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Leaf size modulation by cytokinins in sesame plants

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ABSTRACT

Phytohormones play important roles in controlling leaf size and in the modulation of various stress responses, including drought. In this study, hormone profiling analyses by ultra high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry (UHPLC-MS/MS) was performed in leaves collected at three stages of active leaf growth to unravel which phytohormones modulate leaf size in sesame (*Sesamum indicum* L.) plants, an important oil-rich crop. Furthermore, endogenous contents of phytohormones were measured in parallel to various stress markers in sesame plants exposed to mild water deficit conditions by withholding water in potted plants for one week. Results revealed a major role of cytokinins and auxin in the modulation of leaf growth in sesame plants (which increased by 21.5 and 2.1-fold, respectively, with leaf growth), as well as a putative antagonistic response between jasmonic acid and salicylic acid during leaf development. Furthermore, growth arrest during water deficit stress appeared to be modulated by cytokinins, the endogenous contents of which decreased (by 48%) in parallel with ABA increases (by 59%). Reductions in the contents of the active cytokinin *trans*-zeatin occurred in parallel with increases in isopentenyladenine contents under drought, which suggests a partial metabolic limitation in cytokinin biosynthesis in leaves upon water deficit stress. These results provide useful information for the hormonal modulation of leaf size and the improvement of leaf growth and production in sesame plants through manipulation of the levels of key regulatory phytohormones.

1. Introduction

Leaves are the primary organs for photosynthesis, and as such have a pivotal role in crop performance and productivity. Leaf development is a complex, multifactorial and dynamic process involving many physiological, biochemical, and molecular regulatory processes that modulate leaf size, shape, and differentiation, always in relation to and modulated by the prevailing environmental conditions (Li et al., 2020). Cell division and cell expansion are key physiological processes that drive leaf development and determine crop productivity, and numerous genes have been identified that, when ectopically expressed or downregulated, increase cell number and/or cell size during leaf growth (Vercruysse et al., 2020).

Drought is one of the most serious outcomes of global change (Fahad et al., 2017) and huge agricultural losses associated with reductions in global agriculture land area are related to drought (Meena et al., 2017). Understanding the relationship between the molecular biology, biochemistry and physiology of crops in relation to their environment is essential for the development of stress tolerant crop varieties (Davies and Ribaut, 2017). This kind of information helps in determining the impact of the environment on particular traits influencing crop growth, yield, and quality in order to prevent substantial yield losses of such valuable food crops under the current frame of global change (Davies and Ribaut, 2017; Meena et al., 2017). Interestingly, a prevailing response of crops to rising [CO2] is an increase in leaf area and this increase is not always beneficial for increasing yield, as it has been recently shown in some crops, such as soybean (Srinivasan et al., 2017). Therefore, it is essential to better understand the physiological basis of leaf size modulation, so that the most adequate leaf growth can be modulated either by transgenic and/or non-transgenic approaches.

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Phytohormones are considered the key molecules that modulate leaf growth and differentiation (Davies, 2010) and are a molecular target for studies aimed at improving crop yields and developing new stress-tolerant crop varieties (Ullah et al., 2018; Pavlović et al., 2018; Davies and Ribaut, 2017; Wani et al., 2016; Verma et al., 2016). Phytohormones are small molecules that function at very low concentrations to carry out different biological processes, including growth, development and stress responses in plants (Wani et al., 2016). Water deficit slows down plant growth and development and hence, reduces plant productivity (Ullah et al., 2018). Plants have evolved to activate several molecular, cellular, biochemical and physiological changes as defensive strategies to survive under such unfavourable conditions (Pavlović et al., 2018; Hu and Xiong, 2014). In recent studies, phytohormones were found to have a key role in drought stress tolerance in plants, enabling them to acclimatize to different water deficit conditions (Ullah et al., 2018; Martignago et al., 2020). Under water deficit conditions, a fine-tuned response of endogenous phytohormones is triggered. Classically, the phytohormones ABA, jasmonic acid, and, to a lesser extent, salicylic acid and ethylene have been described to be involved in drought-related processes (Verna et al., 2016; Ullah et al., 2018). However, there is growing evidence that other phytohormones such as cytokinins (CKs), gibberellins (GAs) and auxin, classically considered as the "growth hormones", are also involved in this complex cross-talk in response to abiotic stress (Kohli et al., 2013).

Sesame (*Sesamum indicum* L., Pedaliaceae) is an ancient oilseed crop that has recently gained considerable importance as a genomic-resource rich oil crop (Wang et al., 2014; Dossa et al., 2017). Despite its high production demand due to its nutritional and economic importance, sesame yield is quite low when compared with other economically important oil crops such as rapeseed, peanut, and soybean (Li et al., 2017). The main reason is that in its natural habitat it is constantly exposed to very high temperatures and episodic/terminal drought events in arid and semi-arid lands (Dossa et al., 2019). Although it is known to be a "survivor crop" or "drought-tolerant" crop due to having a deep root system and better adaptability to water deficits over other oil crops, sesame can still be highly sensitive to prolonged drought periods after germination and/or during flower anthesis (Dossa et al, 2016, 2019; Nadeem et al., 2015; Tahir et al., 2014). The expected high demand for rich edible oils, which is estimated to be 240 million tons by 2050 (Dossa et al., 2016) together with the harsh environmental conditions that oil crops such as sesame have to endure, opens new avenues of research on its drought tolerance (Dossa et al., 2019).

Here, we aimed at better understanding the mechanisms of leaf size control in sesame plants, with emphasis on the possible role of phytohormones in the regulation of leaf growth. We were interested in identifying possible phytohormones involved in the modulation of leaf growth in sesame plants growing under optimal growth conditions. Furthermore, we also performed a hormonal profiling analysis by ultra high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry (UHPLC-MS/MS) to identify possible hormones involved in the modulation of leaf growth arrest under water deficit conditions in sesame plants.

2. Materials and methods

2.1. Plant material

Seeds from sesame (*Sesamum indicum* L.), which were obtained at National Agriculture Research Center (NARC, Islamabad, Pakistan) were used for experiments. Upon arrival to the University of Barcelona, seeds were treated with 2% NaOCl for 2–3 min to sterilize them prior to germination. Then, they were thoroughly washed three times with distilled water, and after sterilization, they were kept for overnight soaking (~24 h) in distilled water at 4 °C to induce germination. Afterwards, seeds were air-dried and sown in plastic trays filled with perlite to germinate at 22–25 °C in an environmentally-controlled growth chamber. After germination, five seedlings per pot were transferred to pots containing a fine mixture of soil, peat and vermiculite (1:1:1, v/v/v) and the pots were kept at 25 °C with a 16-h day/8-h night photoperiod in a growth chamber (light intensity ~120 μmol $m^{-2}s^{-1}$) until used.

For experiment 1, all plants were watered regularly with Hoagland solution for optimal growth during the study and samples were taken during a very active growth period. Three stages of growth were selected as Initial, Middle and End of the study, with a separation of two days between them. Plants were in a very active period of growth during the experiment, which was performed on three-week old plants in the same environmentally-controlled growth chamber as described before. For experiment 2, plants were grown under optimal conditions for four weeks and then, two water irrigation treatments were imposed on plants: well-watered (WW, control), where plants received a Hoagland nutrient solution (Hoagland and Arnon, 1950); and water-stressed (WS), where plants were exposed to seven days of water deficit by withholding water.

For both experiments, leaf samples were collected to estimate leaf growth and water content, as well as to measure the hormonal profiling of leaves through UHPLC-MS/MS. For experiment 2, the maximum efficiency of photosystem II photochemistry (F_v/F_m ratio) of leaves were also measured as additional markers of the degree of stress imposed on plants. The experiments always included five biological replicates per sampling point and treatment, and each biological replicate included the measurement of four leaves from each of the five plants in each pot.

2.2. Leaf growth, water contents and F_v/F_m ratio

Changes in leaf growth were examined by estimating leaf biomass, leaf area, and leaf mass per area (LMA) ratio throughout the experiment. Leaves were weighed to estimate leaf biomass, and then leaf area was measured using a flatbed scanner (model Officejet Pro 8610, HP, California, USA). Dry mass was estimated by weighing the samples after oven drying at 65 °C until constant weight and then, leaf mass per area (LMA) was calculated as dry mass/area. The leaf relative water content (RWC) was calculated as (fresh mass-dry mass)/(turgid mass-dry mass) x 100, where the turgid mass was obtained after hydrating the leaves at 4 °C in the dark for 24 h and the dry mass was estimated after oven-drying the sample at 80 °C to constant mass. The maximum photosystem II efficiency (Fv/Fm ratio) was measured with a Mini-PAM II (Photosynthesis Yield Analyser, Walz, Germany) in dark-adapted leaf samples *in situ*.

2.3. Hormonal profiling of leaves

For hormonal profiling, samples were collected and immediately frozen in liquid nitrogen and then stored at −80 °C for subsequent analyses. Determination of phytohormones, which included a hormonal profiling including ABA, salicylic acid, jasmonic acid, auxin (indole-3-acetic acid), cytokinins (including *trans*-zeatin, *trans*-zeatin riboside, isopentenyl adenine and isopentenyl adenosine), gibberellins (GA1, GA3, GA4 and GA7) and melatonin was performed using UHPLC-MS/MS as described by Müller and Munné-Bosch (2011). Deuterium-labelled compounds (Olchemim, Olomuc, Czech Republic) for all phytohormones were used as internal standards to estimate recovery rates for each sample.

2.4. Statistical analyses

For experiment 1, all data were subjected to ANOVA analyses. Mean differences between time points were determined by Tukey's test at $P < 0.05$. For experiment 2, differences between irrigation treatments were tested by using the Student's t-test and differences were considered significant at $P < 0.05$.

3. Results

3.1. *Influence of phytohormones on the control of leaf size*

Optimal growth conditions in the growth chamber resulted in a rapid growth of plants. As indicated by increases in the leaf biomass and leaf area, plant growth was characterized by an increase in leaf size (Fig. 1). This increase in leaf size was due to both increases in leaf biomass between the initial and the middle points of measurements, and to increases in leaf area only during the latest phase of growth. Leaf biomass almost doubled during the first phase of growth from 3.4 to 6.2 mg dry matter to kept unaltered later, while the leaf area increased both from the initial to the middle phase (by 81%), and from the middle to the end phase (by 40%). It is noteworthy that these phases were separated by two days only, so very rapid growth occurred during this period in sesame plants, with a first phase (from initial to middle) characterized by both cell division and expansion, and a second phase (from middle to end) mostly characterized by leaf expansion. During this growth period, the LMA and RWC were not significantly altered, the latter being always above 80% (Fig. 1).

The hormonal profiling of leaves by UHPLC-MS/MS during this very active growth period revealed significant differences in the endogenous contents of several phytohormones, including changes not only in cytokinins and salicylic acid (Fig. 2) but also in the auxin indole-3-acetic acid and melatonin (Fig. 3). Among the cytokinins measured, which included the active cytokinins *trans*-zeatin and isopentenyl adenine, as well as their respective precursors, *trans*-zeatin was the major cytokinin, which levels reached up to 11 ng/g dry matter (Fig. 2). *Trans*-zeatin riboside and isopentenyl adenosine were also present but at lower amounts, followed by isopentenyl adenine, which was found below the detection limit. Interestingly, *trans*-zeatin increased as growth progressed and increased during both growth phases reaching the highest contents at the end of the studied growth period (Fig. 2). This trend was not observed for the ribosylated cytokinins, although both *trans*-zeatin riboside and isopentenyl adenosine decreased in the middle of the study relative to the other two time points. In absolute amounts, this decrease accounted for a metabolically significant part of the increase in *trans*-zeatin (between the initial and middle phase the sum of the decrease in both ribosylated compounds [1.97 + 0.31 ng g dry matter] accounted for the 60% increase in *trans*-zeatin [3.35 ng g dry matter], Fig. 2).

The other phytohormones whose contents were altered during leaf growth were the chorismate-derived phytohormones salicylic acid (Fig. 2), indole-3-acetic acid and melatonin (Fig. 3). Among these phytohormones, the one that was present at the highest amounts was salicylic acid, which reached up to 300 ng/g dry matter at the end of the growth period, followed by auxin, which attained 110 ng/g dry matter, and finally melatonin, whose highest levels were 12 ng/g dry matter at the start of the growth period. Interestingly, auxin contents increased progressively during the study, but more specifically during the initial stages of growth, salicylic acid contents increased during the latest phase of active leaf growth, and melatonin decreased at the initial stages (Figs. 2 and 3). Among the gibberellins measured, we could only quantify GA4, the contents of which kept unaltered during leaf growth (Fig. 3). Finally, jasmonic acid contents tended to decrease with the progression of leaf growth, and although changes were not significant ($P = 0.65$), a significant negative correlation was found between the contents of salicylic acid and jasmonic acid ($P < 0.05$).

3.2. *Hormonal profiling changes under water deficit*

Under water deficit stress conditions after withholding water for seven days, sesame plants showed a reduction in leaf area by 15%, while leaf biomass was not significantly altered in water-stressed plants compared to the well-watered treatment (Fig. 4). This resulted in an increase in the LMA by 13% in water-stressed plants relative to controls, all coincident with reductions in the RWC from 84% to 72% (Fig. 4).

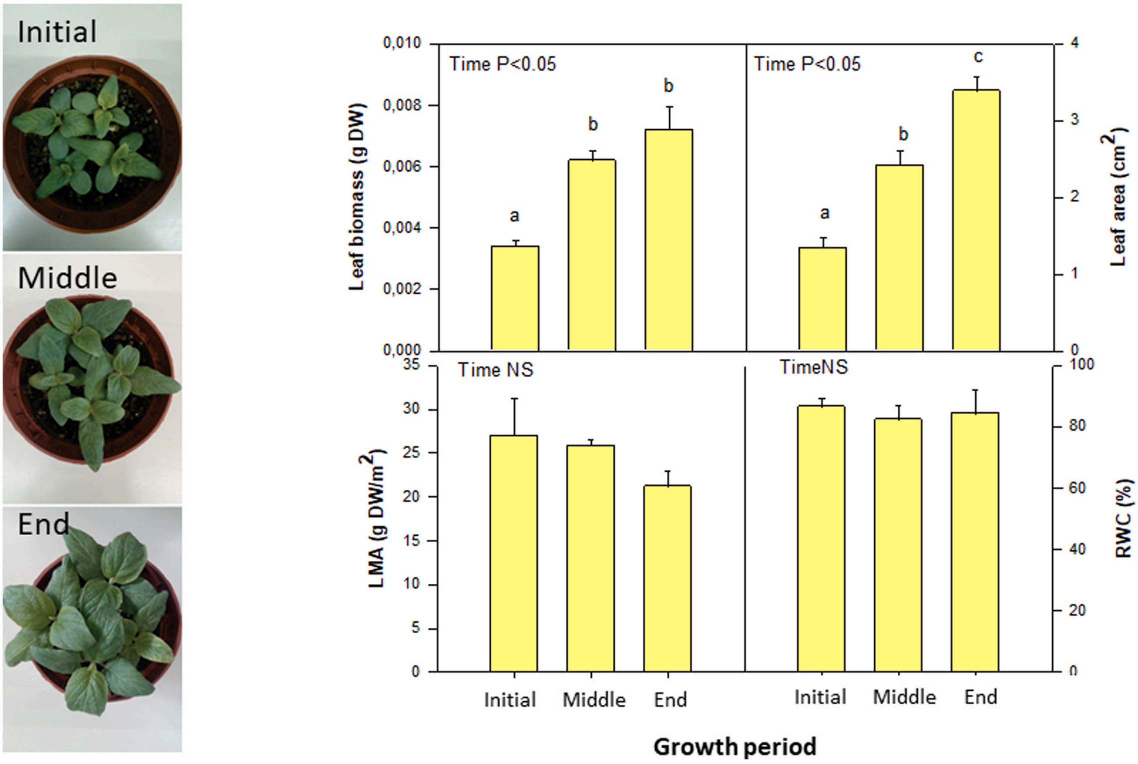

Fig. 1. Changes in leaf size, given as leaf biomass and leaf area, the leaf mass per area ratio (LMA) and relative leaf water content (RWC) in sesame plants during a very active growth period. Photographs of plants during the growth period are also shown. Data represent the mean ± standard error of 5 replicates. Results of statistics are shown in the inlets. NS denotes non-significant differences.

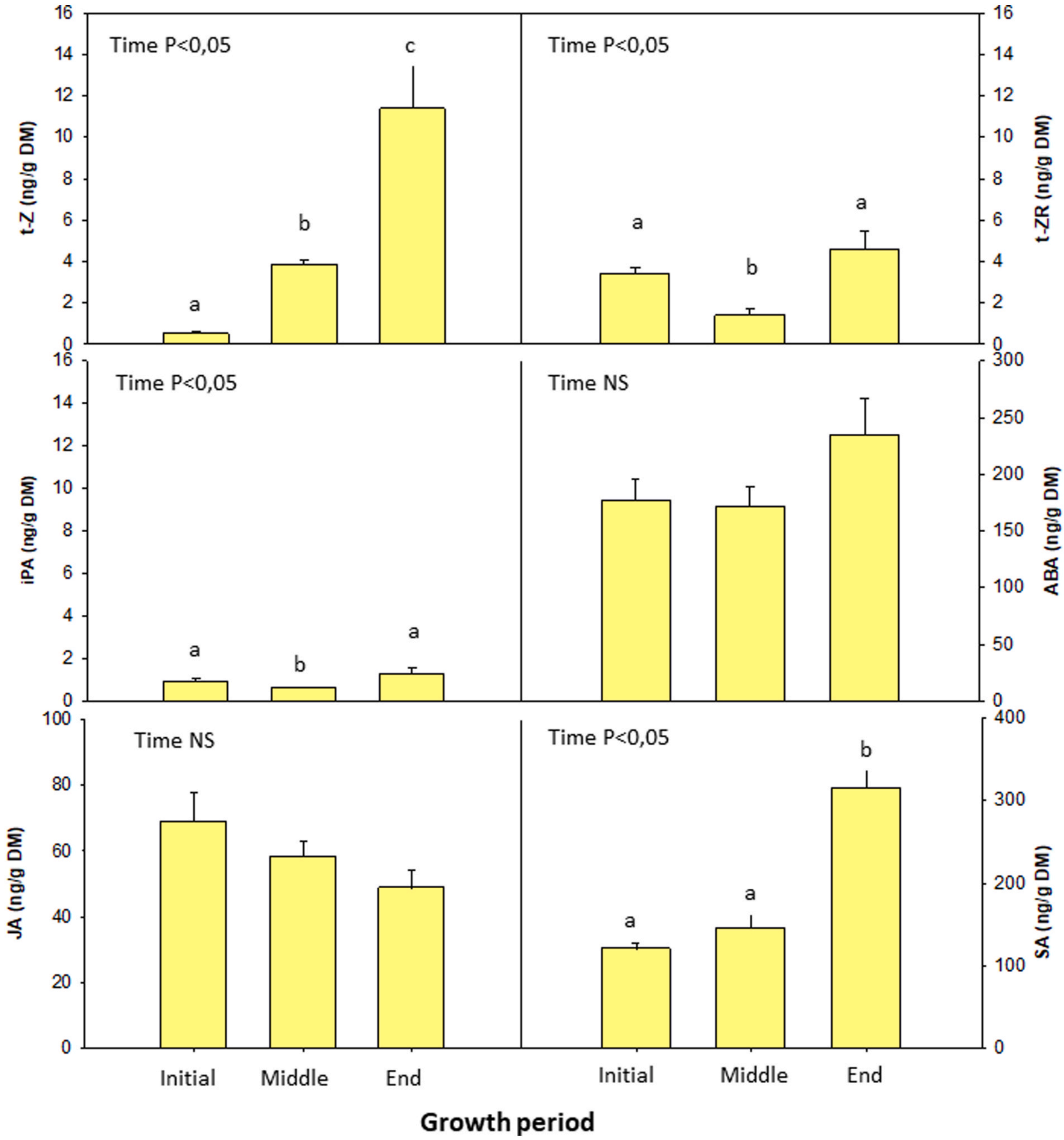

Fig. 2. Changes in the endogenous contents of cytokinins, including *trans*-zeatin (t-Z), *trans*-zeatin riboside (t-ZR) and isopentenyl adenosine (iPA), ABA, salicylic acid (SA) and jasmonic acid (JA) in sesame plants during a very active growth period. Data represent the mean ± standard error of 5 replicates. Results of statistics (one-way ANOVA) are shown in the inlets. Different letters indicate significant differences between leaf growth stages (Tukey posthoc test, $P < 0.05$). NS denotes non-significant differences.

Under these drought stress conditions; the hormonal profiling of leaves revealed a major putative role for cytokinins and ABA. While *trans*-zeatin was reduced by half in water-stressed plants compared to controls, ABA levels increased by 60% in response to water deficit (Fig. 5). The endogenous contents of all other measured phytohormones remained unchanged in response to water deficit, except for isopentenyl adenosine the levels of which doubled in response to water deficit. However, the increases in the latter hormone only accounted for the 9% decrease of *trans*-zeatin in absolute amounts. Although water deficit resulted in significant reductions in the RWC and a reduction in leaf area (Fig. 4), it is worthy to note that sesame plants continued to grow during this period of water deficit, and this was observed in parallel with no differences in the endogenous contents of the auxin indole-3-acetic acid between well-watered and water-stressed plants (Fig. 5).

4. Discussion

Sesame is a very valuable oilseed crop, but its growth and productivity are significantly compromised if it encounters water deficit conditions (drought), most particularly if stress occurs at very early plant growth stages (as the present study shows) or during flowering. Like other plants, sesame is subjected to growth adjustments and several physiological, biochemical, and molecular changes during water deficit stress, particularly in photosynthesis, hormone signal transduction, and metabolism (Egea et al., 2018; You et al., 2019; Wang et al., 2017; Ma et al., 2016). The present study supports the idea that cytokimins and

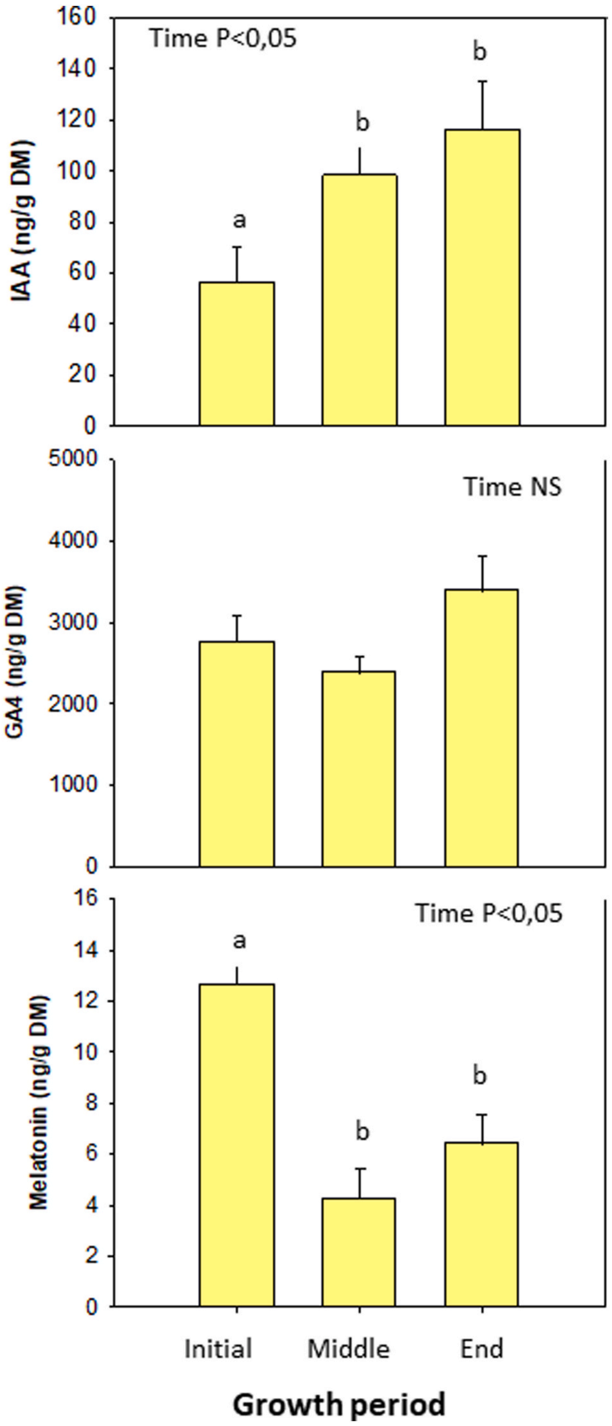

Fig. 3. Changes in the endogenous contents of the auxin indole-3-acetic acid (IAA), gibberellin 4 (GA4) and melatonin in sesame plants during a very active growth period. Data represent the mean ± standard error of 5 replicates. Results of statistics (one-way ANOVA) are shown in the inlets. Different letters indicate significant differences between leaf growth stages (Tukey posthoc test, $P < 0.05$). NS denotes non-significant differences.

auxin synergically modulate leaf growth under optimal growth conditions, while ABA and cytokinins may be antagonistically involved in plant response to water deficit stress, in relation to reductions in leaf growth, to our knowledge for the first time in sesame plants. This kind of information is meaningful to better understand the underlying adaptive mechanisms associated with the control of leaf size and therefore potential productivity, as well as drought tolerance in sesame, essential for the introduction of new drought tolerant sesame varieties through genetic crop improvement or the manipulation of drought tolerance through the application of plant growth regulators and/or biostimulants (e.g., algal extracts rich in phytohormones).

Leaf growth, either due to both increases in leaf biomass and area during the first phase and to increases in leaf area only during the second phase could be evaluated here in sesame plants in relation to the changes in the endogenous contents of phytohormones. The results suggest that both cytokinins and auxin play a major role in controlling leaf size in sesame plants. Cytokinins are very well known for their control of the cell cycle and auxin in modulating leaf expansion, both hormones interacting between them in both processes providing a positive feedback regulation of leaf growth (Davies, 2010). Interestingly, chorismate-derived hormones (see Pérez-Llorca et al., 2019) were also all affected by leaf growth, including changes in salicylic acid, indole-3-acetic acid, and melatonin, although to a different extent and with a different timing of events. It appears that besides the metabolism of cytokinins, which was affected by leaf growth leading to a prevalence of the active *trans*-zeatin during active leaf growth, the chorismate pathway is also modulated in such a way that salicylic acid accumulation is predominant as the leaf reaches full expansion, thus probably providing enhanced protection against potential biotrophic pathogens by salicylic acid once the cell expansion role of auxin has been accomplished. The competitive nature of metabolic routes derived from chorismate and how they are affects by leaf development deserve further investigations at the molecular level. Another interesting aspect that requires further research is the antagonistic behaviour found between the contents of salicylic acid and jasmonic acid, both correlating negatively. Salicylates and jasmonates may act synergically or antagonically depending on the plant growth conditions, biotic and abiotic stress factors to which plants are exposed to, and the tissue and plant species (Pérez-Llorca et al., 2019a). Here, while JA tended to decrease progressively, SA contents increased with leaf growth, more specifically during the second growth phase. Furthermore, SA contents were 6.5-fold higher compared to those of JA at the end of leaf growth. Aside from a putative antagonistic behaviour between the two plant hormones, it seems that SA might play a predominant role of protection over JA in fully-expanded sesame leaves.

Plant growth was not severely affected by the given regime of water deficit stress during the experiment, 7 days of withholding water led to slight reductions in leaf area in water-stressed plants compared to well-watered plants, but both leaf biomass and area increased in both plant groups during the study period. The maintenance of auxin contents might play here a role, despite in this case the endogenous contents of the active cytokinin *trans*-zeatin were significantly reduced. Reductions in the endogenous contents of *trans*-zeatin might result from a slightly lower conversion of isopentenyl adenosine to *trans*-zeatin riboside, but it might mostly be caused by an activation of cytokinin oxidase, which has been shown to increase upon water-deficit stress in other plant species (Brugière et al., 2003). This response may be an important acclimatory response to drought since reductions in leaf size result in a lower overall amount of transpiration area, which together with ABA increases helps preserve leaf water contents at relatively high levels. It is also interesting to mention here that, despite reductions in RWC, the Fv/Fm ratio was not altered by water deficit stress and no significant differences were observed between water-stressed and control plants (values were always kept between 0.81 and 0.82 throughout the study), thus indicating that the given stress was not severe (Pavlović et al., 2018).

Drought is one of the most alarming and common abiotic stresses hitting the yield of food crops worldwide (Daryanto et al., 2016), and it is evident from the present and previously reported studies that plants have evolved various mechanisms for sensing and responding to drought in order to adapt to water-restricted environments (Zwack and Rashotte, 2015). As shown here, it is a widely accepted concept now that plants growth and development as well as their interactions with the environment like drought stress responses are regulated by plant hormones,

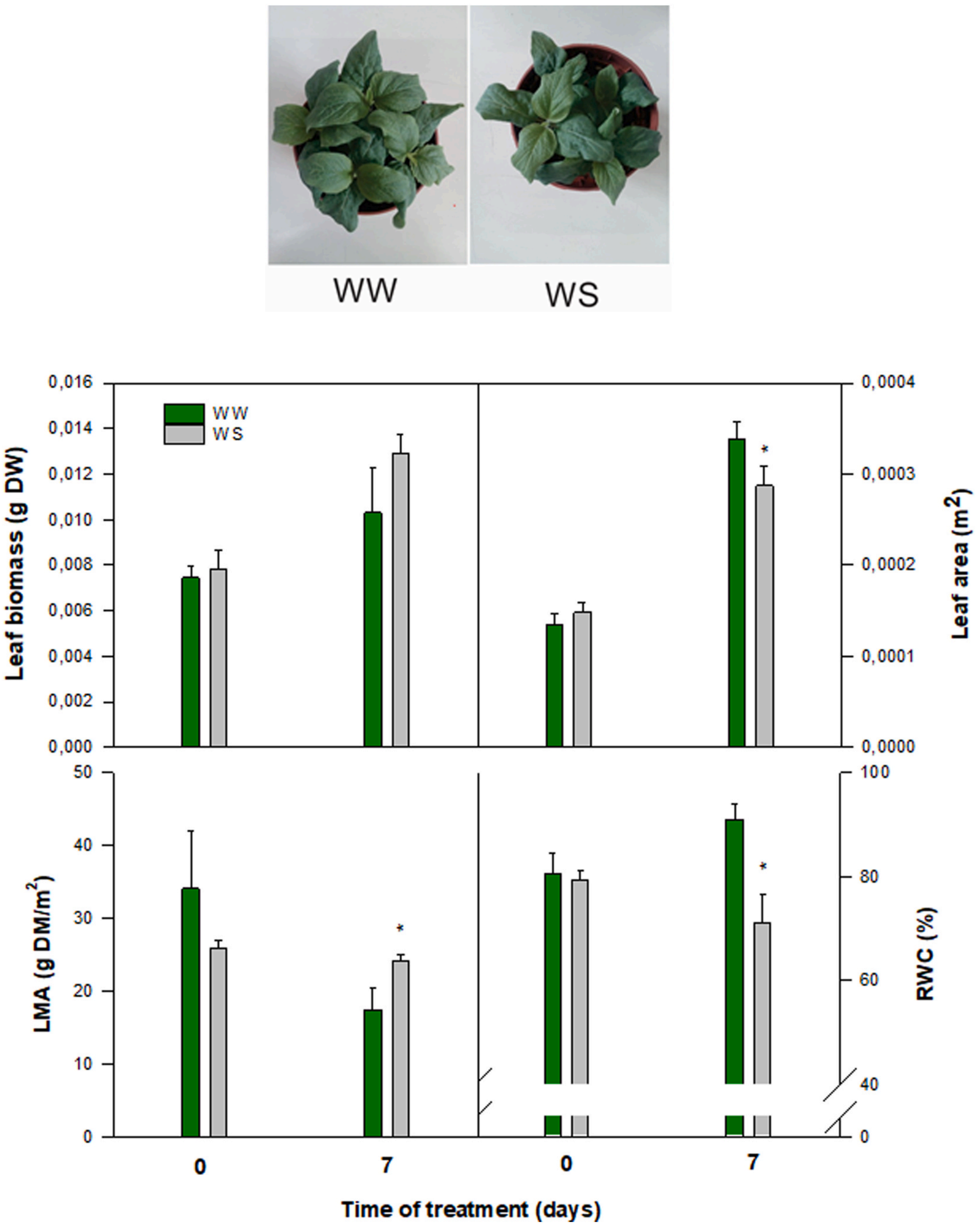

Fig. 4. Changes in leaf size, given as leaf biomass and leaf area, the leaf mass per area ratio (LMA) and relative leaf water content (RWC) in well-watered (WW) and water-stressed (WS) sesame plants. Water deficit was imposed on plants by withholding water for seven days (see materials and methods for details). Photographs of WW and WS plants at 7 days of treatments are also shown. Data represent the mean ± standard error of 5 replicates. An asterisk indicates significant differences between WW and WS plants (Student's *t*-test, $P < 0.05$).

which interact between them to provide the most adequate adaptive response (Ha et al., 2012). As reported in previous studies, water deficit stress responses in plants are mainly mediated by highly complex molecular mechanisms involving hormone signaling and metabolism, particularly abscisic acid (ABA) as the most important stress hormone along with ABA-dependent gene expression (Urano et al., 2017). However, other hormones can also play a role. ABA, together with jasmonates, salicylates, auxins, cytokinins, and GAs may all play a role depending on the species studied, the plant organs investigated or the severity of the stress (Urano et al., 2017). Albeit the predominant role of ABA in abiotic stress responses including drought, an inevitable function of cytokinins enhancing drought tolerance is catching particular attention in recent years (Khandal et al., 2020; Li et al., 2020; Werner et al., 2010; Nishiyama et al., 2011; Rivero et al., 2009). In the present study, reduced contents of cytokinins, particularly *trans*-zeatin in water-stressed plants as compared to their respective well-watered plants suggests a role for CKs in the modulation of growth arrest and hence drought tolerance, which is in agreement with previous studies (Rulcová and Pospíšilová 2001; Rivero et al., 2007). Moreover, cytokinins have been reported to be key regulators of plant growth and stabilization of photosynthetic machinery during the progression of stress (Prerostova et al., 2018; Müller and Munné-Bosch, 2021, and references therein). Thus, according to these results and previously reported studies, the cytokinin-ABA interaction has a pivotal role in plant

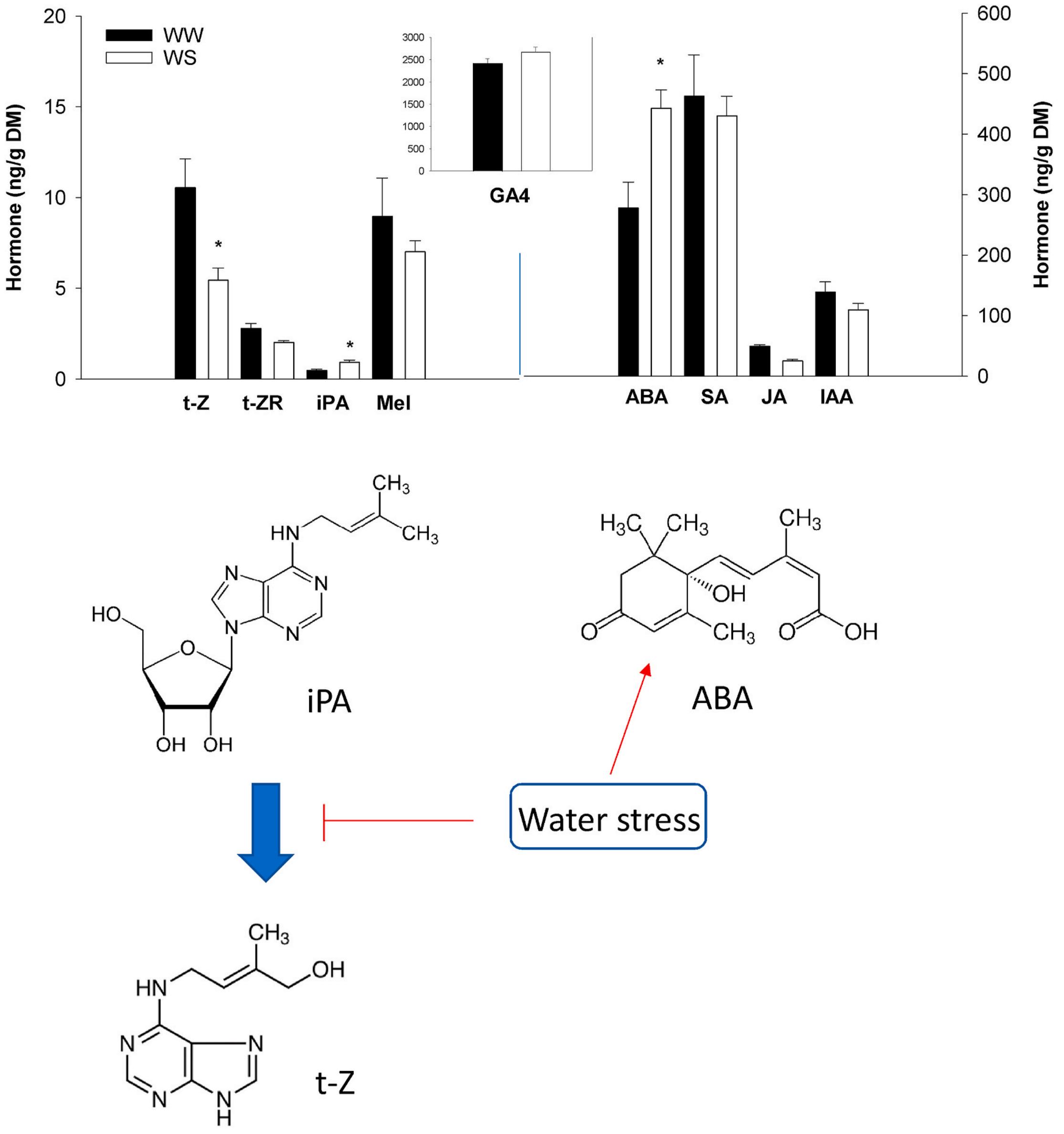

Fig. 5. Differential hormonal profile (including the contents of the cytokinins, *trans*-zeatin [t-Z], *trans*-zeatin riboside [t-ZR] and isopentenyl adenosine [iPA], melatonin [Mel], gibberellin 4 [GA4], ABA, salicylic acid [SA], jasmonic acid [JA] and the auxin indole-3-acetic acid [IAA]) in sesame plants exposed to water-stressed conditions for 7 days (WS, *white bars*) compared to controls (well-watered [WW] plants, *black bars*). Data represent the mean ± standard error of 5 replicates. An asterisk indicates significant differences between WW and WS plants (Student's t-test, $P < 0.05$).

adaptation to drought stress (Hai et al., 2020; Huang et al., 2018). Although still unexplored in sesame plants, the molecular basis of this antagonistic relationship has been recently unraveled in Arabidopsis demonstrating SnRK2.2, SnRK2.3, and SnRK2.6 (kinases) as the key positive regulators of the ABA signaling pathway which directly interacts with and phosphorylates ARR5, a negative regulator of cytokinin signaling as a negative feedback loop (Huang et al., 2018). Besides this, more elaboration on ABA and cytokinin interactions entail both direct and indirection interplays of ABA-responsive genes (such as MYB and DREB transcription factors) mediating the regulation of pathways that impact cytokinin homeostasis (Guo and Gan, 2011). Thus, the inhibition of cytokinin signaling by ABA pathway results in the reduction of cytokinin levels leading to a wide range of morphological and biochemical changes enabling plants to cope up with water deficit stress (Li et al. 2019).

It is concluded that (i) leaf size might be modulated by cytokinins and auxin, their intertwined function controlling cell division and expansion to promote leaf growth under optimal conditions, and (ii) ABA and cytokinins might play a role in the activation of drought tolerance mechanisms in sesame plants, including among others, a reduction in leaf growth to modulate leaf size, which ultimately results in better control of the overall plant transpiration rate. The outcomes of this study provide basic molecular information for future crop improvement by (i) genetic manipulation of key regulatory molecules (such as phytohormones, related drought-responsive genes, or transcription factors), and/or (ii) the application of plant growth regulators and/or biostimulants that may serve to control leaf size and help activate drought tolerance mechanisms in sesame plants.

Author contribution statement

M.M. and S.M.B. designed the experiments; M.M., M.P.L. and A.C. performed experiments; S.M.B. wrote the manuscript with the help of all authors; S.F. and S.M.B. supervised the work.

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.

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