Plant Physiology and Biochemistry 212 (2024) 108779

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Melatonin mediated tolerance to benzalkonium chloride phytotoxicity through improved growth, photochemical reactions, and antioxidant system in wild-type and *snat2* mutant *Arabidopsis* lines

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ARTICLE INFO

Keywords:
Antioxidant system
Arabidopsis
Benzalkonium chloride
Melatonin
Serotonin N-Acetyltransferase

ABSTRACT

Melatonin (Mel) is a phytohormone that plays a crucial role in various plant processes, including stress response. Despite numerous studies on the role of Mel in stress resistance, its significance in plants exposed to benzalkonium chloride (BAC) pollution remains unexplored. BAC, a common antiseptic, poses a threat to terrestrial plants due to its widespread use and inefficient removal, leading to elevated concentrations in the environment. This study investigated the impact of BAC (0.5 mg L^{-1}) pollution on wild-type *Col-0* and *snat2* knockout mutant *Arabidopsis* lines, revealing reduced growth, altered water relations, and gas exchange parameters. On the other hand, exogenous Mel (100 μM) treatments mitigated BAC-induced phytotoxicity and increased the growth rate by 1.8-fold in *Col-0* and 2-fold in *snat2* plants. *snat2* mutant seedlings had a suppressed carbon assimilation rate (A) under normal conditions, but BAC contamination led to further A repression by 71% and 48% in *Col-0* and *snat2* leaves, respectively. However, Mel treatment on stressed plants was successful in improving F_v/F_m and increased the total photosynthesis efficiency by regulating photochemical reactions. Excessive H_2O_2 accumulation in the guard cells of plants exposed to BAC pollution was detected by confocal microscopy. Mel treatments triggered almost all antioxidant enzyme activities (except POX) in both *Arabidopsis* lines under stress. This enhanced antioxidant activity, facilitated by foliar Mel application, contributed to the alleviation of oxidative damage, regulation of photosynthesis reactions, and promotion of plant growth in *Arabidopsis*. In addition to corroborating results observed in many agricultural plants regarding the development of tolerance to environmental stresses, this study provides novel insights into the action mechanisms of Mel under the emerging pollutant benzalkonium chloride.

1. Introduction

Melatonin (Mel, N-acetyl-5-methoxytryptamine) is a phytohormone and a signaling molecule synthesized in chloroplasts and mitochondria of plant cells (Sharma et al., 2024). Mel plays a key role in flowering and circadian rhythm (Lee et al., 2019), regulation of germination and growth (Back, 2021), and formation of the defense response against biotic and abiotic stresses (Arnao and Hernández-Ruiz, 2020; Kakkar, 2023). In plants, Mel can be synthesized by two different pathways. While the formation of serotonin from L-tryptophan proceeds normally, in healthy plants it is acetylated in the chloroplasts via serotonin N-acetyltransferase (SNAT) and then methylated by the activity of N-acetylserotonin methyltransferase (ASMT), completing its conversion to Mel (Back et al., 2016). In plants under stress, serotonin first undergoes a methylation reaction, and then Mel synthesis occurs with SNAT activity (Xie et al., 2022). While these two different biosynthesis metabolisms allow the plant to switch between normal and defense modes, it also reveals the evolutionary signal role of Mel in the formation of the stress response (Gao et al., 2023).

There are two SNAT isogenes in the *Arabidopsis* genome (SNAT1 and SNAT2). In the study performed with *Arabidopsis* SNAT1 and SNAT2 knockout mutants (*snat1* and *snat2*), a significant decrease in Mel levels,

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https://doi.org/10.1016/j.plaphy.2024.108779
Received 23 February 2024; Received in revised form 15 May 2024; Accepted 27 May 2024
Available online 29 May 2024
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especially under stress conditions, and sensitivity to environmental variables were determined compared to wild-type plants (Yang et al., 2021). Mutant *Arabidopsis* lines overexpressing the *Vitis vinifera* SNAT1 gene showed increased Mel production during stress and resistance to salt stress (Wu et al., 2021). It is known that Mel accumulates in tissues and chloroplasts under adverse conditions and has a direct scavenging effect on reactive oxygen (ROS) and reactive nitrogen species (RNS) (Khan et al., 2023; Manchester et al., 2015). Moreover, it was observed that Mel exhibited a protective effect on cells against oxidative stress by stimulating the transcription of genes associated with key antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), and glutathione-S-transferase (GST). Furthermore, Mel enhanced the activity of the ascorbate-glutathione (AsA-GSH) cycle and the functions of related enzymes, such as ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR), and monodehydroascorbate reductase (MDHAR) via signal transduction pathways (Back, 2021; Lee and Back, 2018).

Numerous reports state that exogenous Mel application provides resistance to plants under stress by maintaining photosynthetic efficiency with its ROS scavenging capacity, regulates growth through crosstalk with other hormones, alleviates oxidative damage by increasing the activities of antioxidant enzymes, and stimulates the expression of responsive genes (Lee and Back, 2021; Zhao et al., 2022). The protective effects of Mel in plants have been shown under drought (Campos et al., 2019), heat (Shi et al., 2015), light (Yang et al., 2021), salinity (Su et al., 2021), and heavy metal stress (Cao et al., 2019), and its mechanisms of action have been reported depending on these stress factors. However, to the best of our knowledge, no study elucidates the importance and action mechanism of Mel in plants exposed to benzalkonium chloride (BAC) pollution.

BAC is a chemical that is frequently used in hand soaps, surface cleaners, medical applications, personal care products, and eye drops due to its antiseptic, antimicrobial, and antibacterial activity (Zhang et al., 2020). It has started to take place in our daily lives, especially after the global COVID-19 epidemic. BAC is a cationic detergent composed of homologues of different alkyl chain lengths from C8 to C18. The surface activity of BACs occurs by very rapid and prolonged incorporation of the hydrophobic alkyl tail into cell lipid membranes (Barber and Hartmann, 2021). The charged part of the molecule interacts with membrane proteins with high affinity and disrupts many cellular processes. For example, it has been shown that BAC has anti-proliferative properties and affects the DNA synthesis phase in the cell cycle (Thakur and Vashistt, 2021). The US Food and Drug Administration (FDA) has banned some antiseptics due to their minimal consumer benefits as well as worries about their detrimental impacts on human and environmental health. However, the decision on the use of BAC was delayed due to insufficient data on environmental effects (Merchel and Tagkopoulos, 2019). However, both the widespread use and inefficient removal of BAC cause high concentrations of BAC to be released into the environment. Especially pharmaceutical factories and hospital wastes are considered the main points of BAC discharge to the environment (Kim et al., 2020). For example, hospital waste in Austria has been shown to contain approximately 700-fold more BAC (2800 μg L^{-1}) compared to domestic wastewater (Kreuzinger et al., 2007). As a result, BAC, which has become one of the emerging pollution in water resources, threatens terrestrial plants.

There are limited studies on the effects of BAC contamination in plant systems. Khan et al. (2018) reported that the dry weight of *Lactuca sativa* and *Lepidium sativum* plants, which were exposed to BAC for 12 days, decreased by 68% and 75%, respectively, and toxicity symptoms such as necrosis and chlorosis appeared. In addition, it was observed that the shoot length decreased in parallel with the increasing BAC concentration in lettuce, which was applied BAC in the range of 0.5–100 mg L^{-1} during germination. Investigating the phytotoxicity of pollutants from wastewater, researchers demonstrated the toxic effects of BAC on the growth and development in *Brassica napus* (Richter et al., 2016). However, these studies were insufficient to reveal the phytotoxicity mechanism of BAC contamination. It is known that BAC exposure causes excessive accumulation of ROS in mammalian cells and triggers cell death in later stages (Jeon et al., 2019). A recent study showed that BAC pollution significantly reduced photosynthesis efficiency and cellular metabolism in *Microcystis* lines (Jia et al., 2024). Our hypothesis in this study was that Mel would provide tolerance to BAC pollution in *Arabidopsis* with its ROS scavenging, antioxidant activity-triggering, and protective effects on photosynthesis reactions. In order to clearly observe the phytotoxicity mechanism of BAC contamination and the role of Mel in the defense response, exogenous Mel (100 μM) was applied to wild-type *Col-0* and *snat2* knockout mutant lines under normal and BAC stressed conditions. Growth rate, water relations, and gas exchange parameters were examined in wild and mutant *Arabidopsis* seedlings under BAC pollution (0.5 mg L^{-1}) with or without Mel treatment. To understand the role of Mel in BAC phytotoxicity, photosystem II photochemistry, chlorophyll *a* fluorescence parameters, oxidative stress markers, and enzymatic and non-enzymatic antioxidant profiles were revealed in *Col-0* and *snat2* plants.

2. Material and methods

2.1. Experimental methodology

Wild-type Colombia 0 (*Col-0*) and knockout line SALK_062388C (*snat2*) *Arabidopsis thaliana* seeds were sourced from the Arabidopsis Biological Resource Center (Ohio State University, USA) (Alonso et al., 2003). *Arabidopsis* seeds underwent surface sterilization and were subsequently placed onto Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) in petri plates for a 21-day cultivation period in a controlled growth chamber. To induce stress, the growth medium was supplemented with 0.5 mg L^{-1} benzalkonium chloride (BAC) based on the previous reports (Khan et al., 2018). *Arabidopsis* seedlings exposed to BAC stress were treated with a 5 ml solution of melatonin (Mel, 100 μM) via foliar spraying, since Kamiab (2020) tested 0–150 μM concentrations of Mel, revealing that the 100 μM treatment dose was the most effective dose in triggering the stress response and alleviating oxidative stress. Seedlings were harvested after a 3-day treatment period.

2.2. Measurement of physiological parameters

Physiological parameters, including the relative growth rate (RGR), relative water content (RWC) of the leaves, and osmotic potential (Ψ_Π), were recorded after three days of treatment. The relative growth rate (RGR) of wheat leaves was determined according to the previously-described method (Hunt et al., 2002). Six plants were used for the control group and each treatment group. After the samples were oven-dried, dry weights (DW) were measured. RGR values were calculated according to the following formula:

$$RGR = \frac{[\ln(DW_2) - \ln(DW_1)]}{(t2 - t1)}$$

Where t_1 and t_2 were initial and final harvest times, respectively DW_1 was dry weight (g) at t_1; DW_2 was dry weight (g) at t_2.

RWC was calculated using a proposed algorithm (Maghsoudi et al., 2019). Six leaves were harvested and their fresh weight (FW) was determined. The leaves were floated on de-ionized water for 6 h and the turgid tissue was blotted dry prior to determining turgid weight (TW). Dry weight (DW) was determined after oven drying at 70°C. The leaf relative water content (RWC) was calculated by the following formula (Smart and Bingham, 1974):

RWC (%) = [(FW-DW) / (TW-DW)] x 100

Leaves were extracted by crushing the material with a glass rod and Ψ_Π was measured using a Vapro Vapor Pressure Osmometer 5600. Ψ_Π

was converted to MPa according to Santa-Cruz et al. (2002) by multiplying by a coefficient of 2.408 × 10^{-3}. Carbon assimilation rate (A), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), stomatal limitation (L_s) and transpiration rate (E) were measured with a portable gas exchange system (LCpro+; ADC, Hoddesdon, UK). Gas exchange parameters were measured in 6 replicates by selecting leaves of similar size from each treatment group at the end of the experimental setup. The stomatal limitation value (L_s) was measured as 1 – C_i/C_a (Ma et al., 2011).

2.3. Analysis of chlorophyll a fluorescence parameters

Handy PEA (Plant Efficiency Analyser, Hansatech Instruments Ltd.) was used to calculate OJIP test parameters. The descriptions for the estimated parameters are included in Supplementary Table S1.

2.4. Determination of gene expressions by qRT-PCR

Qiagen RNeasy kit was used for RNA isolation from samples. Any remaining genomic DNA contamination was cleaned using DNase I (NEB). After determining the amount and quality of the total RNA isolated, cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific). *Arabidopsis Actin* gene was used to normalize transcript levels of samples, and relative expressions were calculated by the 2^{-DDCT} method (Livak and Schmittgen, 2001). Before starting the qPCR experiment, cDNA libraries and primer sets were tested to ensure amplification efficiency. Final results presented the fold change of gene expressions in treatment groups relative to control plants. The primers and PCR conditions used in this study are given in Supplementary Table S2.

2.5. Analysis of oxidative stress biomarkers

Determination of H_2O_2 content was measured according to Liu et al. (2010). Leaves were homogenized in cold acetone and centrifuged. The supernatant was mixed with titanium reagent and then ammonium hydroxide was added to precipitate the titanium-peroxide complex. The reaction mixture was centrifuged. The pellet was washed with cold acetone and dissolved. The absorbance of the solution was measured at 410 nm. H_2O_2 concentrations were calculated using a standard curve prepared with known concentrations of H_2O_2.

Lipid peroxidation (thiobarbituric acid reactive substances (TBARS) content) was determined according to Rao and Sresty (2000). TBARS concentration of leaf samples was calculated from the absorbance at 532 nm, and measurements were corrected for nonspecific turbidity by subtracting the absorbance at 600 nm. The concentration of TBARS was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

H_2O_2 concentration in guard cells was observed using 2,7-dichlorofluorescein diacetate (H_2DCF-DA), as detailed in previous studies (Ahammed et al., 2020).

2.6. Analysis of enzyme/non-enzyme antioxidants

0.5 g leaf samples were extracted in Tris-HCl (25 mM Tris, 1% Triton-X100, pH: 7.4) and centrifuged at 14000 g for 30 min. Supernatants were collected, and total protein contents were measured by the Bradford (1976) method.

For SOD (EC 1.15.1.1) isozyme activity, samples were subjected to non-denaturing polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (1970). Total SOD activity assay was based on the method of Beauchamp and Fridovich (1971). CAT isozymes were detected according to Woodbury et al. (1971). Total CAT (EC 1.11.1.6) activity was estimated according to the method of Bergmeyer (1970). The isozymes and enzyme activity of POX (EC 1.11.1.7) were based on the method described by Seevers et al. (1971) and Herzog and Fahimi (1973), respectively. NADPH oxidase (NOX) isozymes were identified as described by Sagi and Fluhr (2001). NOX (EC 1.6.3.1) activity was measured according to Jiang and Zhang (2002). The enzyme/isozyme activities of glutathione S-transferase (GST, EC 2.5.1.18) and glutathione peroxidase (GPX, EC 1.11.1.9) were determined (Hossain et al., 2006; Ricci et al., 1984).

Electrophoretic APX separation was performed according to Mittler and Zilinskas (1993). APX (EC 1.11.1.11) enzyme activity was measured according to Nakano and Asada (1981). GR (EC 1.6.4.2) activity was measured according to Foyer and Halliwell (1976). Isozymes compositions of GR were determined by native PAGE analysis (Hou et al., 2004).

Monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) activity was assayed by the method of Miyake and Asada (1992). Dehydroascorbate reductase (DHAR; EC 1.8.5.1) activity was measured according to Dalton et al. (1986). Total and reduced ascorbate (AsA) contents were done according to the method of Dutilleul et al. (2003) with modifications. The oxidized form of ascorbate (DHA, dehydroascorbate) was measured using the formula DHA = Total AsA-Reduced AsA. The glutathione (GSH) was assayed according to Paradiso et al. (2008). Oxidized glutathione (GSSG) was determined after the removal of GSH by 2-vinylpyridine derivatization. GSH redox state (%) was determined by calculating the ratio of GSH to total glutathione (GSH + GSSG) according to Shi et al. (2013).

Gels stained for SOD, CAT, POX, APX, GR, GST, and NOX activities were photographed with the Gel Doc XR + System and then analyzed with Image Lab software v4.0.1 (Bio-Rad, California, USA). Known standard amounts of enzymes (0.5 units of SOD and 0.2 units of CAT and POX) were loaded onto gels. For each isozyme set/group, the average values were significantly different at $p < 0.05$ using Tukey's post-test.

2.7. Statistical analysis

The experiments were repeated thrice independently and each data point was the mean of six replicates. All data obtained were subjected to a one-way analysis of variance (ANOVA). Statistical analysis of the values was performed by using SPSS 20.0. Tukey's post-test was used to compare the treatment groups. Comparisons with $p < 0.05$ were considered significantly different. In all figures, the error bars represent standard errors of the means.

3. Results

3.1. Mel-induced changes in growth, water relations and gas exchange parameters of Arabidopsis

BAC stress caused over 60% growth retardation in wild-type and mutant lines (Fig. 1A). Mel + BAC combined treatment increased RGR by 88% in *Col-0* plants, while RGR in *snat2* plants increased 2-fold compared to stressed plants. Treatment with 100 μM foliar Mel resulted in increased RWC of *Arabidopsis* lines (Fig. 1B). Mel application to stressed plants (Mel + BAC groups) enabled the recovery of RWC in both genotypes. The Ψ_Π of Mel groups increased by approximately 9% in *Col-0* plants compared to the control (Fig. 1C). The highest Ψ_Π level was noted in *snat2* under Mel + BAC, with a 20% increase.

Approximately 25% higher A was determined in both *Col-0* and *snat2* leaves after Mel treatment (Fig. 2A). BAC contamination significantly suppressed A by 71% and 48% in *Col-0* and *snat2* plants, respectively. Mel treatment to *Col-0* plants exposed to BAC contamination (Mel + BAC) induced A up to 3-fold. In *snat2* plants, the Mel + BAC group showed a 63% increment in A compared to the stress-only group. BAC stress caused a 72% increase in g_s compared to the control (Fig. 2B). g_s of the *snat2* groups responded to treatments differently than the wild type, and Mel and BAC application resulted in 30% and 67% reduced g_s, respectively. On the other hand, a 6-fold increased g_s was detected in Mel + BAC group plants compared to stress. Mel and BAC applications caused a significant C_i decrease of 24% and 37% in *snat2* plants, respectively (Fig. 2C). A 31% decreased C_i was determined in the Mel +

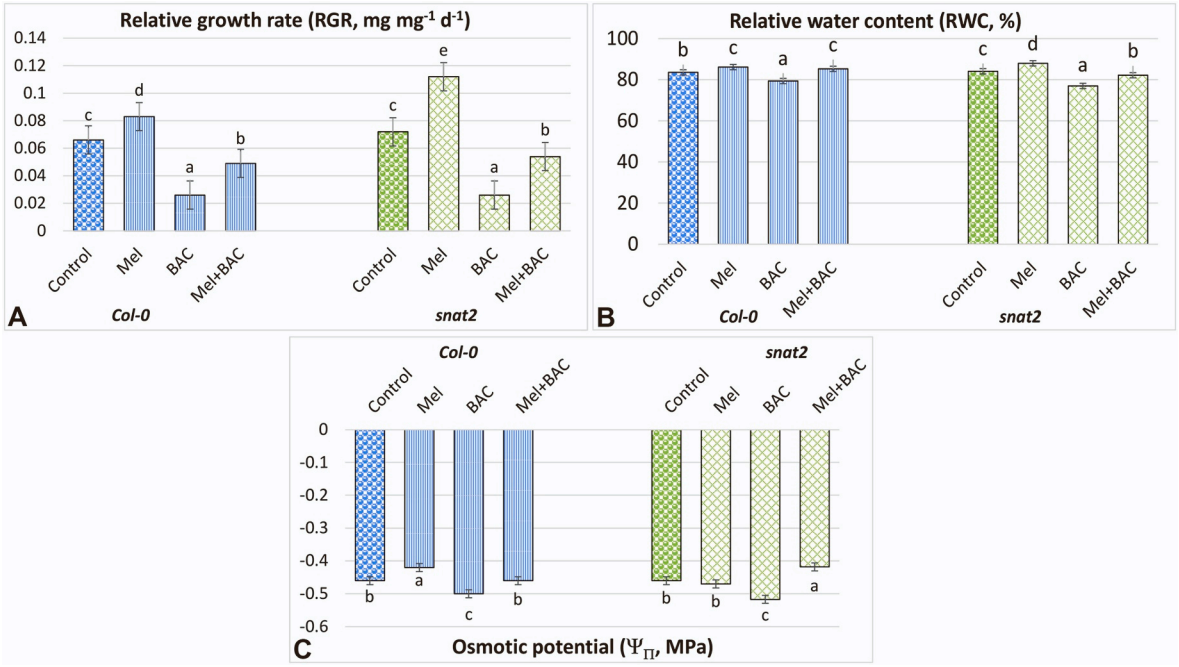

Fig. 1. The relative growth rate (RGR, **A**), relative water content (RWC, **B**), and osmotic potential (Ψ_{Π}, **C**), in the leaves of *Arabidopsis thaliana* wild-type (*Col-0*) and SNAT2 knockout mutant (*snat2*) lines treated with melatonin (Mel, 100 μM) under benzalkonium chloride (BAC, 0.5 mg L^{-1}) stress. All data obtained were subjected to a one-way analysis of variance (ANOVA). Differences were considered to be significant at $p < 0.05$.

BAC applied wild-type plants. Mel + BAC combined application caused a 60% decrease in the E of *Col-0* plants compared to the stress group (Fig. 2D). On the contrary, a 4.5-fold increase in E of the Mel + BAC group was detected in the *snat2* line. L_s of wild-type leaves increased by 52% after Mel treatment and decreased by 56% with BAC stress (Fig. 2E). Mel + BAC combined application resulted in 5.5-fold more L_s compared to plants under stress alone. Exposure to BAC contamination resulted in a significant decrease in the A/C_i ratio of 73% in wild-type plants compared to control plants (Fig. 2F). While Mel + BAC combined treatment did not create a significant difference in the *snat2* line compared to the stress group, it provided an approximately 5-fold increase in *Col-0* plants.

3.2. Effects of Mel on photosynthesis and chlorophyll a fluorescence in Col-0 and snat2 lines

BAC stress caused the F_v/F_m value to decrease by more than 10% and the F_v/F_o ratio to decrease by approximately 40% in both lines (Fig. 3A–B). F_o/F_m of BAC-exposed *Col-0* and *snat2* plants increased by 51% and 45% compared to the control, respectively (Fig. 3C). However, Mel treatment on stressed plants (Mel + BAC group) was successful in providing recovery close to control in F_v/F_m, F_v/F_o, and F_o/F_m values in both lines. When the radar plots of the OJIP test were examined, it was observed that BAC stress reduced the total performance indexes (PI_{ABS} and PI_{total}), especially by causing a loss in the electron transport flux (Et_o/RC) (Fig. 4A–B).

3.3. Changes in SNAT1 and SNAT2 gene expression profiles under BAC treatments

T-DNA insertion in the SNAT2 gene and RT-PCR analysis of SNAT1-2 mRNA in wild-type (*Col-0*) and *snat2* mutant plants are represented in Fig. 5A–B. SNAT1 gene expression in wild-type *Arabidopsis* leaves under BAC stress was reduced 0.7-fold relative to control (Fig. 5C). In Mel + BAC combined application, increased SNAT1 gene expression was observed compared to stress. It was determined that SNAT1 gene expression increased 1.2-fold in *snat2* mutant plants under BAC stress.

3.4. Oxidative stress markers and antioxidant enzyme profiles in Arabidopsis exposed to BAC

H_2O_2 accumulation in stomatal guard cells was examined under a confocal microscope using H_2DCF-DA dye (Fig. 6A). BAC stress increased H_2O_2 accumulation by 26% in wild-type plants (Fig. 6B). In the Mel + BAC combined group, H_2O_2 content decreased by 20% compared to stressed *Col-0* plants. Similarly, in the *snat2* line, H_2O_2 increased by 7% umder the BAC group and decreased by 13% at the Mel + BAC group. Lipid peroxidation level (TBARS content) increased by 61% and 63% in *Col-0* and *snat2* plants exposed to BAC pollution, respectively (Fig. 6C). In wild-type plants, the Mel + BAC group had a 31% reduced TBARS content compared to the stress-alone group, while this rate was 27% in *snat2* after Mel + BAC exposure.

Native-PAGE analysis revealed the presence of five SOD isoenzyme bands in *Arabidopsis* leaves (Mn-SOD1-2 and Fe-SOD1-3) (Fig. 7A). In *Col-0* plants, Mel treatment increased total SOD activity by 12%, while BAC stress resulted in 11% decreased activity (Fig. 7B). On the other hand, Mel + BAC treatment provided 27% higher SOD activity in the *snat2* line. The presence of four different CAT isoenzymes detected in wild-type and mutant *Arabidopsis* plants (CAT1-4) (Fig. 7C). While all *Col-0* treatment groups showed an increase in total CAT activity, the highest activity was reported in the Mel + BAC combined application with an 11% increase compared to the stress group (Fig. 7D). BAC stress caused 43% suppressed CAT activity compared to control. Recovery of CAT activity was achieved with a 2.6-fold increase in the Mel + BAC group. Three POX isoenzymes were determined in the experimental groups (POX1-3) (Fig. 8A). 100 μM Mel spraying increased total POX activity by 24% in *Col-0* leaves and 37% in *snat2* (Fig. 8B). Nine isozyme bands belonging to GST were obtained in gel electrophoresis (GST1-9) (Fig. 8C). While BAC stress did not cause a change in GST activity in both lines, Mel application to stressed plants (Mel + BAC groups) induced GST activity by 57% in *Col-0* and 82% in *snat2* (Fig. 8D). Three NOX isozymes were detected in mutant and wild-type *Arabidopsis* leaves (NOX1-3) (Fig. 9A). A 25% suppressed NOX activity was reported in *Col-0* leaves and 16% in *snat2* plants exposed to BAC pollution (Fig. 9B). However, the stress-induced loss of NOX activity was recovered in the

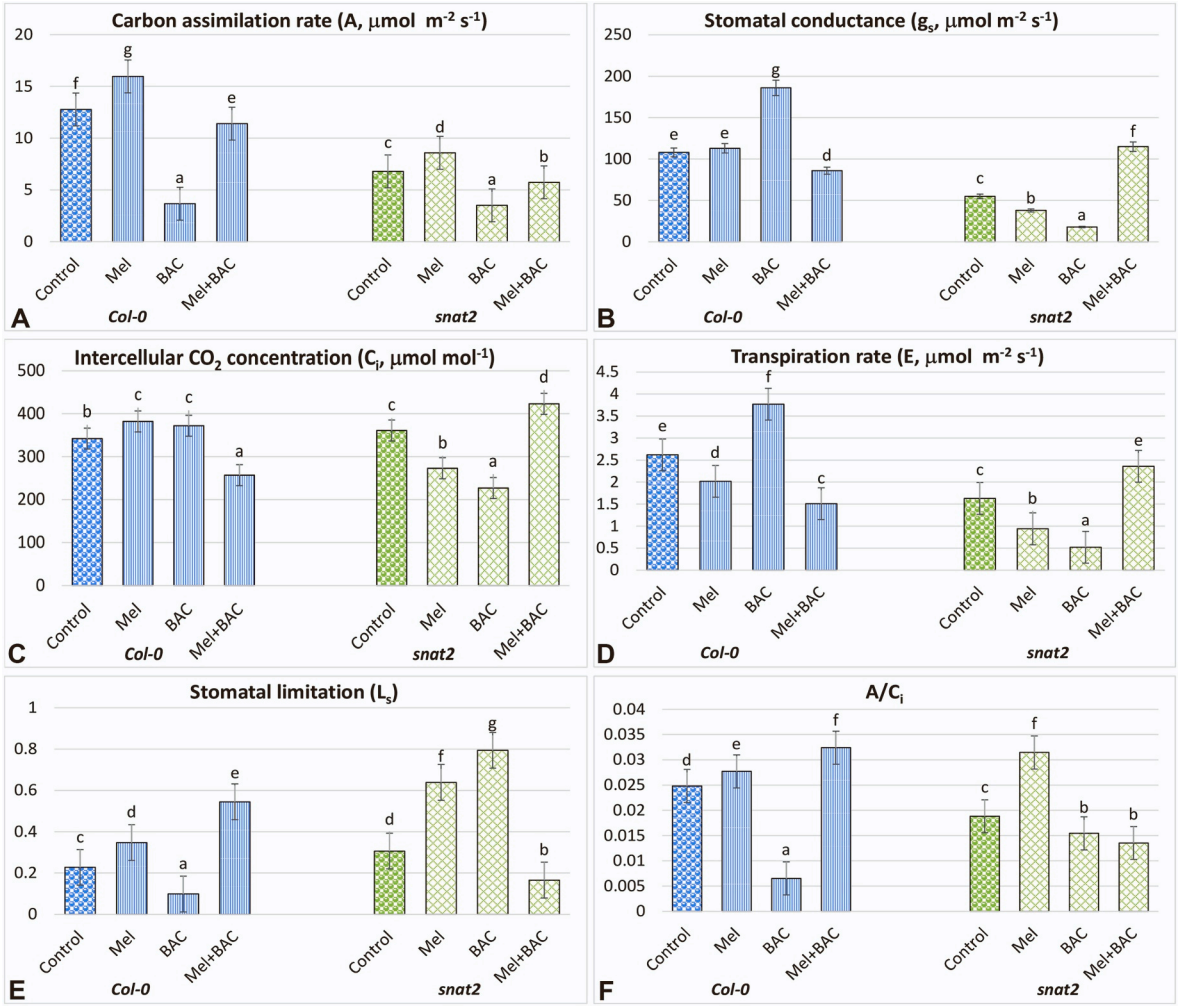

Fig. 2. Carbon assimilation rate (A, **A**), stomatal conductance (g_s, **B**), intercellular CO_2 concentrations (C_i, **C**), transpiration rate (E, **D**), stomatal limitation rate (L_s, **E**), and A/C_i value (**F**) in the leaves of *Arabidopsis thaliana* wild-type (*Col-0*) and SNAT2 knockout mutant (*snat2*) lines treated with melatonin (Mel, 100 μM) under benzalkonium chloride (BAC, 0.5 mg L^{-1}) stress. All data obtained were subjected to a one-way analysis of variance (ANOVA). Differences were considered to be significant at $p < 0.05$.

Mel + BAC groups. As presented in Fig. 9C, two isozyme bands of GPX were identified (GPX1-2). While BAC stress caused a 32% decrease in total GPX activity in *Col-0* leaves, the GPX activity increased by 34% in *snat2* plants (Fig. 9D). Mel treatments had a triggering effect on GPX activity under both normal and stress conditions.

3.5. Interaction of Mel with AsA-GSH cycle-related enzymatic and non-enzymatic antioxidants in Arabidopsis under BAC stress

Gel analysis results shown in Fig. 10A reveal the presence of two APX isozymes in *Arabidopsis* leaves (APX1-2). BAC exposure significantly limited APX activity in both lines (Fig. 10B). Mel application to stressed plants resulted in increased APX activity by 55% in *Col-0* plants and 78% in *snat2* plants. Three isoenzyme bands of the GR enzyme were determined (GR1-3) (Fig. 10C). Total GR activity in *Col-0* leaves increased by 87% under BAC stress and further increased by 41% in Mel + BAC combined application (Fig. 10D). BAC stress resulted in a loss of 16% and 18% MDHAR activity in *Col-0* and *snat2* plants, respectively, compared to the control (Fig. 11A). While Foliar Mel application increased MDHAR activity in all groups, the highest MDHAR activity increase in the stressed plants was observed in the *Col-0* Mel-BAC group with 2.16-fold. Similarly, DHAR activity was detected to decrease by 25% in *Col-0* and by 42% in *snat2* plants exposed to BAC (Fig. 11B). Mel treatment under control conditions did not change DHAR activity but provided 26% more DHAR activity in the Mel + BAC applied *Col-0* plants compared to stressed plants. A 65% decreased DHAR activity was noted in the *snat2* Mel + BAC group. While the total AsA content of *Arabidopsis* leaves decreased by 34% in *Col-0* plants with BAC exposure, it did not change in *snat2* plants (Fig. 11C). Mel + BAC combined application did not cause any difference in the tAsA content of *Col-0* compared to plants treated with stress alone but resulted in a 2.3-fold increased tAsA in *snat2* plants. DHA content increased 1.3- and 2.3-fold in *Col-0* and *snat2* leaves under BAC pollution, respectively (Fig. 11D). An opposite profile occurred in the Mel + BAC groups of the two *Arabidopsis* genotypes, and the combined treatment resulted in a 32% DHA increase in *Col-0* and a 35% DHA decrease in *snat2* compared to the stressed plants. Mel treatment resulted in increased GSH only in *Col-0* plants under control conditions (Fig. 11E). While BAC stress reduced the GSH levels of *Col-0* and *snat2* leaves by 31% and 19%, respectively, Combined application of Mel + BAC to *snat2* plants resulted in a small induction in GSH content. GSSG content of *Col-0* Mel and BAC groups increased by 19% and 81%, respectively (Fig. 11F). BAC exposure also caused a 53% increased GSSG accumulation in *snat2* leaves. In stressed plants, Mel treatment resulted in a 16% reduced GSSG content in *Col-0*, while there was no statistical difference *in snat2*. The tAsA/DHA ratio decreased by over 50% in *Col-0* and *snat2* leaves under BAC pollution (Fig. 11G). Mel + BAC treatment in the *snat2* line resulted in a 3.6-fold increased tAsA/DHA ratio compared to plants under stress alone. When

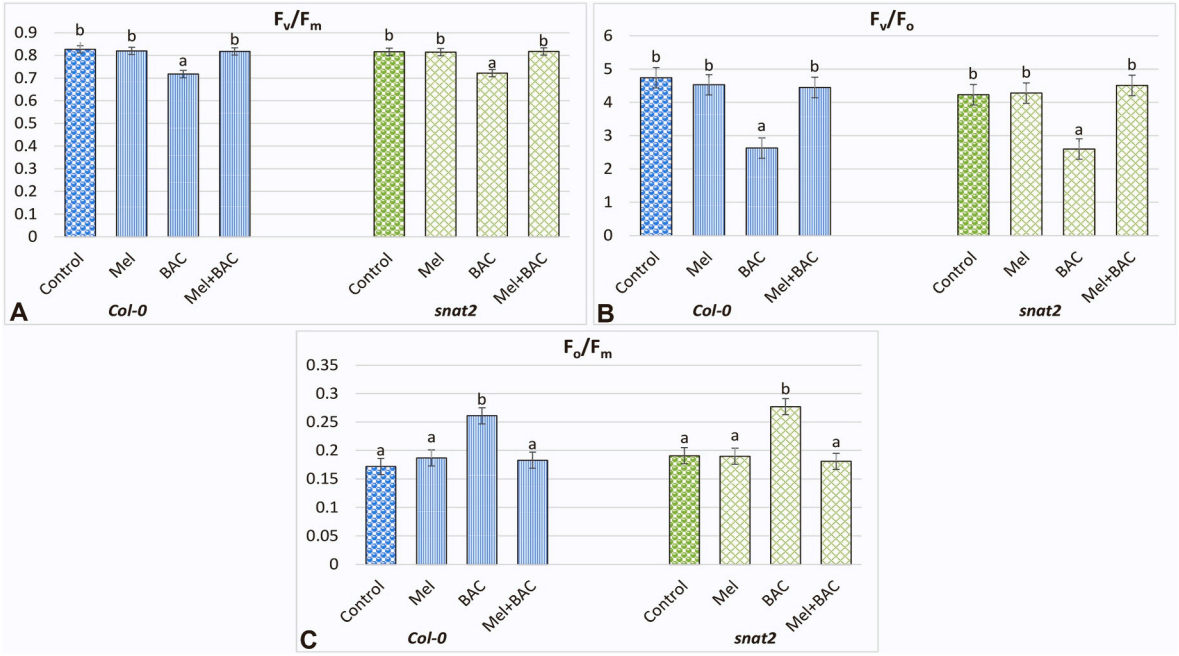

Fig. 3. The maximal quantum yield of PSII photochemistry (F_v/F_m, **A**), potential photochemical efficiency (F_v/F_o, **B**), and physiological state of the photosynthetic apparatus (F_o/F_m, **C**) in the leaves of *Arabidopsis thaliana* wild-type (*Col-0*) and SNAT2 knockout mutant (*snat2*) lines treated with melatonin (Mel, 100 μM) under benzalkonium chloride (BAC, 0.5 mg L^{-1}) stress. All data obtained were subjected to a one-way analysis of variance (ANOVA). Differences were considered to be significant at $p < 0.05$.

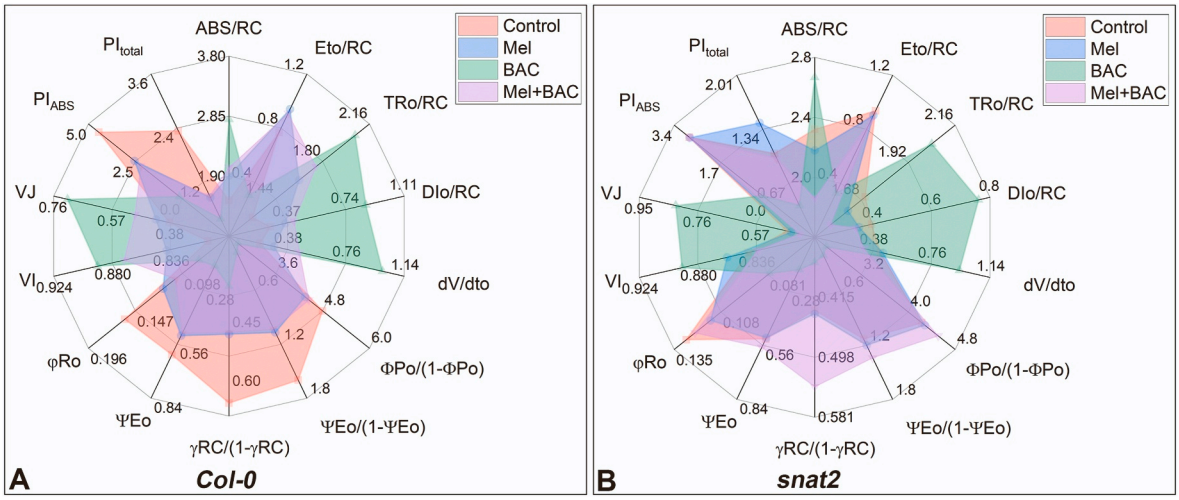

Fig. 4. OJIP transient radar plots (**A, B**) in the leaves of *Arabidopsis thaliana* wild-type (*Col-0*) and SNAT2 knockout mutant (*snat2*) lines treated with melatonin (Mel, 100 μM) under benzalkonium chloride (BAC, 0.5 mg L^{-1}) stress.

all experimental groups were examined, it was observed that the GSH redox state was suppressed in stressed plants and Mel treatments did not make a difference in both conditions (Fig. 11H).

4. Discussion

Melatonin (Mel), categorized among indoleamines, plays a pivotal role in various plant processes, ranging from seed germination (Wang et al., 2024) to stress response (Gao et al., 2023). Consequently, leveraging Mel as a plant growth regulator to enhance agricultural productivity and induce stress tolerance in plants holds significant promise (Sharma et al., 2024). The growth-promoting effects of Mel in plants were initially unveiled by Hernandez-Ruiz et al. (2004), demonstrating parallels between the effects of Mel and indole acetic acid (IAA), which shares a similar chemical structure. Our findings indicate that Mel treatments increased the relative growth rate (RGR) in both *Arabidopsis* lines (*Col-0* and *snat2*) under both control and BAC stress conditions. This increase in RGR may be due to Mel triggering growth by activating hormonal pathways. One of the most important toxic effects of BAC contamination in cells is that it inhibits cell proliferation by causing loss of mitochondrial functions and DNA damage (Zhang et al., 2020). The decrease in RGR and relative water content (RWC) observed in *Col-0* and *snat2* plants exposed to BAC stress may be directly due to damage to the cells at the root tips. When the uptake of water and nutrients from the roots is interrupted, limitations in growth, photosynthesis efficiency, and gas exchange are determined in many plant species (Deans et al., 2020). There were significant changes in RGR, RWC, and osmotic potential (Ψ_Π) of *Arabidopsis* plants exposed to BAC in this study. Similar

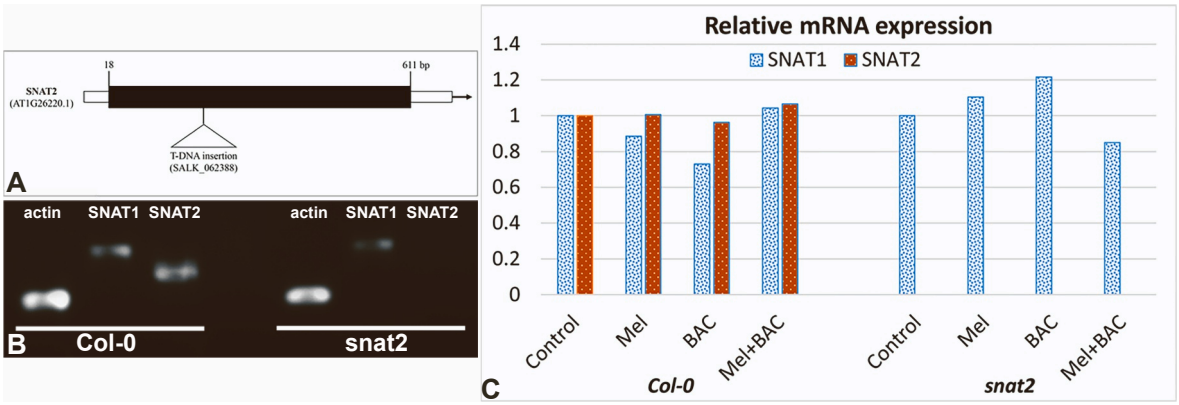

Fig. 5. Schematic diagram of *Arabidopsis snat2* knockout mutation, exon (solid box) and intron (open box) are indicated. The positions of the T-DNA insertions are indicated by arrows (not to scale) (**A**), RT-PCR analysis of SNAT mRNA in wild-type (*Col-0*) and *snat2* mutant plants (**B**), and the expression of SNAT1 and SNAT2 genes in the leaves of *Arabidopsis thaliana* wild-type (*Col-0*) and SNAT2 knockout mutant (*snat2*) lines treated with melatonin (Mel, 100 μM) under benzalkonium chloride (BAC, 0.5 mg L^{-1}) stress (**C**).

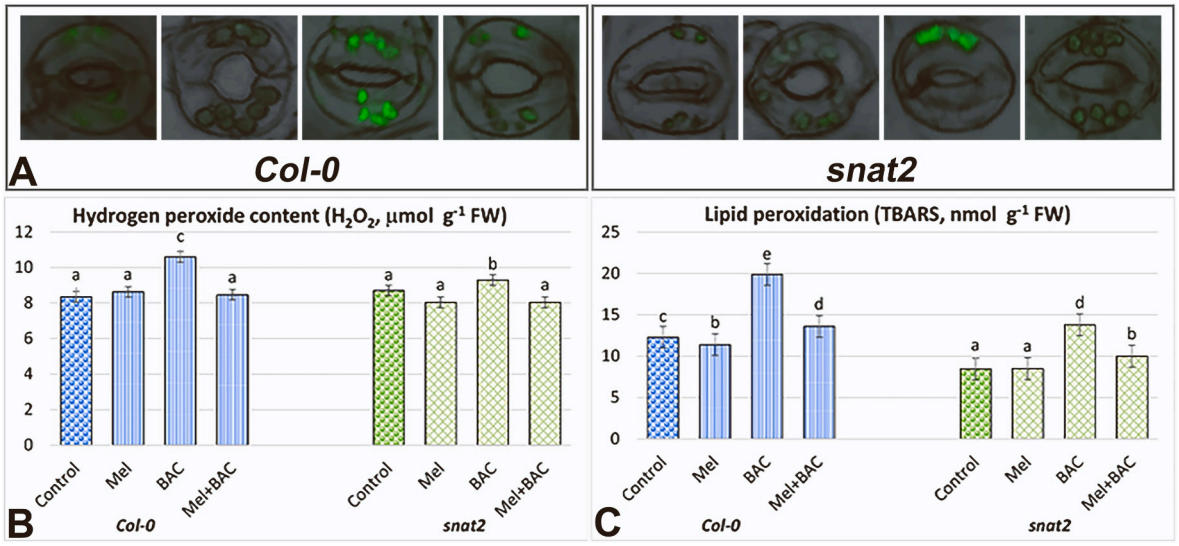

Fig. 6. Accumulation of H_2O_2 in guard cells identified with the 2,7-dichlorofluorescein diacetate (H_2DCF-DA) under confocal microscopy (**A**), the changes of hydrogen peroxide content (H_2O_2, **B**) and lipid peroxidation level (TBARS content, **C**) in the leaves of *Arabidopsis thaliana* wild-type (*Col-0*) and SNAT2 knockout mutant (*snat2*) lines treated with melatonin (Mel, 100 μM) under benzalkonium chloride (BAC, 0.5 mg L^{-1}) stress. All data obtained were subjected to a one-way analysis of variance (ANOVA). Differences were considered to be significant at $p < 0.05$.

results were demonstrated in lettuce plants exposed to increasing concentrations of BAC contamination, with a 68% loss in plant dry weight in parallel with a nearly 50% decrease in the content of N and Mg nutrients (Khan et al., 2018). However, in both *Col-0* and *snat2* plants, Mel treatments regulated growth and water relations by relieving BAC stress-induced inhibitions. An increase in plant growth and vascular development was also observed with higher Mel content in mutant rice plants that overexpressed the caffeic acid O-methyltransferase gene, which plays a role in Mel biosynthesis (Huangfu et al., 2022).

Due to the close relationship between Mel biosynthesis and photosynthesis reactions, the lower carbon assimilation rate (A) observed in Mel deficient *snat2* mutants compared to *Col-0* plants aligns with previous findings (Hwang et al., 2020). Exposure to BAC stress also caused serious decreases in A in both lines. In their study on *Microcystis* strains, Jia et al. (2024) showed that BAC contamination of 0.2 mg L^{-1} and above led to decreases in photosynthesis efficiency along with decreases in chlorophyll *a* content. The authors also reported that BAC exposure causes many metabolic changes such as pentose phosphate pathway, fatty acid biosynthesis, and amino acid metabolism. In plants under stress, chloroplasts become the center of excessive ROS formation. Especially, electrons escaping from light reactions in photosystem II (PSII) and disruption of electron flow in the electron transport chain (ETC) directly lead to the formation of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). Hwang et al. (2020) showed that Mel protects photosynthetic reactions and increases stress tolerance by preventing excessive ROS accumulation in chloroplasts. Similarly, our results showed that Mel treatment increased the maximum quantum yield of PSII (F_v/F_m) by preventing ROS accumulation in chloroplasts under normal and BAC stress conditions. Huangfu et al. (2022) also reported that induced Mel synthesis increased photosynthesis efficiency by reducing chlorophyll degradation in *Oryza sativa*. The study conducted on wild-type and *snat1* mutant *Arabidopsis* seedlings reported that exogenous Mel application provided osmotic stress tolerance by its ROS scavenging effect and by triggering the activity of antioxidant enzymes SOD and CAT (in correlation with our results), and in addition, Mel-induced stress tolerance was directly linked to the CAND2 receptor (Wang et al., 2021). However, there are conflicting data regarding the function of CAND2, the Mel-binding plasma membrane protein. Wei

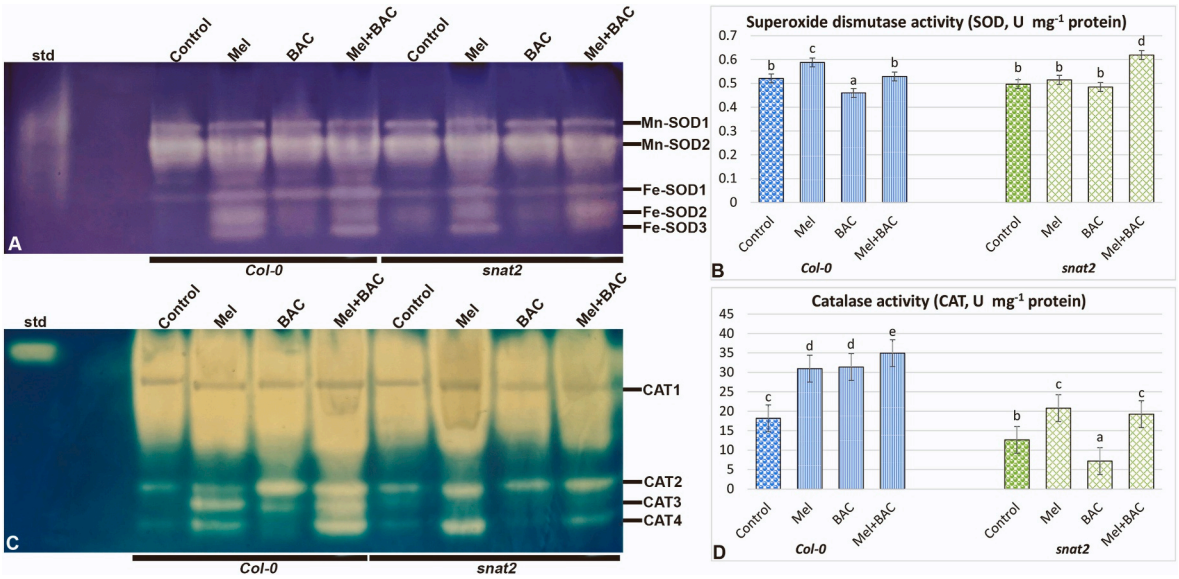

Fig. 7. The relative band intensity of different types of superoxide dismutase isoenzymes (SOD, **A**), total SOD activity (**B**), catalase isoenzymes (CAT, **C**) and total CAT activity (**D**) in the leaves of *Arabidopsis thaliana* wild-type (*Col-0*) and SNAT2 knockout mutant (*snat2*) lines treated with melatonin (Mel, 100 μM) under benzalkonium chloride (BAC, 0.5 mg L^{-1}) stress. All data obtained were subjected to a one-way analysis of variance (ANOVA). Differences were considered to be significant at $p < 0.05$.

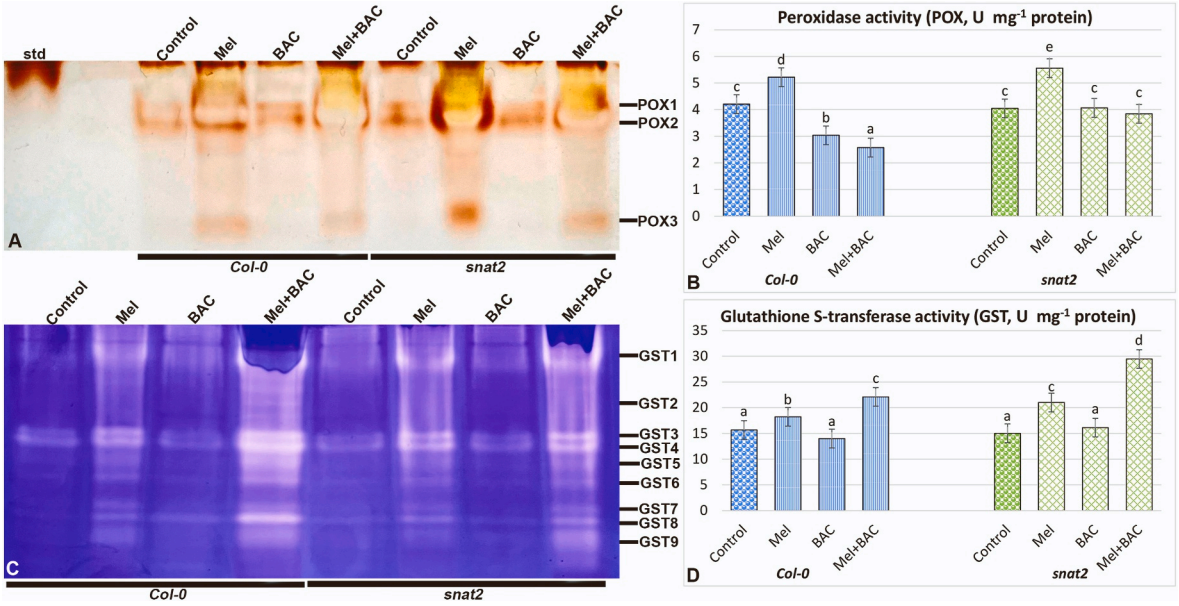

Fig. 8. The relative band intensity of different types of peroxidase isoenzymes (POX, **A**) and total POX activity (**B**), glutathione S-transferase isoenzymes (GST, **C**) and total GST activity (**D**) in the leaves of *Arabidopsis thaliana* wild-type (*Col-0*) and SNAT2 knockout mutant (*snat2*) lines treated with melatonin (Mel, 100 μM) under benzalkonium chloride (BAC, 0.5 mg L^{-1}) stress. All data obtained were subjected to a one-way analysis of variance (ANOVA). Differences were considered to be significant at $p < 0.05$.

et al. (2018) suggested that CAND2, by binding with Mel, activates NADPH oxidase (NOX) dependent H_2O_2 production, promoting ion flow and eventually leading to stomatal closure. In contrast, later it has been shown that CAND2 is not involved in Mel-induced defense (Back and Lee, 2020). However, in our results, all Mel treatments resulted in an increase in NOX enzyme activity in *snat2* mutant and *Col-0* plants under control and BAC contamination conditions. It was observed that this increase was followed by stomatal limitations in plants treated with Mel under control conditions. The study of gas exchange parameters in response to BAC stress revealed distinct responses in *Col-0* and *snat2* plants. While a stress response was created by increasing transpiration rate (E) and stomatal conductance (g_s) in wild-type plants exposed to BAC contamination, g_s increased and intercellular CO_2 content (C_i) decreased in *snat2* plants. This differential response may be attributed to increased oxidative stress in guard cells due to Mel deficiency in *snat2* plants. Notably, Mel treatment under stress conditions resulted in decreased g_s in *Col-0* plants and increased g_s in *snat2* plants, deviating from the primary stress response in both treatment groups. This may be attributed to the inability of *snat2* plants to mount a robust stress response due to the loss of SNAT2 function located in chloroplasts (Lee et al., 2019). In addition, when the expression profiles of SNAT1 and SNAT2 in *Col-0* plants were examined, it was determined that the SNAT2 gene had higher expression under BAC stress, and although there was a small increase (1.2-fold) in the expression of the SNAT1 gene under BAC

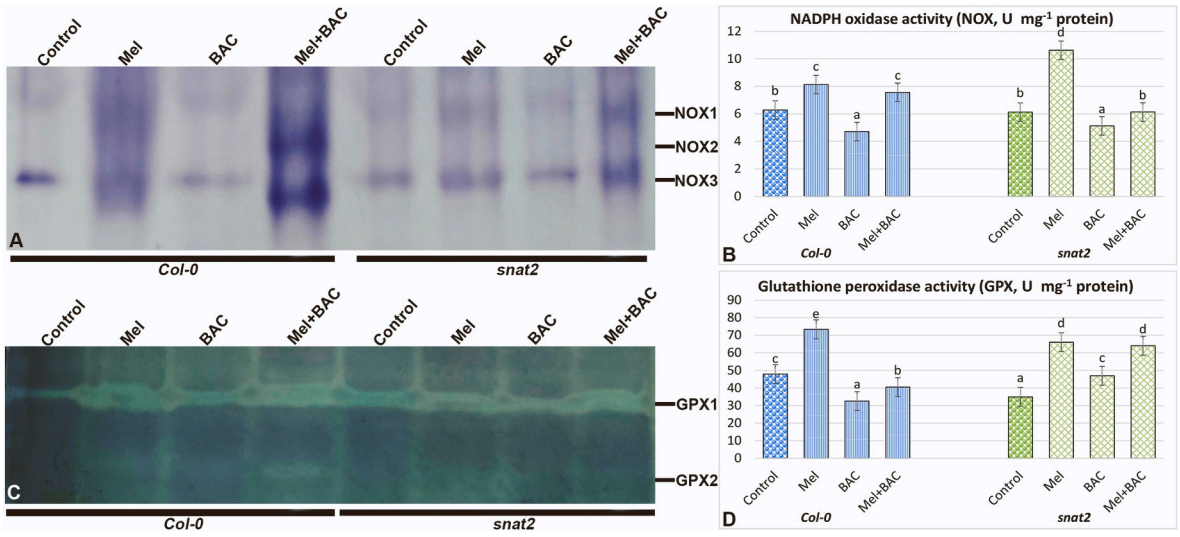

Fig. 9. The relative band intensity of different types of NADPH oxidase isoenzymes (NOX, **A**) and total NOX activity (**B**), glutathione peroxidase isoenzymes (GPX, **C**), and total GPX activity (**D**) in the leaves of *Arabidopsis thaliana* wild-type (*Col-0*) and SNAT2 knockout mutant (*snat2*) lines treated with melatonin (Mel, 100 μM) under benzalkonium chloride (BAC, 0.5 mg L^{-1}) stress. All data obtained were subjected to a one-way analysis of variance (ANOVA). Differences were considered to be significant at $p < 0.05$.

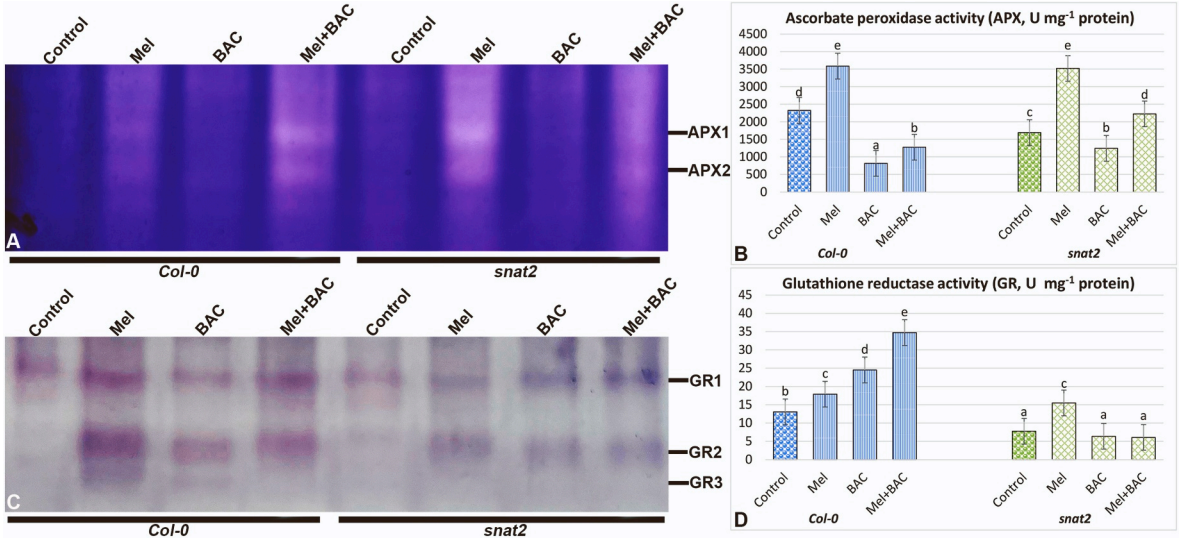

Fig. 10. The relative band intensity of different types of ascorbate peroxidase isoenzymes (APX, **A**) and total APX activity (**B**), glutathione reductase isoenzymes (GR, **C**), and total GR activity (**D**) in the leaves of *Arabidopsis thaliana* wild-type (*Col-0*) and SNAT2 knockout mutant (*snat2*) lines treated with melatonin (Mel, 100 μM) under benzalkonium chloride (BAC, 0.5 mg L^{-1}) stress. All data obtained were subjected to a one-way analysis of variance (ANOVA). Differences were considered to be significant at $p < 0.05$.

stress in *snat2* plants, it was not at a level to compensate for the SNAT2 deficiency. These results prove the previously reported Mel deficiency in *snat2* mutant *Arabidopsis* plants especially under environmental stress (Yang et al., 2021). However, a detailed examination of chlorophyll-*a* fluorescence parameters showed that Mel treatments reversed the decreased performance indexes (PI_{total}) caused by BAC stress by regulating photochemical reactions and reducing the dissipated energy flow (DI_o/RC) in both lines. Similar results were shown in *snat1* and *snat2* *Arabidopsis* mutants under high light stress, and it was reported that the net photosynthesis rate along with chlorophyll *a and b* contents increased with exogenous Mel treatment (Yang et al., 2021).

BAC exposure causes oxidative damage following excessive ROS production in cells (Jeon et al., 2019). Our study also delves into the oxidative damage caused by stress, including increased hydrogen peroxide accumulation and lipid peroxidation levels (TBARS content) in BAC-contaminated *Col-0* and *snat2* plants. Additionally, it was observed that the enzymes of the antioxidant defense system were mostly not triggered in *Arabidopsis* lines under BAC contamination. However, foliar Mel treatments in stress groups resulted in an increase in the activities of almost all antioxidant enzymes of wild-type and mutant plants (except POX). The increase in the expression of antioxidant response genes due to exogenous Mel applications under abiotic stress was shown by Lee and Back (2018) in wild-type, *snat1*, and *asmt* mutant *Arabidopsis* lines. This increase in activity of the antioxidant defense system that we encounter with Mel treatments may be due to NOX signaling (Wei et al., 2018) and the role of Mel in hormonal signal transduction (Kanwar et al., 2018), as discussed above. Compatibly, Mel is known to trigger phenolic and flavonoid biosynthesis and accumulation in plants (Ebrahimi et al., 2023; Ullah et al., 2023). Vafadar and Ehsanzadeh (2023), in their studies on *Dracocephalum kotschyi* genotypes under salt stress,

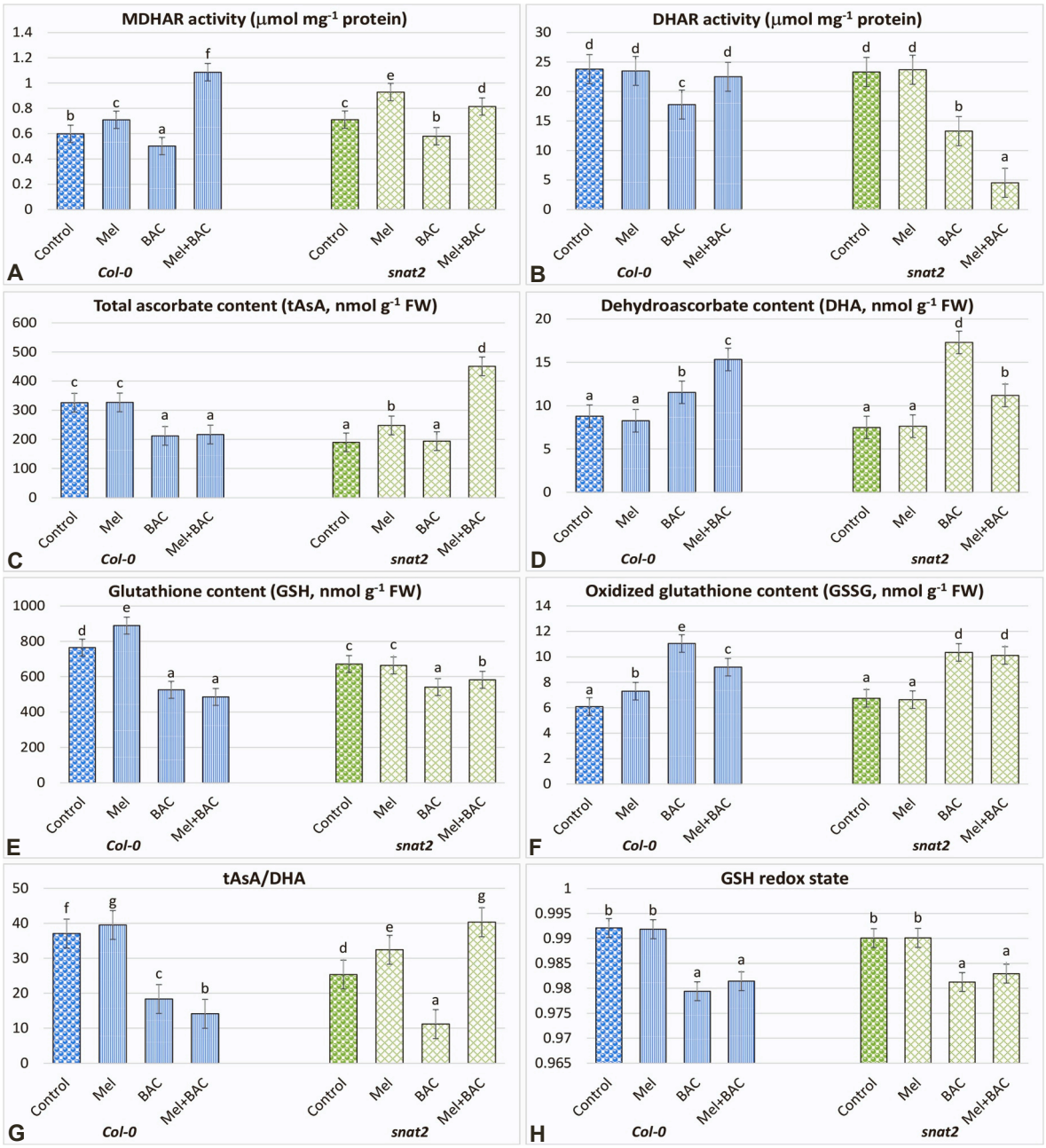

Fig. 11. The monodehydroascorbate reductase activity (MDHAR, **A**), dehydroascorbate reductase activity (DHAR, **B**), total ascorbate content (AsA, **C**), dehydroascorbate content (DHA, **D**), glutathione content (GSH, **E**), oxidized glutathione content (GSSG, **F**), AsA/DHA (**G**) and GSH redox state (**H**) in the leaves of *Arabidopsis thaliana* wild-type (*Col-0*) and SNAT2 knockout mutant (*snat2*) lines treated with melatonin (Mel, 100 μM) under benzalkonium chloride (BAC, 0.5 mg L^{-1}) stress. All data obtained were subjected to a one-way analysis of variance (ANOVA). Differences were considered to be significant at $p < 0.05$.

showed that 100 μM Mel treatments increased total phenolic and flavonoid contents, improved radical scavenging capacity and provided resistance to salt stress. Therefore, the stress response, we obtained with Mel treatment in this study, may have been achieved by Mel increasing the production of secondary metabolites that neutralize oxidative stress.

Mel + BAC treatment in *Col-0* plants reduced oxidative damage by inducing APX, GR, MDHAR, and DHAR enzyme activities involved in the ascorbate-glutathione (AsA-GSH) cycle and maintained cellular redox homeostasis. Bai et al. (2020) have shown that enzymes involved in Mel biosynthesis directly interact with APX, resulting in increased activity. Mel application in *snat2* plants enabled the clearance of ROS accumulation by renewing the AsA pool, but GSH redox status did not increase due to the deficiency in high GR activity. Overall, the high antioxidant activity provided by the Mel application helped to alleviate oxidative damage, regulate photosynthesis reactions, and promote plant growth in *Arabidopsis* under BAC stress. In addition to the results demonstrated in many agricultural plants in the development of tolerance to environmental stresses(Khan et al., 2020), this study has revealed for the first time the action mechanisms of Mel under the emerging pollutant benzalkonium chloride.

CRediT authorship contribution statement

Evren Yildiztugay: Writing – review & editing, Writing – original draft, Methodology, Investigation. **Busra Arikan Abdulveli:** Writing – review & editing, Writing – original draft, Methodology, Investigation.

Ceyda Ozfidan-Konakci: Writing – review & editing, Writing – original draft, Methodology, Investigation. **Ismail Turkan:** Writing – review & editing, Writing – original draft, Methodology, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

Acknowledgements

This work was supported by Selcuk University Scientific Research Projects Coordinating Office (Grant Number: 23408008).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2024.108779.

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