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The response of *Petunia* × *atkinsiana* 'Pegasus Special Burgundy Bicolor' to mechanical stress encompassing morphological changes as well as physiological and molecular factors

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In 1973, Jaffe identified and characterized the phenomenon of thigmomorphogenesis, also referred to as mechanical stress (MS) or mechanical stimulation in plants. Previous studies on petunia plants demonstrated that MS significantly affects growth dynamics. As a response to MS, petunias exhibit increased levels of indole-3-acetic acid (IAA) oxidase and peroxidase, although the active transport of endogenous IAA remains unaffected. Furthermore, earlier research has shown that MS inhibits the synthesis of IAA and gibberellin (GA_3), with noticeable effects on the 14th day of mechanical stimulation. The current experiment made on *Petunia* × *atkinsiana* 'Pegasus Special Burgundy Bicolor' focused on evaluating the morphological and physiological responses to MS, along with the expression of specific touch-responsive genes such as GH3.1, which is involved in auxin metabolism, and calmodulins (CaMs), playing an important role in stress responses. GH3.1 expression was found to be negatively correlated with IAA synthesis while positively correlated with GAs synthesis and IAA oxidase activity. Variable expression patterns were observed in the calmodulins: CAM53 and CAM81 expression positively correlated with IAA synthesis and plant height, whereas CAM72 expression was positively associated with GAs levels and IAA oxidase activity in plants touched 80× per day, but all of them were negatively related to IAA content and shoot increment, while positively related to GAs synthesis and IAA oxidase activity.

Keywords Touch stress, Thigmomorphogenesis, Auxin synthesis, Calmodulin, Gene expression

Under natural conditions, plants are exposed to touch stress caused by wind, rainfall, snowfall, and contact with neighbouring plants[1]. The phenomenon of plant responses to such mechanical stresses was first described by Jaffe[2], and named thigmomorphogenesis. This refers to the ways in which plants adapt their growth and development in response to mechanical stimuli, including touch stress, encompassing alterations in shoot elongation, root development and their mechanical reforcement, branching patterns, flowering etc.[3–9].

Plants subjected to prolonged touch stress undergo changes in their growth characteristics such as a reduction in growth rate leading to compact growth, inhibition of internode elongation, and increased diameter[1,3,5,10–14]. However, the plant's response depends the type of touch stimulus (wind, brushing, pressure, vibration, or shaking), its frequency and duration, and its species, or even variety, specific[2,5,12,13,15]. Moreover, the age of the tissue or organ on which mechanical stress acts is crucial. The younger the tissue or organ and the shorter the

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terminal internode, the greater the potential for growth inhibition under touch stress[16]. Young plants respond more quickly, and when exposed to touch stress, they become more resistant to physical stimulis through shortening and thickening their stems[17–19]. In the experiment by Jędrzejuk et al.[5], touching *Petunia* × *atkinsiana* 'Pegasus Velvet Picotee' 80 times a day for a month resulted in a 43.2% reduction in plant size compared to the control. According to[13–15], tomato growth under touch (wind) stress was stunted, and plants were 26–36% shorter than controls. Growth inhibition in tomatoes under brushing was also observed in the experiments of Duman and Düzyaman[4].

Growth inhibition is primarily associated with auxin synthesis[3,12,20–23]. Auxins play crucial roles in various growth and developmental processes, including cell elongation, differentiation, and regulating plant responses to biotic and abiotic stresses[24]. Particularly, Indole-3-acetic acid (IAA), serves as a common substrate for Gretchen Hagen 3 proteins (GH3)[25]. The GH3 protein family, including GH3.1, possesses auxin amino acid synthase activity, converting active IAA into an inactive state by binding free IAA to amino acids[26,27]. Through the treatments and degradation of the amino acid molecule and IAA, the plant can maintain homeostasis[28]. The GH3.1 gene is responsible for the negative feedback regulation of IAA concentration[29]. Excessive IAA levels lead to an upregulation of GH3 expression, resulting in the storage or degradation of amino acid-bound IAA. Upregulation of GH3 genes was observed in plants subjected to salt, heavy metal, cold, and drought stresses[30–37]. The decreased free IAA content and plant dwarfing was observed in transgenic rice plants upregulating the OsGH3.1 gene of transgenic rice plants[38]. Upregulation of the GH3-13 gene in rice subjected to drought stress reduced the level of free IAA and was an indicator of increased plant tolerance to stress[39,40]. The effect of touch stress on GH3 gene expression has not yet been shown.

The inhibited plant growth is associated also with disruption of auxin synthesis and activation of IAA oxidase (IAAO) and peroxidase (POD)[9,12,41]. IAAO activity might play a pivotal role in controlling endogenous IAA levels, and the relationship between the levels of IAA and IAAO activity is negatively correlated[42–44].

The interaction between IAA and gibberellins (GAs) has been also extensively studied[45–48]. It was shown that auxins regulate GAs biosynthesis and signalling and both together, IAA and GAs, are responsible for regulating the elongation of the shoot while GAs are mainly responsible for flowering[48–50]. Touch stress can lead to delayed flowering and a fewer flowers, thereby impacting the adjustment of flowering dates and flower intensity[5,51–53]. Auxins likely mediate the floral transition through a positive interaction with GAs signalling by modulating GA levels or promoting DELLA protein degradation[46,48,54]. In the recent experiment by Jędrzejuk[12], it was also observed that prolonged touch stress caused by 120 and 160 touches a day resulted in the arrest of IAA and gibberellic acid (GA_3) in plants.

One of the first measurable changes under touch stress involves an elevation in intracellular Ca^{2+} ions, serving as a secondary transmitter of mechanical signals in plants responding to various abiotic factors[55–59]. Alterations in Ca^{2+} levels are thought to establish a connection between the mechanical stimulus and plant growth responses. Mechanical stimuli prompt the expression of diverse genes, including those encoding the CALcium MODULationg protein (calmodulin; CaM) and CaM-related proteins[10,60,61]. There is a common agreement that calmodulins play crucial roles in plant adaptation to stress and contribute significantly to plant defense[62–64].

Braam et al.[10] discovered the existence of touch-induced genes (TCH), which encode for various proteins, including CaM, CaM-related, and a xyloglucan endotransglycosylase. TCH1 encodes CaM, TCH2 and TCH3 encode CaM-related proteins[10,65,66]. An increased expression of these genes was observed 10–30 min after the onset of touch stress, but the expression was not suppressed after 1 h[10]. The expression levels of TCH2 and TCH4, encoding xyloglucan endotransglycosylase increased in inflorescence stems when increased weight was applied at the apex, resulting in enhanced secondary growth[65]. Consequently, TCH regulation takes place not solely in response to mechanical stimuli but can also be triggered by mechanical strains potentially produced during morphogenesis[67,68]. The unique regulatory property of the TCH proteins may elicit physiological and morphological adaptations in plants in response to environmental factors[67].

The purpose of the present work was to determine how touch stress affects the expression of selected touch stress-related genes, responsible for the plant reaction, aspecially the growth and flowering dynamics of *Petunia* × *atkinsiana*. Additionally, the study was designed to explore how touch stress affects these parameters over time and with varying levels of intensity.

Materials and methods

Four-week-old seedlings of *Petunia* × *atkinsiana* 'Pegasus Special Burgundy Bicolor', with purple–white rays on the petals, were obtained from Volmary Polska Company. In early February, the plants were planted into 11 cm diameter pots into substrate dedicated for bedding plants and composed of high peat and wood fibers Ecofibrex, pH 5.5 (Kronen, Poland). The plants were placed on a table with an ebb and a flow bench (95 × 480 cm), in a greenhouse at the Warsaw University of Life Sciences, Poland.

The average temperature during the day was 22 °C and at night 18 °C. The relative air humidity was 75%. Conditions in the greenhouse were controlled by the HortiMax (Synopta software) climate computer.

Experimental design

Touch stress (mechanical stimulation) was applied to plants by brushing them 80 or 160 times a day, using a brushing instrument described in details previously[5], for 56 days, from February 22nd till April 20th when the brushing instrument was turned off. Plants were further evaluated for the next 15 days until the 71st day of the experiment. The control plants were growing at the same time without brushing also for 71 days.

Evaluation of plant growth and flowering and sampling for biochemical analyses and determination of gene expression were done five times during the experiment, at the beginning (day 0) and 7, 14, 56 and 71 days after experiment started. Due to small size of the terminal shoot fragments at the beginning of experiments, IAA

content, IAAO activity and gene expression were not determined at day 0. In one vegetation season 90 plants were used, 30 plants in each of three treatments (control, 80× and 160× touching a day). The frequency of 80 and 160 touches per day was selected based on previous studies by Jędrzejuk et al.[5,12]. In earlier research, plants subjected to 80 touches per day exhibited the most pronounced response to the treatment compared to those receiving 40 or 60 touches (see[5]). To further investigate the effect of touch intensity, subsequent experiments applied 80, 120, and 160 touches per day. The 120-touch treatment was excluded due to inconsistent plant responses, whereas plants exposed to 80 and 160 touches per day demonstrated clear and measurable reactions to stress.

In each treatment, 15 random plants were labeled by consecutive numbers and consistently evaluated for their growth and flowering in each of the indicated above five dates during the experiment, while the remaining 15 plants were sampled for analyses.

The experiments were conducted for 3 seasons (2020–2022) considered as independent replications. The results of biometric measurements and analyses of IAA content and IAAO activity are means from 3 seasons. The GAs content was determined in one season.

Biometric evaluation

The length of the main shoots of the plant was measured at each evaluation date and the increments between consecutive dates were calculated. The mean shoot increment per plant was then calculated.

The number of fully open flowers was counted at the beginning of flowering e.g. in the 30th day of the experiment, at the end of brushing (day 56), and at the end of the experiment (day 71).

Number of branches

Lateral shoots (side branches) were defined as shoots emerging from leaf axils along the main stem. Only shoots exceeding [e.g., 1 cm in length] were considered for counting. Each plant was inspected visually, and the number of lateral shoots on the main stem was recorded manually. The counting was performed from the base of the stem upwards, ensuring that no lateral shoots were missed. The count was done using a hand-held counter to maintain accuracy. The process was repeated for 15 plants per treatment, and the average number of lateral shoots per plant was calculated. Replications were carried out to ensure statistical reliability.

Biochemical analyses

For biochemical analyses, 1.5 cm shoot terminal fragments of 1.5 cm in length, including the apical meristems, were taken from three random selected plants at each observation date. The samples were frozen in liquid nitrogen and stored at − 20 °C until analysis.

The dry weight (DW) was determined by drying samples at 105 °C until a constant weight was obtained.

IAA content

The content of free IAA was determined according to a method with the Salkowski reagent Gang et al.[69] and by measuring absorbance at 520 nm using the Shimadzu UV-1280 (Shimadzu, Japan) spectrophotometer. The IAA content was expressed in $ng \cdot g^{-1}$ DW.

IAAO activity

The IAA oxidase activity was determined according to Zhang[70]. Absorbance was measured at 530 nm using the UV1600 spectrophotometer (AOE Instruments, Shanghai). Three replicates were performed for each treatment. Results were expressed in μg IAA per g^{-1} DW per hour. It is assumed that 1 unit [1U] of IAA oxidase degrades in 1 h.

General gibberellins content

Total GAs content was determined according to the method of Graham and Thomas[71]. The method is effective in determining endogenous GA_3, GA_1, and GA potassium salts. The method does not determine auxins and cytokinins. Absorbance was measured spectrophotometrically at 430 nm by using the UV1600 instrument (AOE Instruments, Shanghai). The blank sample was distilled water. The content of total GAs was determined according to the curve for GA_3 and expressed in $ng \cdot g^{-1}$ DW.

Statistical analysis

Statistical analysis was performed using the General Linear Model program Statgraphics Centurion XIX 2019. ANOVA1 was used for biometric and biochemical analyses, and means were compared using the LSD test. Statistical analysis was made in each term separately. The significance level was $\alpha = 0.05$.

Correlations between variables were assessed using Pearson's correlation coefficient (r). Correlation analysis were performed using Statgraphics Centurion XIX 2019, with a significance level set at $p < 0.05$. The results of the correlation have been included in the supplementary (supplemetary Table S1 and S2).

Gene expression

Gene expression was determined by real-time quantitative PCR. Plant material was collected on 4 dates: 7 and 14 days after the experiment started, 56 days after experiment started (end of MS process) and 71 days after the experiment started (15 days after MS was turned off, to check plant behaviour). 1.5 cm stem apical meristems were taken. Material was collected from 9 plants (divided into three blocks of three plants in each treatment) into sterile cryoprobes. Samples were immediately frozen in liquid nitrogen. The analysis was carried out in 2022.

Total RNA was isolated using the Direct-zol RNA MiniPrep Plus kit with TRI Reagent (Zymo Research, Irvine, CA, USA) and followed by DNase I treatment (Thermo Fisher Scientific). RNA purity were estimated on the NanoDrop ND-1000 (NanoDrop Products, Wilmington, DE, USA). RNA purity was checked by PCR and

qPCR on the RT control. cDNA was synthesized using the NG dART RT kit (EURx). Quantitative RT-PCR was performed in triplicate using the QuantStudio 3 Real-Time PCR (Applied Biosystems) with the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific), with the primers listed in Supplementary Figure (Table S3). Primers were validated for single product specificity and their effectiveness to range between 90 and 105%

Real-time PCR cycling conditions were as follows: 3 min denaturation at 95 °C and 40 cycles of amplification (10 s at 95 °C, 45 s at 55 °C). Product melting curves were generated following PCR to ensure purity of the amplification products.

Normalization was done to the expression of the *PhCYP* gene. The Pfaffl method was applied to calculate the relative gene expression and the randomization test with default 10.000 random reallocations to test the statistical significance of calculated expression ratios of the sample to the control was performed using the REST 2009 (Qiagen) software[72]. The difference between mean expressions was considered significant at $p < 0.05$.

Results

Touch stress affected shoot growth but did not arrest a number of flowers

Touch stress significantly affected plant architecture and number of flowers (Figs. 1 and 2). The habitat of stressed plants was more compact, with a higher number of later shoots compared to control. The shoots of stressed plants were thicker (unpublish data).

In this study, the *Petunia* × *atkinsiana* 'Pegasus Special Burgundy Bicolor' plants of all treatments reached full flowering on the 30th day of the experiment. On this date, plants stressed with 160 × had statistically the highest number of flowers (ca. 3 flowers/plant) (Fig. 2). On the 56th day of the experiment, the differences in flowering between the stressed and control plants was more significant. Plants stressed 80 and 160 times a day had significantly more flowers (12.67 and 16.27, respectively) than control plants. At the last measurement date (71st day of the experiment), there were also more flowers in stressed plants (ca. 29 flowers in both treatments) than control plants (24 flowers/plant).

The effect of mechanical stress (MS) on the number of branches was observed (Fig. 3). Thirty days after the onset of MS, the highest number of branches was recorded in plants subjected to 160 touches per day, with an average of 13.53 branches. Fifty-six days after the experiment began, plants exposed to touch stress exhibited a significantly higher number of branches compared to the control group (Fig. 3). However, fifteen days after the cessation of MS, no significant differences in branch numbers were observed across all treatment groups.

The effect of mechanical stress on plant growth dynamics was observed (Fig. 4). The first differences in plant height were visible from the 7th day of the experiment (Fig. 4). On the 7th day of stress, plants touched 80 times a day were shorter than the control, which was not seen in plants stressed 160 times a day. The increase in height for plants touched 80 times a day was 1.66 cm, while in plants stressed most intensively (160 times) the increase was almost twice as much (3.02 cm) (Fig. 5).

On the 14th day of stress, plants touched 80 times a day were the shortest compared to the other treatments (Fig. 4). There were statistically significant differences in shoot increment between treatments (Fig. 5). The smallest increment was observed in plants treated 160× per day (4.5 cm), while the increment in control plants was the highest (5.21 cm).

On the 56th day of stress, the stressed plants were statistically shorter (ca. 22 cm) than the control plants (26.9 cm) (Fig. 4). In addition, there were no statistically significant differences between those stressed 80 and 160 times a day. When analyzing the shoot increment, there was a statistically significant difference between all examined plants (Fig. 5).

Fig. 1. *Petunia* × *atkinsiana* 'Pegasus Special Burgundy Bicolor' subjected to mechanical stimulation, on the 30th day of the experiment (beginning of full flowering). From left: control (without touch stress), 80 and 160 touches per day.

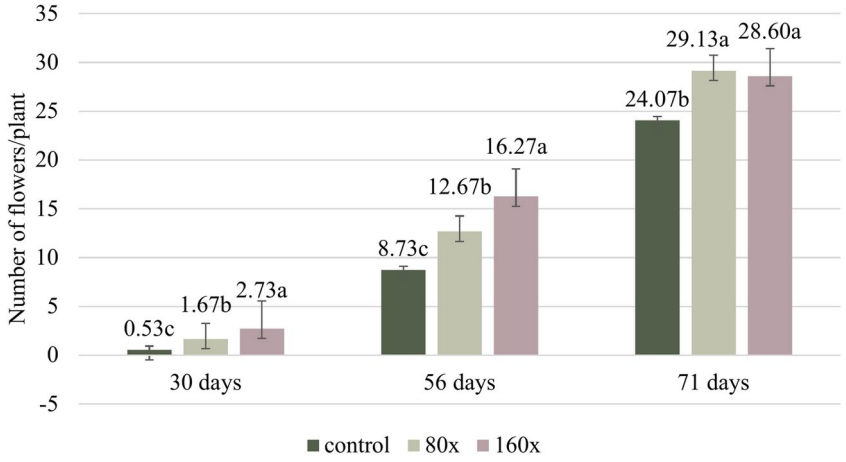

Fig. 2. Effect of mechanical stress on flowering dynamics. Number of petunia flowers subjected to mechanical stimulation (MS) depending on the intensity of stress; subjected to control, 80 and 160 cycles per day of mechanical stimulation, 30th day of the experiment (beginning of full flowering). 15 plants in each block from each treatment were measured. The letters represent the statistical differences ($\alpha \leq 0.05$) between the treatments; means labeled with the same letter do not differ significantly ($P = 0.05$); a represents the highest value. Data and standard error bars are representative of three independent replications, where each replication is an average from each season (2020, 2021, 2022). Across all seasons, the data trends were consistent.

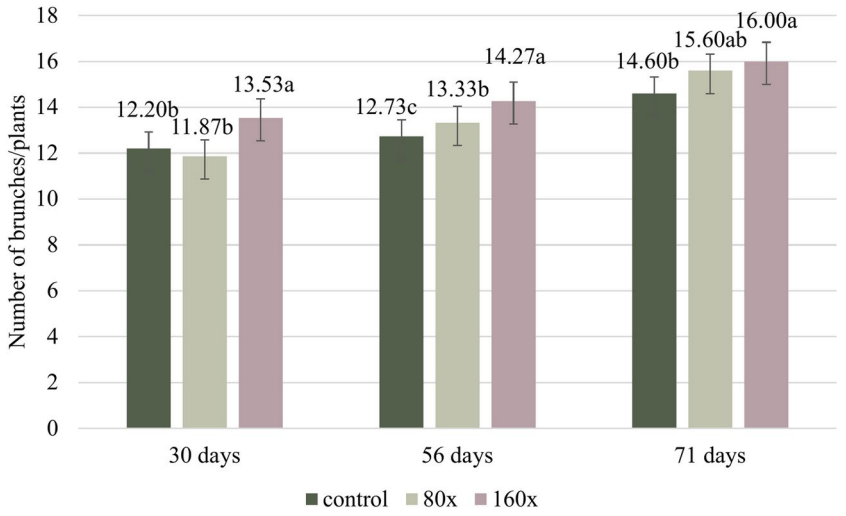

Fig. 3. Effect of mechanical stress on the number of branches. 15 plants in each block from each treatment were measured. The letters represent the statistical differences ($\alpha \leq 0.05$) between the treatments; means labeled with the same letter do not differ significantly ($P = 0.05$); a represents the highest value. Data and standard error bars are representative of three independent replications, where each replication is and average from each season (2020, 2021, 2022). Across all seasons the data trends were consistent.

On the 71st day of the experiment, plants stressed 160 times a day were the tallest among all treatments (37 cm), while those touched 80 times a day were the shortest (30.1 cm). At the same time, there were also statistically significant differences in shoot growth dynamics (Fig. 5).

IAA content, IAA oxidase activity, and GH3.1 gene expression

IAA content varied irrespective of stress duration and the intensity of stimulation (Fig. 6). Significant statistical differences were observed on day 14 of the experiment. Stressed plants had significantly lower IAA content relative to the control. Plants stressed 80 times a day had the lowest IAA content of all treatments (190.38 $ng \cdot g^{-1}$ DW). Fifteen days after the end of the experiment (71th day), no statistically significant differences were observed between all treatments.

On day 14 of the experiment, a significant increase in IAAO activity was observed in stressed plants compared to the control (Fig. 7). On the 56th day of the experiment, plants touched 80 times a day had the lowest IAAO

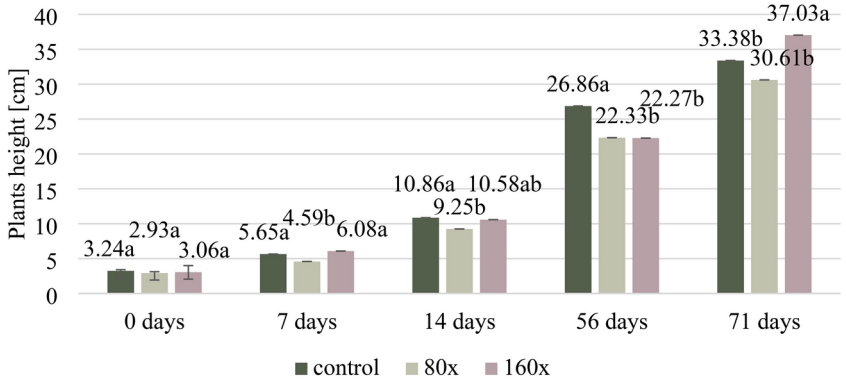

Fig. 4. Growth dynamics of stressed plants- Plant height [cm]. 15 plants in each block from each treatment were measured. The letters represent the statistical differences ($\alpha \leq 0.05$) between the treatments; means labeled with the same letter do not differ significantly ($P = 0.05$); a represents the highest value. Data and standard error bars are representative of three independent replications, where each replication is an average from each season (2020, 2021, 2022). Across all seasons, the data trends were consistent.

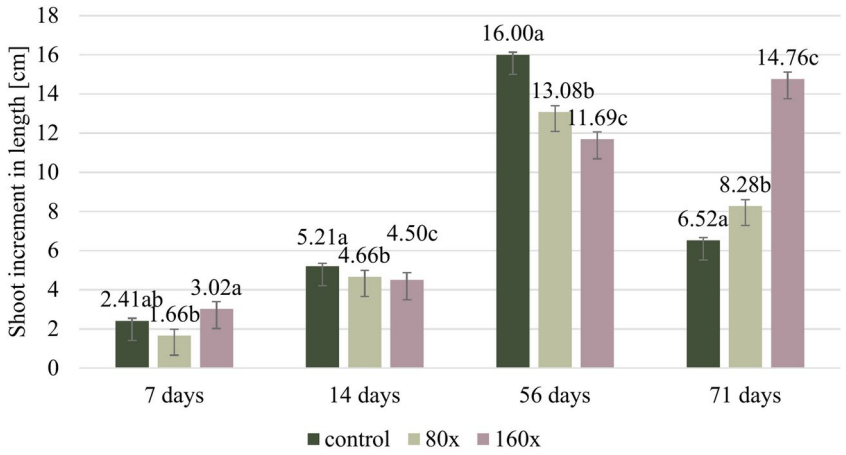

Fig. 5. Growth dynamics of stressed plants- Shoot increment [cm]. 15 plants in each block from each treatment were measured. The letters represent the statistical differences ($\alpha \leq 0.05$) between the treatments; means labeled with the same letter do not differ significantly ($P = 0.05$); a represents the highest value. Data and standard error bars are representative of three independent replications, where each replication is an average from each season (2020, 2021, 2022). Across all seasons, the data trends were consistent.

activity of all the treatments and at the same time low IAA content (Fig. 6). Fifteen days after the end of the experiment (71th day), no statistically significant differences were observed between the treatments.

On the 14th day of the experiment, GH 3.1 was up-regulated under stress in each treatment (Fig. 8). Simultaneously, on this date, plants subjected to 80 and 160 stress cycles per day exhibited higher IAA oxidase activity than control plants (Fig. 7). On day 56 of the experiment, plants stressed 160 times a day showed up-regulation of the GH3.1 gene. On day 71 of the experiment, plants stressed 160 times a day were characterized by upregulation of the GH3.1, despite similar IAA oxidase activity as in the other treatments (Fig. 7) and a marked difference in growth compared to the control (Fig. 4).

Total gibberellins content

The total content of gibberellins exhibited variations based on the duration of stress and the treatment (Fig. 9). On day 14 of the experiment, the stressed plants also showed a statistically higher total gibberellins content. Plants stressed 80 per day had the statistically highest content of total gibberellins (11.04 ng·g s.m.$^{-1}$) (Fig. 10).

However, on day 56 of stress, an inverse relationship was observed. The control plants had the statistically highest content of total gibberellins (9.64 ng·g s.m.$^{-1}$). The lowest content was recorded in plants stressed 160 times daily (5.48 ng·g s.m.$^{-1}$). On day 71 (after the stress), the stressed plants had higher or comparable total gibberellins content (8.93 and 7.61 ng·g s.m.$^{-1}$, respectively) compared to the control (7.86 ng·g s.m.$^{-1}$).

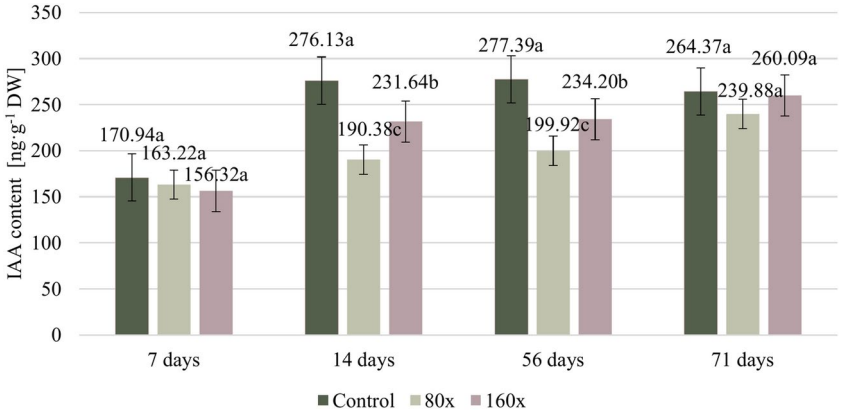

Fig. 6. IAA content (ng·g^{-1} DW), analyzed separately, of petunias subjected to MS depending on stress intensity duration. 15 plants in each block from each treatment were measured. The letters represent the statistical differences ($\alpha \leq 0.05$) between the treatments; means labeled with the same letter do not differ significantly ($P = 0.05$); a represents the highest value. Data and standard error bars are representative of three independent replications, where each replication is and average from each season (2020, 2021, 2022). Across all seasons, the data trends were consistent.

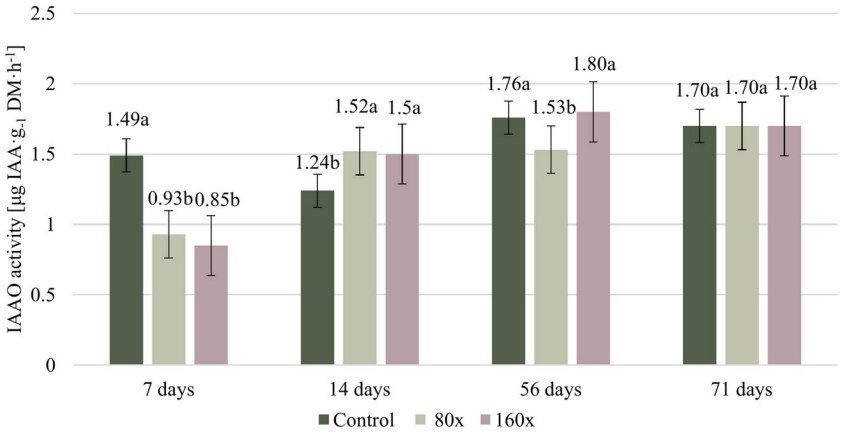

Fig. 7. IAAO activity (µg IAA·g $^{-1}$ DM·h^{-1}) of petunias subjected to MS depending on stress intensity duration (analyzed separately). 15 plants in each block from each treatment were measured. The letters represent the statistical differences ($\alpha \leq 0.05$) between the treatments; means labeled with the same letter do not differ significantly ($P = 0.05$); a represents the highest value. Data and standard error bars are representative of three independent replications, where each replication is an average from each season (2020, 2021, 2022). Across all seasons, the data trends were consistent.

Calmodulin-encoding genes (CaM72, CaM53 and CaM81)

In the current experiment, the expression of three genes (CaM72, CaM53 and CaM81) encoding calmodulin synthases was investigated. On the 7th day of stress, an increase in the expression of CaM53 and CaM81 was observed in plants touched 80 times per day (Figs. 11, 12). In contrast, no expression of the CaMs tested was observed in plants stressed 160 times a day.

After next 7 days, upregulation of the CaM72 and CaM53 gene was observed in all treatments (Figs. 10, 11). Moreover, plants subjected to 160 touches per day showed an upregulation in CaM81 gene expression.

Following the 56th day of touch stress, all the examined calmodulin-encoding genes in all treatments displayed upregulation (Figs. 10, 11, 12). Fifteen days after the machine was turned off, both the CaM72 and CaM53 genes in all treatments were also observed to be upregulated (Figs. 10, 11).

III Correlation of biochemical parameters and gene expression in plants stressed 160 times a day

A strong correlation was observed between plant appearance and changes at the physiological and molecular levels in plants stressed 160 times a day.

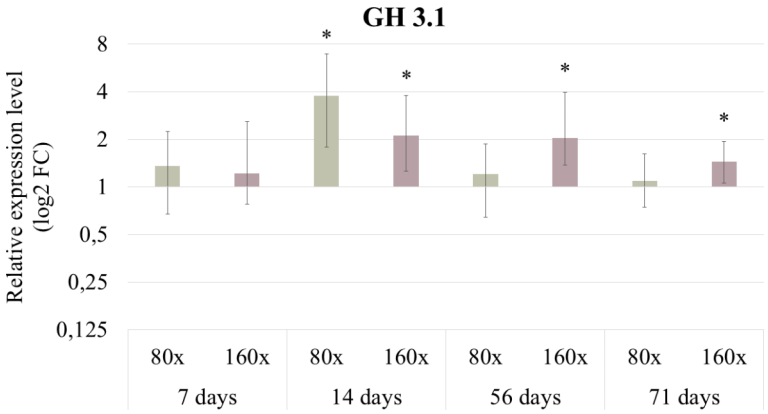

Fig. 8. Relative expression of the GH 3.1-synthetase gene IAA (EST884899). The fold change (FC) in relative expression level was normalized to nontreated samples (control). Error bars represent mean ± standard deviation (SD) with three biological replicates and three technical replicates. Values are the mean ± SD. ∗ $P < 0.01$.

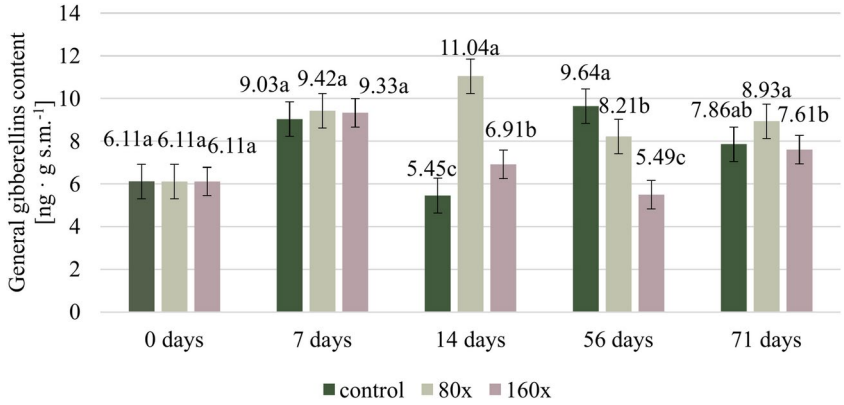

Fig. 9. General gibberellins content (ng·g s.m.$^{-1}$) of petunias subjected to MS depending on stress intensity duration. 15 plants in each block from each treatment were measured. The letters represent the statistical differences ($\alpha \leq 0.05$) between the treatments; means labeled with the same letter do not differ significantly ($P = 0.05$); a represents the highest value. Data and standard error bars are representative of three independent replications, where each replication is an average from each season (2020, 2021, 2022). Across all seasons, the data trends were consistent.

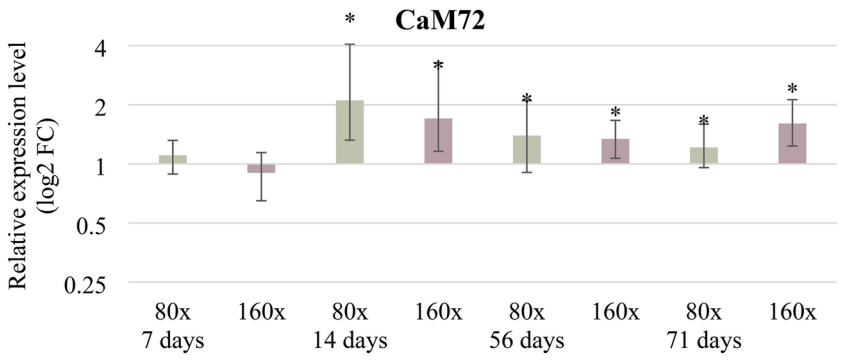

Fig. 10. Relative expression of calmodulin-encoding gene (CaM72). The fold change in relative expression level was normalized to nontreated samples (control). Error bars represent mean ± SD with three biological replicates and three technical replicates. Values are the mean ± SD. ∗ $P < 0.01$.

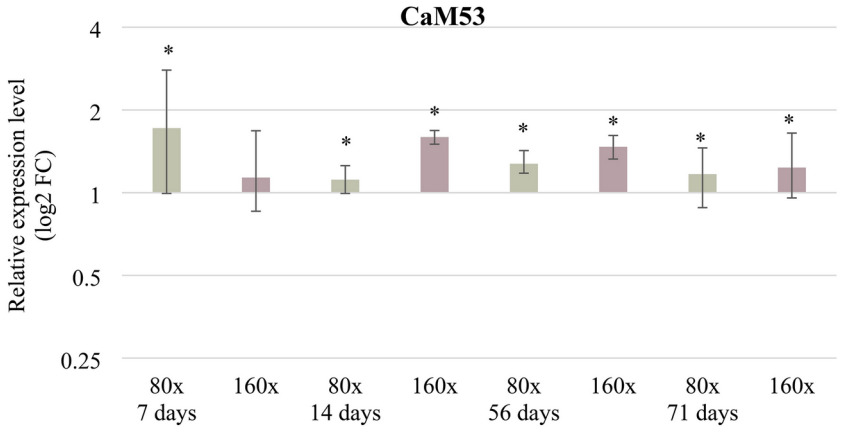

Fig. 11. Relative expression of calmodulin-encoding gene (CaM53). The fold change in relative expression level was normalized to nontreated samples (control). Error bars represent mean ± SD with three biological replicates and three technical replicates. Values are the mean ± SD. ∗ $P < 0.01$.

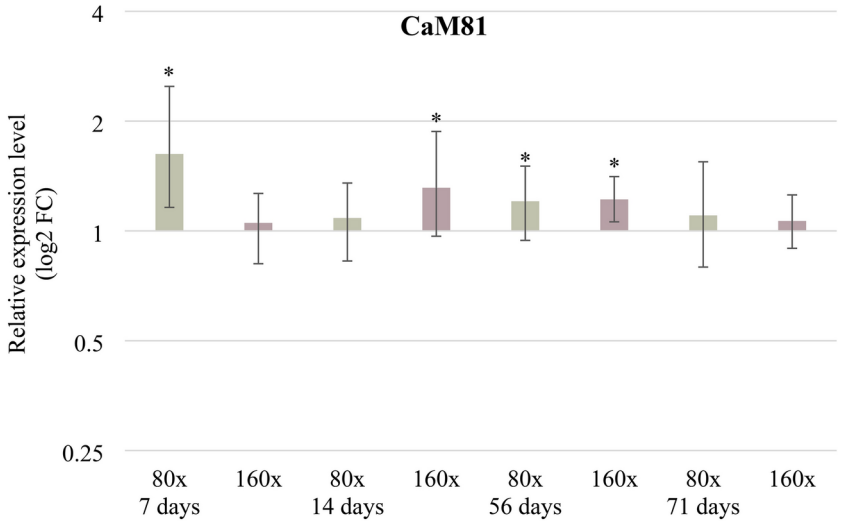

Fig. 12. Relative expression of calmodulin-encoding gene (CaM81). The fold change in relative expression level was normalized to nontreated samples (control). Error bars represent mean ± SD with three biological replicates and three technical replicates. Values are the mean ± SD. ∗ $P < 0.01$.

GH3.1

In plants stressed 160 times a day, a high negative correlation was observed between GH3.1 and IAA content (− 0.803; $p < 0.01$) (Suppl. Tab. S2). Additionally, a very high correlation was observed in the GH3.1 gene and IAA oxidase activity (0.861; $P < 0.01$), as well as a negative correlation between GH3.1 and shoot increments (− 0.969; $P < 0.01$).

Calmodulins

In plants stressed 160 times a day, a very high positive correlation was observed between GH3.1 and CaM81 as well as CaM53 (0.959; 0.985; $p < 0.001$, respectively) (Suppl. Tab. S2). Considering the correlation of CaM81, CaM53, and CaM72 expression with growth dynamics (total plant height and shoot increments), it was found that in plants stressed 160 times a day, CaM53 and CaM81 expression did not affect growth inhibition (0.851; 0.756; $P < 0.01$, respectively). However, a strong negative correlation was observed between CaM81 and CaM53 and shoot increments (− 0.873; − 0.911; $P < 0.01$, respectively).

Discussion

Touch stress affects shoot growth but does not arrest the number of flowers

In response to the touch stimuli, there is an inhibition of growth rate, reduction in internode length, radial growth and more compact growth habits[1,3,5,11–15,73,74]. According to Autio et al.[75], stroking plants for as little as 60 min/day allows for a significant reduction in height. Petunia (*Petunia* × *atkinsiana*) growth was reduced by

18%, while Aster (*Aster dumosus*) by 25%. No such effect was noted in the dusty miller (*Jacobaea maritima*). In the current experiment, after 56 days of stress, a reduction in petunia growth of 16.86% and 17.09% was observed in plants subjected to 80 and 160 touches a day, respectively compared to the control. The experiment by Autio et al.[75] also indicated that the rate of plant response varied among species.

These changes are often linked to alterations in auxin synthesis and distribution, as auxin is crucial in regulating plant growth and response to environmental stimuli. Touch stress can alter auxin pathways, leading to a redistribution of the hormone and consequently modifying growth patterns, especially in height and number of branches[5,76–78]. Several research indicates that the susceptibility of a plant to a stimulus, however, depends on the species, the age of the tissue or the type of mechanical factor[14,16,79,80]. Auxin synthesis and transport are particularly sensitive to these mechanical cues, with increased mechanical stress often inhibiting auxin flow to growing regions, thereby reducing internode elongation and plant height[16,81]. There was a decrease in auxin levels in *Bryonia dioica* as a result of mechanical stress (brushing), and an increase was observed in *Phaseolus vulgaris* as a result of brushing[76]. The increase in auxin levels was explained by slowed transport of IAA in internodes and accumulation of IAA in the stem. In an experiment by Mitchell[77], inhibition of polar auxin transport in stems due to stem rubbing was observed in peas. In an experiment by Jędrzejuk et al.[5], Petunia cv. 'Pegasus Velvet Picotee' stressed 80 times a day showed a high increase in endogenous IAA, and a concomitant inhibition of elongation growth, which was explained by a disruption of polar auxin transport rather than IAA synthesis. In the current experiment, it was observed that the first differences in growth were visible as early as 7 days after the onset of stress. On the 7th day of stress, plants touched 80 times a day were shorter than the control. Interestingly, the growth dynamics of the most intensively stressed plants were twice as much as those stressed 80 times. However, reduction in plant height as a result of mechanical stress and differences in growth dynamics compared to unstressed plants were evident throughout the experiment (except on the 14th and 71st days). On the 14th day of the experiment, no statistically significant differences were observed between the stressed and control plants. However, differences in growth dynamics were apparent. Stressed plants exhibited the smallest growth rates compared to the control. Jędrzejuk et al.[12] reported similar findings, indicating no differences in shoot growth for plants stressed on day 14th. The absence of statistically significant differences in growth on the 14th day of the experiment, despite visible differences in growth dynamics, can likely be attributed to changes in endogenouse auxin content and its regulation. According to the findings, IAA levels were significantly lower in stressed plants, particularly in those touched 80 times a day, by day 14. This decrease in IAA could inhibit elongation growth, but the reduction may not have been large enough to produce statistically significant differences in overall plant height at that point. However, the growth dynamics differ because the plants were still responding to mechanical stress by altering their hormonal balance. During mechanical stress increased activity of IAA oxidase, which degrades IAA, slowing the growth of stressed plants was visible, compared to controls. This disruption in auxin homeostasis may explain why stressed plants had reduced growth increments without showing clear height differences by day 14. Over time, these dynamics would become more pronounced, leading to more significant growth differences later in the experiment.

The number of branches, flowers, and total gibberellin content were closely related and dependent on the duration and intensity of touch stress stimulus, as indicated in the literature[5,12,51,82,83]. According to Fu and Harberd[47] and Paponov et al.[84], gibberellins and auxins, in response to stress conditions, work together to stimulate DELLA accumulation and subsequently increase ROS uptake capacity and abiotic resistance. Touch stress typically stimulates the production of lateral shoots while simultaneously inhibiting the elongation of the main shoot. In the present experiment, by day 56, it was observed that stressed plants exhibited an increase in the number of lateral shoots alongside a reduction in shoot elongation and a slowdown in growth dynamics. This relationship can be explained by the interaction between auxins and gibberellins. On day 56, stressed plants showed an increase in IAA synthesis (compared to day 14), while the content of total gibberellins decreased over time (analyzing data from day 14 and day 56).

An increase in the number of lateral shoots often correlates with an increased number of flowers in stressed plants[85,86]. According to Morel et al.[85] and Vernieri et al.[86], touched plants produced more flowers, although their diameters were smaller. Conversely, in the experiment by Jędrzejuk et al.[5], plants subjected to 40, 60, and 80 touches per day, led to a reduction in flower numbers, decreasing from 36 to 21 in *Petunia* × *atkinsiana* 'Pegasus Velvet Picotee'. However, in the same experiment, the 'Dark Red' variety responded differently: stroking increased the number of flowers from 11 in the control group to 22 in the group touched 80 times. In both varieties, no significant impact of stress on flower diameter was observed (unpublished data). Onguso et al.[53] and Salehi and Salehi[83] also reported no impact of mechanical stress on flower number. In the current experiment, stressed plants flowered earlier and produced more flowers than the control group. This phenomenon may also prove the individual characteristics of genera, but also a variety. The response of studied petunias can be partly attributed to the role of gibberellins in promoting flowering. In the early stages of stress, gibberellin levels increased, promoting flower formation. However, prolonged touch stress, as observed on day 56, led to a significant reduction in GA content in severely stressed plants. According to Colebrook et al.[82] and Castro-Camba et al.[87], reduced GA content enhances abiotic stress tolerance, while increased GA content diminishes it. In current experiment increased GA content leading to increased number of flowers, may be positively found in commercial horticulture.

According to Achard et al.[88], DELLA proteins, key components of GA signalling, contribute to the growth inhibition of stressed plants and enhance stress tolerance through a shared mechanism. Based on the findings of Jędrzejuk et al.[12], growth inhibition and reduced GA_3 content under touch stress are already evident by day 30.

In the current experiment, the content of total GAs was also decreasing alongside touch stress duration, that was evident on day 56. Based on these findings, it may be claimed that decrease of GAs in stroked flowers is a defence against touch stress. Therefore, future investigations should focus on tracking changes in DELLA

protein content under touch stress at higher frequencies, and assessing the levels of free proline, and other osmoprotectants involved in regulating cellular homeostasis.

What are the relations between IAA content, IAA oxidase activity and GH3.1 gene expression in plants subjected to mechanical stimulation?

The alterations in shoot length in response to a touch stimulus are primarily attributed to disruptions in the synthesis of plant hormones, such as auxins[89,90]. Stress conditions can affect both IAA oxidase activity and IAA gene expression[91–93]. It has been proposed that IAA oxidase (IAAO) activities may help regulate IAA content[94,95]. IAAO is usually involved in auxin catabolism and negatively correlated with IAA levels, thereby regulating the concentration of IAA[94,96]. Several authors[97–101], claim that growth reduction during MS is a consequence of the IAA oxidation and an increase in the IAA oxidase, as well as other enzymes activities[101,102].

In the current experiment, mechanical stimulation led to observable changes in both IAA content and IAAO activity, with a significant negative correlation between these two parameters. On the 14th day of the experiment, IAA content was reduced in stressed plants, particularly in those touched 80 times per day, and this reduction was associated with an increase in IAAO activity, indicating that auxin catabolism was actively contributing to the regulation of growth under stress conditions. This relationship aligns with previous studies showing that mechanical stimulation increases IAAO activity, resulting in reduced auxin levels and growth inhibition[97,103,104]. In research made by Jedrzejuk et al.[5] in petunia 'Pegasus Velvet Picotee' the IAA oxidase activity was higher in brushed plants than in the controls during touch stress duration. This effect was not observed in petunia 'Dark Red'.

Alongside IAAO activity, the GH3.1 gene, which encodes an enzyme involved in auxin conjugation, was also upregulated in response to mechanical stress. GH3 expression is associated with enhanced stress tolerance, particularly in plants exposed to drought, cold, or salinity stress[32,33,40]. The GH3 family of enzymes is known to mediate plant responses to various stress factors by regulating auxin homeostasis through conjugation of free IAA to amino acids, thus rendering it inactive[32,35,105]. In the current experiment, it was observed that touch stress affected GH3.1 upregulation, with both treatments yielding a negative correlation with IAA content and shoot increment and a positive correlation with IAAO activity. On the 14th day of MS, the negative correlation between IAA synthesis and IAAO activity as well as GH3.1 upregulation, was observed. On the56th day of the experiment, upregulation of GH3.1 was observed only in plants subjected to 160 strokes per day. There was significant difference in IAAO activity and IAA synthesis between control and plants stroked 160× per day. Increased production of an IAA-conjugating GH3 enzyme was definitely associated with growth inhibition, closely linked to increased stress resistance. Since 56 day of the experiment plants obtained homeostasis in growth dynamics and thus, decreased IAAO activity connected with lower GH3.1 expression may be an explanation. This suggests that the combination of increased auxin oxidation and conjugation serves as a feedback mechanism to modulate auxin levels under prolonged stress, contributing to growth inhibition.

Interestingly, 15 days after stress cessation, touched plants (especially those stroked 160 times per day) showed the highest growth, indicating a delayed compensatory response, likely caused by overproduction of auxin after stress removal. This rebound effect may result from the plant's strategy of overproducing IAA in response to long-term stress, allowing for rapid growth after stress is removed. Abiotic stress including MS, causes overproduction of reactive oxygen species (ROS), detrimental to cell components[106]. ROS overproduction in plant tissues may damage membranes, macromolecules, affect cellular metabolism and play a crucial role in cellular damage[107]. The main ROS generated in plant cells under stress conditions are hydrogen peroxide (H_2O_2) and superoxide (O^{2-}) radicals. Their deleterious effects are usually neutralized by enzymes such as catalases and peroxidases produced by plants[108]. The main role of plant biostimulants in plant production is to improve plant metabolic processes without changing their natural pathway and also by interacting with plant signaling cascades thereby reducing negative plant reactions to stress[109]. In previous experiments made by Jędrzejuk et al.[5,12] on petunia, increased peroxidase levels, during MS were observed, playing the role in arrestment of IAA synthesis, but also defending leading to cellular walls lignification. Although other free radicals scavengers in petunia were not tested in MS, it can be assumed, based on the literature data and the results obtained in the current experiment, that the plants actively defended themselves against oxidative stress[110,111].

Does calmodulin related genes expression modulate GH3.1 activation?

Stresses initiate a signal-transduction pathway, which contributes to increased cytosolic Ca^{2+} that stimulates Ca^{2+}/calmodulin-dependent activity (Ca^{2+}/CaM)[112]. In the experiment of Cholewa et al.[113], it was observed that after exposure of the plant to cold or hypoxia, activation of Ca^{2+}/CaM occurs. The upregulation of calmodulin-related genes (CaM53, CaM72, and CaM81) during mechanical stress highlights the critical role of calcium signaling in plant adaptation to environmental stimuli. Calmodulins act as calcium sensors and mediators, playing a pivotal role in regulating cellular processes during stress[112]. In this experiment, all three calmodulin-related genes showed increased expression by day 56, indicating that the plants experienced significant stress, regardless of the intensity of the mechanical stimulation. This finding aligns with studies that demonstrate the activation of calmodulin pathways in response to various abiotic stresses, such as cold and hypoxia[113].

In the present experiment, a strong correlation was observed between GH3.1 gene expression and some calmodulin genes, with patterns dependent on the intensity of mechanical stimulation. At 80 touches per day, GH3.1 expression was significantly correlated with CaM72, suggesting that this calmodulin plays a critical role in regulating GH3.1 under moderate stress. In contrast, under more intense stress (160 touches per day), GH3.1 expression was correlated with CaM53 and CaM81, indicating that these calmodulins are more active in plants subjected to higher stress levels. The variation in calmodulin-GH3.1 correlations based on stress intensity suggests that different calmodulin-related genes may be activated in response to varying levels of mechanical stress. This could imply a hierarchical response mechanism, where CaM72 is sufficient to regulate auxin homeostasis under

moderate stress, while more severe stress conditions require the involvement of CaM53 and CaM81 to maintain auxin balance and prevent growth inhibition. The upregulation of these calmodulins under severe stress could be necessary to manage the elevated levels of calcium ions triggered by the stronger mechanical stimulus.

Interestingly, even 15 days after the cessation of mechanical stress, upregulation of CaM53 and CaM72 was still observed, suggesting that the plants continued to cope with the residual effects of the stress. This prolonged upregulation may indicate the initiation of a post-stress adaptation process, where calcium signaling remains active to help the plant recover and maintain homeostasis. According to Brenya et al.[114] in the absence of mechanical stress, most transcripts return to abasal expression level, although some can persist to show a modified gene expression for several days poststimulation. Further investigation is required to understand how long calmodulin-related genes remain upregulated after stress and whether this contributes to long-term resilience in plants.

The strength of touch stress clearly influenced the expression of both calmodulin-related genes and GH3.1. Plants subjected to 160 touches per day showed higher levels of GH3.1 and calmodulin gene expression compared to those touched 80 times per day. This suggests that the intensity of the mechanical stimulus plays a crucial role in determining the degree of stress response, with more severe stress leading to greater activation of both calcium signaling and auxin regulation pathways.

Conclusions

- Mechanical stimulation (MS) influences the morphology and physiological responses of Petunia 'Pegasus Special Burgundy Bicolor', including growth, branching, and flowering dynamics. In this study, flowering presents an unexpected outcome. Contrary to most literature, plants exposed to MS exhibited an increase in both the number of branches and flowers. Indole-3-acetic acid (IAA) content was positively correlated with growth dynamics; however, no significant correlation was observed between total gibberellin (GA) content and flowering.
- The expression of studied TCH genes is not significantly correlated with petunia physiological data as a response to touch stress. It also depends on stress impact.
- Calmodulin expression is mostly convergent with GH3.1 beginning of the 14th day of the experiment; the correlation between GH3.1 and subsequent CaMs may differ depending on stress impact. It confirms the thesis that calmodulin activation may modulate auxin-related pathways, which include the expression of genes like GH3.1.

Data availability

Data is provided within the manuscript or supplementary information files (Supplementary Table S4).

Received: 18 April 2024; Accepted: 4 December 2024
Published online: 10 January 2025

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Acknowledgements

This work was supported by the Incubator of Innovation 4.0 (grant supported by Ministry of Higher Education, Poland). The publication was financed by Science development fund of the Warsaw University of Life Sciences—SGGW

Author contributions

N. Kuźma—wrote the research paper, made biometrical, biochemical and RT-qPCR analyses, made statistic M. Klimek-Chodacka- bioinformatics and RT-qPCR, analyzed data. R. Budzyński—constructed the brushing apparatus and was responsible for its work during the experiment. R. Barański—qPCR experiment support, data analysis and manuscript correction. A. Jędrzejuk—designed the experiment, analyzed data. A. Jędrzejuk—designed the experiment, analyzed data.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-024-82364-0.

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