemical agent for developing F^--tolerance in rice seedlings through the down regulation of *CLC*s, efficient MG detoxification and activation of the AsA-GSH cycle.

Author contribution statement

AB and ARC designed the experimental plan. AB performed all the experiments, generated data and drafted the manuscript. ARC supervised the entire work, provided critical comments and suggested and incorporated necessary corrections or modifications within the manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interest in publishing this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.10.033.

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