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Effect of moderate heat stress on *Arabidopsis thaliana* with modified *BPMs* expression

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Abstract – In *Arabidopsis thaliana* (L.) Heynh., a major part of the MATH-BTB protein family (BPM proteins) participates in the ubiquitin-proteasome pathway as substrate-specific adaptors of Cullin 3 dependent E3 ligase. Through targeting of specific proteins for degradation, BPMs are involved in various developmental processes, as well as in the plant's ability to adapt to changing environmental conditions. The aim of this research was to investigate the oxidative stress response of *Arabidopsis* with modified *BPMs* expression to moderate heat stress. Seedlings of the wild-type, a line overexpressing *BPM1* gene (*oeBPM1*), and a line with down-regulation of *BPM1*, *4*, *5,* and *6* genes (*amiR-bpm*) were exposed to 37 °C for six hours. Seedlings were sampled immediately after stress and after a recovery period of 24 h. Control seedlings were grown at 24 °C and were collected at the same time points as treated ones. The level of lipid peroxidation, H_2O_2 and proline content, as well as the activity of antioxidant enzymes (G-POD, APX, CAT, and SOD) were evaluated. Control *amiR-bpm* and *oeBPM1* seedlings generally had lower H_2O_2 and proline content, respectively, than the wild-type. After exposure to 37 °C, *oeBPM1* and *amiR-bpm* lines showed reduced proline content, while no change in lipid peroxidation level and H_2O_2 content was observed. Further, seedlings with modified *BPM* expression showed differences in the activity of G-POD, APX, and SOD, while no change was observed in the activity of CAT. The results obtained suggest involvement of BPM proteins in the response of *Arabidopsis* to moderate heat stress.

Keywords: antioxidant enzymes, *Arabidopsis thaliana*, MATH-BTB, BPM proteins, H_2O_2, lipid peroxidation, PCA analysis, proline

Introduction

Temperatures above or below optimum alter or disrupt plant metabolic homeostasis, leading to various physiological, biochemical, and molecular changes (Mittler et al. 2012), including inhibition of photosynthesis (Greer et al. 2011), destabilization of the membrane by lipid peroxidation, and modification of the antioxidant system to maintain cellular redox balance (Kumar et al. 2012). All these changes affect plant development, reproduction, and productivity (Hasanuzzaman et al. 2020). Therefore, the mechanisms that enable plants to maintain a balance between survival and continued growth are extremely important (Grubb 1998). One of these mechanisms is the ubiquitin-proteasome system (UPS), an important pathway for degradation of abnormal peptides and short-lived regulators in the cell (Zhou et al. 2014). Specificity of proteasomal degradation is facilitated by substrate-specific module of ubiquitin ligases (E3). Proteins containing domains Meprin and TRAF (tumor necrosis factor receptor-associated factor) homology (MATH) and Broad-complex Tramtrack and Bric-à-brac (BTB) are known to be substrate receptors for Cullin 3 (CUL3)-based E3 ligases (Gingerich et al. 2005; Chen et al. 2013). Six genes encoding MATH-BTB (BPM) proteins are present in the model plant *Arabidopsis thaliana* (L.) Heynh. Several studies have suggested that the response of *Arabidopsis* to abiotic stress is regulated through the interaction of BPM proteins with various transcription factors. Lechner et al. (2011) showed that BPM proteins directly interact with transcription factor ATHB6, a negative regulator of abscisic acid (ABA) response. BPM proteins have also been shown to be involved in fatty acid metabolism through their interaction with transcription activator WRI1 (Chen et al. 2013). Moreover, Morimoto et al. (2017) have demonstrated an important role of BPM proteins in the plant's response to heat stress through the interaction of the BPM2 protein with the protein DREB2A, an important transcription factor that controls plant response to dehydration and heat stress by the activation of many stress-inducible target

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genes. This is particularly interesting in the context of the recent results of Škiljaica et al. (2020) showing that BPM protein stabilises and accumulates at high levels at the elevated temperature of 37 °C. Furthermore, Bauer et al. (2019) have shown that some of the proteins interacting with MATH-BTBs are part of the plant antioxidant system.

Plants have evolved a complex enzymatic and non-enzymatic antioxidant network to overcome the negative effects of reactive oxygen species (ROS) overproduction as a result of various types of stress, including heat stress (Mittler et al. 2012; Hasanuzzaman et al 2020). ROS include singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH) (Kumar et al. 2012; Hasanuzzaman et al. 2013). The major ROS-scavenging enzymes in plants include guaiacol peroxidase (G-POD; EC 1.11.1.7), ascorbate peroxidase (APX; EC 1.11.1.11), and catalase (CAT; EC 1.11.1.6), which catalyse the degradation of H_2O_2 to non-toxic components, as well as superoxide dismutase (SOD; EC 1.15.1.1) which catalyses the dismutation of $O_2^{\cdot -}$ to O_2 and H_2O_2 (Mittler 2002). In addition, compatible solutes such as proline and soluble sugars are often accumulated under heat stress to stabilize and protect protein and enzyme structure, maintain membrane integrity, and act as ROS-scavengers (Lv et al. 2011).

The main objective of our study was to investigate the effect of moderate heat stress (37 °C) on the induction of oxidative stress and the activation of antioxidant system in *Arabidopsis* with modified *BPMs* expression, by measuring the level of lipid peroxidation, H_2O_2 and proline content, and the activity of four antioxidant enzymes (G-POD, APX, CAT and SOD). Because knowledge about the possible involvement of BPM proteins in stress response is limited, principal component analysis was used to distinguish similarities and differences in the heat stress response among the wild-type, a line overexpressing *BPM1* gene, and a line with down-regulation of *BPM1*, *4*, *5* and *6* genes. Additionally, since transgene integration could alter various metabolic pathways (Cellini et al. 2004), we analysed and discussed the basic biochemical response of lines with modified *BPMs* expression under favourable growth conditions.

Materials and methods

Plant material, growth conditions and stress treatment

Transgenic plants overexpressing *BPM1* gene (*oeBPM1*) in *Arabidopsis thaliana* (L.) Heynh. ecotype Col-0 were generated and phenotypically characterized by Škiljaica et al. (2020). The line showing significant reduction in transcript accumulation of *BPM1, 4, 5* and *6* genes (*amiR-bpm*) was generated and described by Lechner et al. (2011). *Arabidopsis thaliana* ecotype Col-0 was used as a wild-type.

Seeds were surface sterilized with 70% (v/v) ethanol for 1 min and 1% (w/v) sodium dichloroisocyanurate dihydrate for 10 min, rinsed five times with sterilized distilled water, and planted on plates containing Murashige and Skoog basal salt mix (Murashige and Skoog, 1962) with vitamins and minerals (M5519, Sigma-Aldrich), 0.8% agar, and 2% sucrose. Seeds (120-150 on each plate) were cold treated at +4 °C in the dark for three days and then transferred to a plant growth chamber where they were kept at 24 ± 1 °C for 12 days under fluorescent white bulbs (75-85 µmol m^{-2} s^{-1}) and a photoperiod of 16 h of light and 8 h of darkness.

After 12 days, when the seedlings had developed two rosette leaves (growth stage 1.02 according to Boyes et al. 2001), 20 plates of each *Arabidopsis* line were divided into two groups – treatment and control. The treatment group was exposed to 37 °C for six hours in an incubator (Hood TH 30, Edmund Bühler GmbH). Fluorescent white bulbs (75-85 µmol m^{-2} s^{-1}) were used as a light source. Immediately after the treatment, half of the plates for each line were collected for further analysis (first time point). The other half was collected after a 24 h recovery period (second time point) under growth conditions (24 ± 1 °C, 75-85 µmol m^{-2} s^{-1}, photoperiod of 16 h light and 8 h dark). The control group grew continuously under the growth conditions and was sampled at the same time points as the treatment group.

Determination of hydrogen peroxide, malondialdehyde and proline content

Plant material (200 mg, fresh weight) was frozen in liquid nitrogen and homogenized in 2 mL of cold 0.1% (w/v) trichloroacetic acid (TCA) with the addition of 3% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 15 000 *g* and +4 °C for 10 min, and the supernatant was used as an extract for the determination of H_2O_2, malondialdehyde (MDA) and proline content.

H_2O_2 content was measured according to Velikova et al. (2000). The extract (0.5 mL) was added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide (KI). The absorbance of the reaction mixture was measured at 390 nm, using 0.1% TCA as a blank. H_2O_2 content was calculated using a standard curve and expressed as µmol g^{-1} of fresh weight.

The level of lipid peroxidation was determined by measuring the amount of MDA, a by-product of unsaturated fatty acid peroxidation (Sunkar et al. 2003). Briefly, the extract (0.5 mL) was added to 1 mL of 0.5% (w/v) 2-thiobarbituric acid (TBA) in 20% (w/v) TCA and incubated in a dry block heater at 100 °C for 30 min. After incubation, the tubes were cooled in an ice bath for 5 min. The absorbance of the solution was measured at 532 and 600 nm. As a blank 0.1% TCA was used. MDA content was calculated after subtracting the non-specific absorbance at 600 nm and using an extinction coefficient of 155 mM^{-1} cm^{-1}. MDA content was expressed as nmol g^{-1} of fresh weight.

Proline content was assayed according to the ninhydrin reaction method described by Bates et al. (1973) with some modifications. Briefly, 0.5 mL of extract in 0.1% TCA was added to 0.5 mL (v/v) glacial acetic acid and 0.5 mL acidic ninhydrin (0.14 M ninhydrin in 12 mL glacial acetic acid and 8 mL 6 M phosphoric acid). The reaction mixture was mixed and incubated in a dry block heater at 100 °C for 1 h.

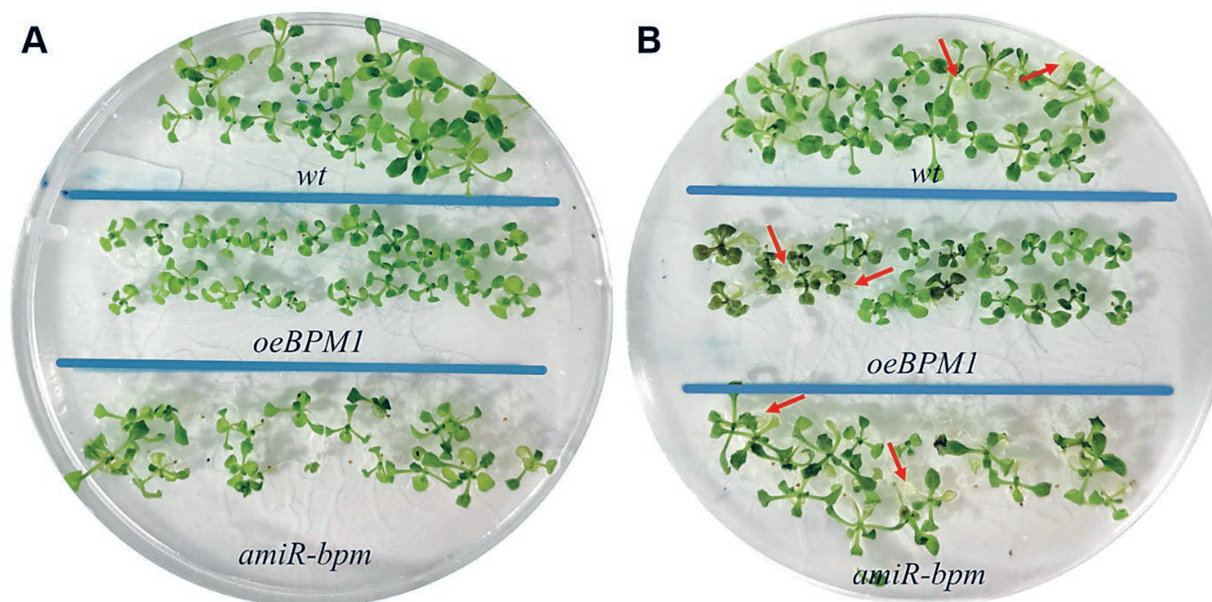

Fig. 1. The growth of *Arabidopsis thaliana* – control seedlings (**A**) and seedlings after exposure to 37 °C (**B**). Three different *Arabidopsis* lines were used – wild-type (*wt*), line overexpressing *BPM1* gene (*oeBPM1*), and line with downregulation of *BPM1, 4, 5* and *6* genes (*amiR-bpm*). Twelve-day-old seedlings were transferred from 24 °C to 37 °C for 6 h and then allowed to recover at 24 °C. Seedlings were photographed five days after exposure to moderate heat stress, along with the control group continuously cultivated at 24 °C. Arrows indicate chlorosis of cotyledons in heat-treated seedlings.

The reaction was stopped by cooling the tubes in an ice bath for 5 min. After cooling, 1 mL of toluene was added to the reaction mixture, mixed thoroughly and after separation of the toluene and aqueous phase, the toluene phase was collected for measurement at 520 nm. Pure toluene was used as a blank. Proline content was calculated using a standard curve and expressed as μmol g^{-1} of fresh weight.

Enzyme activity assays

Plant material (100 mg, fresh weight) was frozen in liquid nitrogen and homogenized in 1 mL of cold 100 mM potassium phosphate buffer (pH 7.0) containing 3% (w/v) PVPP. The homogenate was centrifuged at 20 000 *g* and +4 °C for 30 min, and the supernatant was used as a crude extract for the assays of guaiacol peroxidase (G-POD), ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD) activity. Total soluble protein content was determined according to the Bradford method (Bradford, 1976) using bovine albumin serum as a standard.

APX (EC 1.11.1.11) activity was assayed according to Nakano and Asada (1981) by measuring the decrease in absorbance of H_2O_2 at 290 nm (ε = 2.8 mM^{-1} cm^{-1}) every second for 15 s. The reaction mixture consisted of 0.1 mM ethylenediaminetetraacetic acid (EDTA) in 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM ascorbate, 0.24 mM H_2O_2 and 180 μL of an enzyme extract. APX activity was expressed as nmol of oxidized ascorbate per minute per milligram of total soluble proteins.

G-POD (EC 1.11.1.7) activity was measured using a reaction mixture of 50 mM potassium phosphate buffer (pH 7.0) with 18 mM guaiacol, 4.5 mM H_2O_2 and 20 μL of the enzyme extract (Chance and Maehly 1955). G-POD activity was assessed by measuring the absorbance of tetraguaiacol at 470 nm every 15 s for 3 min. The activity was calculated using the extinction coefficient (ε = 26.6 mM^{-1} cm^{-1}) and expressed as μmol of tetraguaiacol per minute per milligram of total soluble proteins.

CAT (EC 1.11.1.6) activity was determined by the decrease in H_2O_2 absorbance at 240 nm (ε = 36 mM^{-1} cm^{-1}) every 10 s during 1.5 min, as described by Aebi (1984). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM H_2O_2 and 50 μL of an enzyme extract. CAT activity was expressed as nmol of decomposed H_2O_2 per minute per milligram of total soluble proteins.

SOD (EC 1.15.1.1) activity was determined by measuring the reduction of nitro blue tetrazolium (NBT), as described by Beauchamp and Fridovich (1971). The assay is based on a xanthine-xanthine oxidase complex as the generator of superoxide anions which in turn reduce NBT to formazan dye. The addition of SOD reduces superoxide anion levels, thereby lowering the rate of NBT reduction. The reaction mixture consisted of SOD reaction buffer (50 mM potassium phosphate buffer, 75 μM NBT and 0.1 mM EDTA, pH 7.8), 2.5 mM xanthine, 2.5 mU mL^{-1} xanthine oxidase and 5 μL of an enzyme extract. The absorbance of formazan dye at 560 nm was measured every 30 s for 3 min. SOD activity was calculated using a standard curve with known concentrations of SOD enzyme and expressed as units (U) per milligram of total soluble proteins. One U of activity is defined as the amount of enzyme required to inhibit NBT reduction by 50%.

Statistics

Each treatment group, as well as control, was prepared in five replicates and the experiment was repeated four times. Independent Student's *t*-test for unequal variances was used when mean values between two groups were compared, i. e. differences between control and treatment group for each line and time point. To test for differences among control groups of all three lines sampled in the same time point (6 h or 24 h recovery), as well as between treatment groups of these lines, data were compared using analysis of variance (ANOVA) followed by Newman-Keuls test. In both analyses, results were considered significantly different at $P \leq 0.05$.

To visualize the similarities and differences among the lines, a principal component analysis (PCA) was performed. Prior to PCA analysis, to reduce noise in the data, a multi-factorial ANOVA was carried out (de Haan et al. 2007). Statistically significant difference among genotypes, treatment and time points was tested by applying the ANOVA model ($P \leq 0.05$), which showed a significant difference between the seedlings collected at the first and second time point. Therefore, PCA analysis was performed separately for two different time points. The analysis included H_2O_2, MDA and proline content, as well as the activity of four antioxidant enzymes (G-POD, APX, CAT, SOD).

All statistical analyses were performed using TIBCO Statistica 13.5.0.17 software package (TIBCO Software Inc., USA) and PCA analysis was performed using R 3.6.2. software and "*factoextra*" package.

Results

Seedling morphology

Several differences in seedling morphology were observed among the lines grown under control conditions (24 °C). Seedlings (including leaf blades) were smaller in *oeBPM1* than in the wild-type and *amiR-bpm*. There were no prominent differences between wild-type and *amiR-bpm* phenotypes, except for the typically serrated leaves described previously in *amiR-bpm* (Lechner et al. 2011) (Fig. 1A). The exposure to moderate heat stress did not affect survival in any of the lines tested. However, within five days of recovery at 24 °C, the applied stress induced chlorosis of cotyledons in all three lines (indicated by arrows, Fig. 1B). Interestingly, the leaves of *oeBPM1* seedlings exposed to 37 °C darkened during the recovery period.

Hydrogen peroxide, malondialdehyde and proline content

In general, *amiR-bpm* seedlings showed significantly lower H_2O_2 content than wild-type and *oeBPM1* seedlings. Comparison between control and treated group showed that wild-type and *amiR-bpm* seedlings collected at the first time point, together with *oeBPM1* and *amiR-bpm* seedlings collected at the second time point, showed slightly (more than 5%) higher H_2O_2 content than their respective controls, but the differences were not statistically significant (Fig. 2A).

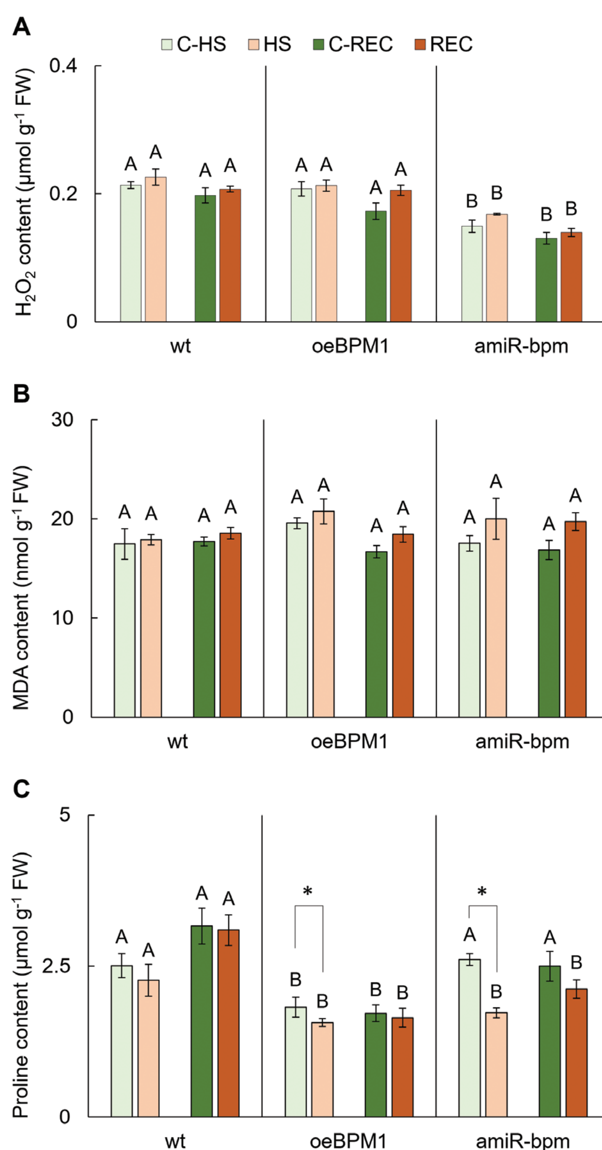

Fig. 2. Hydrogen peroxide (H_2O_2) (**A**), malondialdehyde (MDA) (**B**), and proline (**C**) content in *Arabidopsis thaliana* seedlings exposed to 37 °C. Three different *Arabidopsis* lines were used – wild-type (wt), line overexpressing *BPM1* gene (oeBPM1) and line with downregulation of *BPM1, 4, 5* and *6* genes (amiR-bpm). Plant material was collected at two time points, first - immediately after stress (HS), and second - after 24 h recovery (REC). C-HS and C-REC represent control groups grown at 24 °C. Results are expressed as means of five replicates ± standard error. Columns marked with asterisks indicate a significant difference (Student's *t*-test, $P \leq 0.05$) between control and treatment group for each line and time point. Columns marked with different uppercase letters indicate a significant difference (Newman-Keuls test, $P \leq 0.05$) among control or treatment groups of all three lines sampled at the same time point - first or second.

Overall, lipid peroxidation level, expressed as MDA content, showed no significant difference among the tested *Arabidopsis* lines. However, the treatment resulted in a slight increase in MDA content in *oeBPM1* and *amiR-bpm* seedlings collected at both time points, compared to their respective control (Fig. 2B).

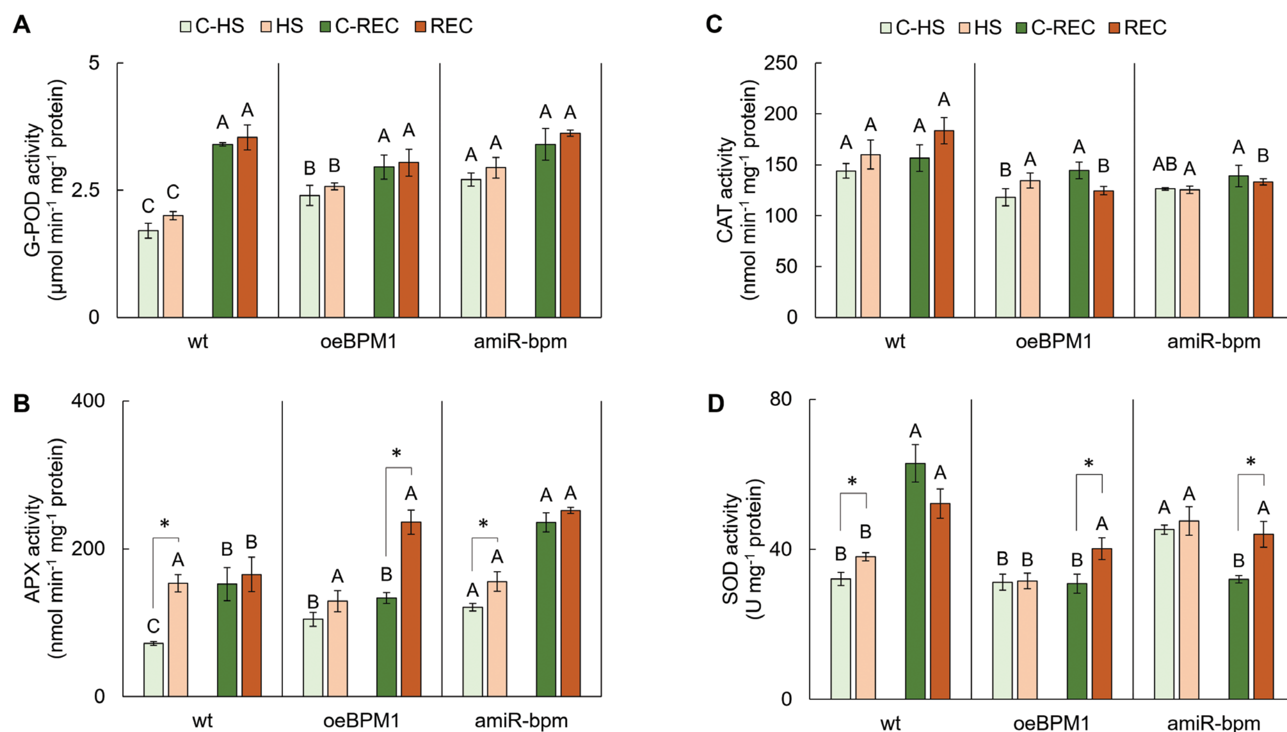

Fig. 3. The activity of guaiacol peroxidase (G-POD) (**A**), ascorbate peroxidase (APX) (**B**), catalase (CAT) (**C**), and superoxide dismutase (SOD) (**D**) in *Arabidopsis thaliana* seedlings exposed to 37 °C. Three different *Arabidopsis* lines were used – a wild-type (wt), a line overexpressing *BPM1* gene (oeBPM1) and a line with downregulation of *BPM1, 4, 5* and *6* genes (amiR-bpm). Plant material was collected at two time points: the first, immediately after stress (HS), and the second, after a 24h recovery period (REC). C-HS and C-REC represent control groups grown at 24 °C. Results are expressed as means of five replicates ± standard error. Columns marked with asterisks indicate a significant difference (Student's *t*-test, $P \leq 0.05$) between control and treatment group for each line and time point. Columns marked with different uppercase letters indicate a significant difference (Newman-Keuls test, $P \leq 0.05$) among control or treatment groups of all three lines sampled at the same time point – first or second.

Proline content was lower in both control groups of *oeBPM1* than in wild-type and *amiR-bpm* control seedlings. When control and treatment groups were compared, exposure to 37 °C caused a significant decrease in proline content in *oeBPM1* and *amiR-bpm* seedlings collected at the first time point. A slight decrease was observed in wild-type seedlings collected at the first time point and in *amiR-bpm* seedlings collected at the second time point (Fig. 2C).

Antioxidant enzyme activity

In general, G-POD activity in both control and treated seedlings collected at the first time point showed significant differences among all three *Arabidopsis* lines. Both *oeBPM1* and *amiR-bpm* showed higher activity than wild-type seedlings. In addition, a comparison between control and treated group showed slightly increased G-POD activity in all three lines sampled immediately after the stress. In contrast, after 24 h recovery period, a slightly increased activity was observed only in treated *amiR-bpm* seedlings (Fig. 3A).

Comparing the control groups of all three *Arabidopsis* lines collected at the first time point, the basic APX activity was higher in *oeBPM1* and *amiR-bpm* than in wild-type seedlings. When control groups collected at the second time point were compared, *amiR-bpm* showed higher activity than wild-type and *oeBPM1* seedlings, whereas there was no significant difference between *oeBPM1* and wild-type seedlings. A prominent reaction to elevated temperature, observed as a significant increase in the APX activity of treated seedlings, was revealed in wild-type and *amiR-bpm* seedlings sampled at the first time point, and in *oeBPM1* seedlings collected at the second time point (Fig. 3B).

CAT activity in control groups sampled at the first time point was lowest in *oeBPM1* seedlings. The activity in *amiR-bpm* seedlings was similar to that in both wild-type and *oeBPM1* seedlings. On the other hand, treated *oeBPM1* and *amiR-bpm* seedlings showed lower activity than wild-type after 24 h recovery period. Comparison between control and treated group showed slightly increased CAT activity in wild-type and *oeBPM1* seedlings collected immediately after the stress, and in wild-type seedlings collected after a recovery period of 24 °C. Slightly decreased activity was observed in *oeBPM1* seedlings after recovery (Fig. 3C).

In general, a comparison of SOD activity in seedlings collected at the first time point showed higher activity in both control and treated group of *amiR-bpm* than in wild-type and *oeBPM1*. At the second time point, control groups of *oeBPM1* and *amiR-bpm* showed lower activity than the wild-type, while there were no differences in heat-treated

seedlings of all three lines. Moreover, comparison between control and treated group of each line showed significantly increased SOD activity in wild-type seedlings immediately after the stress. On the other hand, after a 24h recovery period a significantly increased activity was observed in *oeBPM1* and *amiR-bpm* seedlings exposed to 37 °C, while the activity slightly decreased in wild-type (Fig. 3D).

Principal component analysis

Principal component analysis (PCA) was performed in order to analyse similarities and differences in response to moderate heat stress among three *Arabidopsis* lines – wild-type, *oeBPM1* and *amiR-bpm*. Since multifactorial ANOVA revealed a significant difference between the response of seedlings collected at two time points, PCA analysis was performed separately for each time point.

For seedlings collected at the first time point, the scree plot indicated that the first three dimensions accounted for 98.02% of the total variance (data not shown). Although the PCA3 dimension represented 18.57% of the total variance, it was not included in the further analysis as it did not provide any additional information. The PCA biplot (Fig. 4A) showed the grouping of seedlings based on a different genotype. Wild-type seedlings were characterized with moderate positive loadings of proline, CAT and H_2O_2 content. Furthermore, *amiR-bpm* seedlings differed from wild-type based on strong negative loading of G-POD and moderate negative loadings of SOD, APX and MDA. *oeBPM1* line was characterized by moderate negative loadings of MDA and H_2O_2 content.

As for the seedlings sampled at the second time point, the scree plot indicated that the first three dimensions accounted for 93.19% of the total variance (data not shown). The PCA3 dimension represented 17.59% of the total variance, however it was not included in the further analysis because it did not provide any additional information. As with seedlings collected at the first time point, the PCA biplot (Fig. 4B) of seedlings sampled at the second time point showed grouping based on different genotypes. Both the control and treatment groups of the wild-type line were grouped according to their moderate positive loadings of APX, SOD and proline content. In addition, *oeBPM1* differed from *amiR-bpm* seedlings according to moderate positive loading of H_2O_2 content and moderate negative loading of G-POD, respectively.

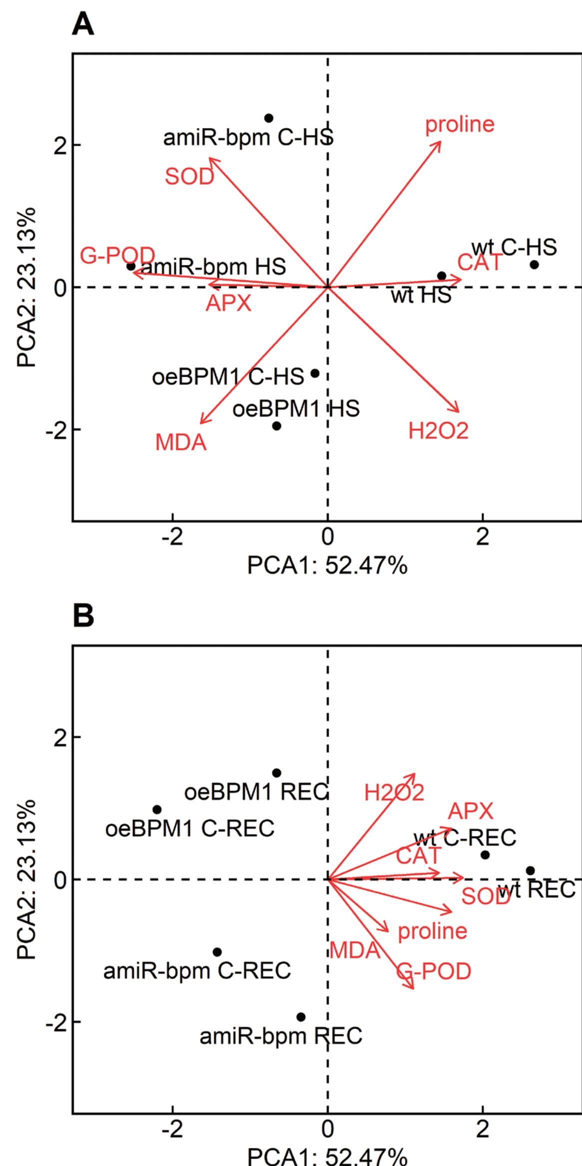

Fig. 4. Principal component analysis (PCA) biplot of seedlings collected at the first (**A**) and at the second (**B**) time point. Three different *Arabidopsis thaliana* lines were used – wild-type (wt), line overexpressing *BPM1* gene (oeBPM1) and line with down-regulation of *BPM1, 4, 5* and *6* genes (amiR-bpm). The abbreviations HS and REC represent the plant material collected immediately after stress (first time point) and after 24 h recovery period (second time point), respectively. C-HS and C-REC represent control groups grown at 24 °C. The parameters measured were: H_2O_2 (hydrogen peroxide content), MDA (level of lipid peroxidation), proline (proline content), G-POD (guaiacol peroxidase), APX (ascorbate peroxidase), CAT (catalase), and SOD (superoxide dismutase).

Discussion

In this study, we have shown the differences in the response of *Arabidopsis thaliana* with modified *BPM* genes expression to moderate heat stress. The temperature of 37 °C was chosen based on the knowledge that BPM1 protein is stabilized and accumulated under elevated temperatures (Škiljaica et al. 2020). The applied temperature, which was approximately 13 °C above the optimal growth temperature for *Arabidopsis*, is considered a moderate heat stress, as the survival percentage was not affected in any of the treated lines. Certain morphological variations were already observed in the seedlings of the lines grown under optimal growth conditions, i.e. seedlings of the *oeBPM1* line were slightly smaller than wild-type and the *amiR-bpm* line. This is consistent with previously reported morphological variation between lines, with adult *Arabidopsis* plants overexpressing *BPM1* having smaller and more compact rosettes and leaves with shorter petioles (Škiljaica et al. 2020). When

the phenotypes of *amiR-bpm* and wild-type were observed, there were no prominent differences between them, except for the typically serrated leaves previously described in *amiR-bpm* (Lechner et al. 2011). In our experiment, exposure to 37 °C for six hours induced chlorosis of cotyledons in all three lines, indicating accelerated senescence. Leaf chlorosis due to a decrease in chlorophyll content is a typical symptom of heat stress in many plants, including *Arabidopsis* (Wang et al. 2018). It was found that the activities of chlorophyll-degrading enzymes, including chlorophyllase and chlorophyll-degrading peroxidase, increased significantly after heat stress (Rossi et al. 2017).

Hydrogen peroxide is the major redox metabolite important for redox sensing, signalling and redox regulation (Sies 2017). It may act as a signalling molecule in promoting the expression of stress-response genes, which could increase the stress tolerance of the plant (Mittler et al. 2012). On the other hand, excessive accumulation of H_2O_2 could be a sign of oxidative damage in the cell (Ozden et al. 2009). Based on the results of H_2O_2 as well as MDA, an indicator of oxidative damage to membranes (Larkindale and Knight 2002), exposure to 37 °C did not cause significant oxidative stress in any of the lines or time points studied. Similar results were shown in the study of Kumar et al. (2012), in which different growth stages of wheat were exposed to a range of elevated temperatures. The most prominent H_2O_2 accumulation was observed at higher temperatures (above 40 °C), while moderate heat stress (35 °C) did not significantly affect H_2O_2 content. Our results suggest that 37 °C is a moderate heat stress for *Arabidopsis* as it caused only low oxidative stress, which could be overcome with a plant antioxidant network (Gür et al. 2010). Indeed, in order to suppress excessive accumulation and possible toxic effects of ROS, plants can activate antioxidant enzymes G-POD, APX, CAT and SOD (Mittler 2002; Zhou et al. 2019). Mittler (2002) suggested that increased APX activity could have a role in the fine regulation of H_2O_2 levels during its involvement in various signalling pathways. In our research, increased APX activity was observed in wild-type and *amiR-bpm*, the line with down-regulation of *BPM1*, *4*, *5* and *6* genes, immediately after exposure to 37 °C. Intriguingly, even under non-stressed conditions, *amiR-bpm* showed higher activity of G-POD and APX, and lower H_2O_2 content than wild-type and *oeBPM1*, suggesting that *BPM* genes could be involved in the control mechanisms of intracellular redox status. This could possibly be explained by the study of Morimoto et al. (2017), which shows that all BPM proteins interact with DREB2A, a key transcription factor involved in the activation of many heat stress-inducible genes. In other words, it is possible that due to the reduced expression of *BPM1*, *4*, *5* and *6* genes, a significant increase in DREB2A protein level could occur, leading to the induction of various genes involved in heat stress response. Indeed, the *amiBPM* line with reduced expression of all *BPMs* (Morimoto et al. 2017) shows upregulation of *Heat stress transcription factor A-3* (*HSFA3*) gene after five hours of exposure to 45 °C. HSFA3 is known for its involvement in the regulation of different oxidative stress-inducible genes, including L-ascorbate peroxidase 2 (APX2) (Hwang et al. 2012). APX2 protein is a key enzyme that converts H_2O_2 to H_2O, thereby reducing H_2O_2 levels in plants.

On the other hand, no significant increase in CAT activity was observed in any of the lines tested. This is not unexpected since CAT removes excess H_2O_2 accumulation during oxidative stress (Mittler 2002), and no increased H_2O_2 content was observed after exposure to 37 °C in any of the three lines studied. Gür et al. (2010) found increased CAT activity after cotton leaves were exposed to a temperature of 45 °C, but not 38 °C which is in line with our results.

Higher activity of SOD, an important scavenger of toxic superoxide radical, was observed immediately after heat stress in wild-type seedlings. However, after a recovery period of 24 h, increased activity was also found in *oeBPM1* and *amiR-bpm*, indicating a delayed activation of SOD which could be due to altered *BPMs* expression in these lines. Increased SOD activity as a result of heat stress is consistent with the work by Kumar et al. (2012), in which increased enzyme activity after exposing wheat plants to 35 °C for two hours was detected. Moreover, *oeBPM1* showed considerably lower SOD activity compared to wild-type and *amiR-bpm*. Sandhya et al. (2021) showed that rice plants overexpressing *DREB2A* gene exhibited increased SOD activity during drought. Thus, it is possible that lower levels of SOD detected in *oeBPM1* could be due to the negative regulation of DREB2A through BPM1-mediated proteasomal degradation. Reduced SOD level in *oeBPM1* is interesting in light of the findings of Guan et al. (2013), who showed that down-regulation of *CSD* genes, i.e. genes encoding Cu/Zn-SOD, increases thermotolerance in plants. Specifically, this implies that *oeBPM1* could be less heat-sensitive than wild-type and *amiR-bpm*. Interestingly, although the increased SOD activity usually results in higher accumulation of H_2O_2, this was not observed in any of the lines exposed to 37 °C. Increased activity of APX, observed in our research, could explain this observation, since it is an enzyme which catalyses degradation of H_2O_2. Moreover, we could assume the participation of non-enzymatic antioxidants which also contribute to the regulation of ROS content, including H_2O_2 (Mittler et al. 2002; Hasanuzzaman et al. 2020). Different G-POD, APX and SOD activity in wild-type seedlings sampled at first and second time point could be surprising because these two groups (C-HS and C-REC) were not heat-treated and the only difference between them was 24-h delayed sampling of the second group (C-REC). However, these differences in antioxidant enzyme activity are not unexpected considering the dynamic developmental processes in seedlings, in which ROS are involved (Swanson and Gilroy 2010; Huang et al. 2019). As mentioned earlier, ROS levels are controlled by antioxidant system meaning that any variation in ROS content could cause a change in antioxidant enzyme activity.

Numerous studies (Ozden et al. 2009; Gill et al. 2013; Li et al. 2018) have detected enhanced levels of proline during various types of abiotic stress, suggesting its role in osmo-

regulation, free radical scavenging, protein stabilization, cellular redox and cytosolic pH balance. However, our results showed decreased proline content in all three lines after exposure to 37 °C. Similar results were observed in the study of Gür et al. (2010) and Kumar et al. (2012), after cotton and wheat plants, respectively, were exposed to a series of elevated temperatures. Rizhsky et al. (2004) hypothesized that accumulated proline could have a harmful effect on plants during heat stress. This hypothesis was confirmed by the research of Lv et al. (2011), in which a transgenic *Arabidopsis* line with ectopic expression of *P5CS1* gene was used. Since P5CS1 is part of the proline biosynthesis pathway, this line accumulates a higher level of proline than wild-type. It was shown that plants with increased proline content had lower chlorophyll content and lower rate of survival, but higher MDA content and antioxidant enzyme activity after exposure to elevated temperatures, suggesting that increased proline content causes reduced thermotolerance. This finding is particularly interesting in the context of our results, where *oeBPM1* generally had lower proline content, indicating a better ability to resist elevated temperatures than wild-type and *amiR-bpm*. In addition, exposure to 37 °C induced the dark green colour of *oeBPM1* leaves which could be correlated to the "stay green" phenotype associated with heat tolerance. For instance, lower activities of chlorophyll-degrading enzymes were detected in heat-tolerant transgenic lines of bentgrass (*Agrostis* spp.) (Rossi et al. 2017). The lower proline content in *oeBPM1* could be explained by negative BPM1-DREB2A regulation. According to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways database, DREB2A is involved in proline metabolism by down-regulating proline dehydrogenase and prolyl 4-hydroxylase, enzymes involved in proline catabolism. This was confirmed by a study of Xiu et al. (2016), in which *Robinia pseudoacacia* L. plants overexpressing *DREB2A* gene were exposed to drought treatments and showed a higher level of proline compared than the wild-type. Overall, it is possible that due to overexpression of *BPM1*, DREB2A levels are reduced in *oeBPM1*, resulting in increased proline catabolism rate and leading to a generally lower proline content in this line.

PCA analysis summarized the measured biochemical parameters and highlighted similarities and differences in the biochemical responses of *oeBPM1*, *amiR-bpm* and wild-type *Arabidopsis* lines to moderate heat stress. At both time points – immediately after exposure to the stress and after 24 h of recovery, grouping based on expression levels of *BPM* genes was observed. These results suggest that the studied lines use different strategies to cope with adverse environmental conditions, i.e. elevated temperature, indicating that BPM proteins are part of the plant response to heat stress. Our hypothesis is consistent with research by Morimoto et al. (2017), in which BPM proteins were found to modulate plant thermotolerance through negative regulation of DREB2A.

The present study has revealed differences in the biochemical response of *Arabidopsis thaliana* with modified expression of *BPMs* to moderate heat stress. Although the temperature value used did not cause a significant level of oxidative stress, different dynamics in H_2O_2 and proline content, as well as in antioxidant enzyme activity were observed. However, to understand the molecular mechanisms of BPMs involvement in stress responses and the strategies used by particular lines better, additional analyses should be performed.

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