Indian Journal of Experimental Biology
Vol. 60, February 2022, pp. 112-120

Stomatal behaviour and endogenous phytohormones promotes intrinsic water use efficiency differently in cotton under drought

Raghvendra Dubey[1,2], Shivanand Suresh Dudhagi[1,2#], Shiv Narayan[1,2#], Anil Kumar[1], Abhishek Niranjan[1] & Pramod Arvind Shirke[1,2]*

[1]Plant Physiology Laboratory, CSIR**-National Botanical Research Institute (NBRI), Lucknow-226 001, Uttar Pradesh, India

[2]Academy of Scientific and Innovative Research (AcSIR), Ghaziabad-201 002, Uttar Pradesh, India

Received 13 November 2020; revised 09 June 2021

The coordination between phytohormones regulation, stomatal behaviour (stomatal index and opening/closing) and gas exchange are potent determinants of plant survival under drought stress. However, we found a knowledge gap in the mechanism regulating the fine-tuning of these features during drought. In order to address this we evaluated gas exchange, stomatal behaviour and endogenous phytohormones content in two cotton varieties (LRA-5166 and NBRI-67) differing in drought sensitivity during water deficit conditions. Variety specific differences were recorded in net photosynthesis rate (A), transpiration rate (E) and stomatal conductance (g_s) with significantly less decrease in drought tolerant LRA-5166 than drought sensitive NBRI-67. The abscisic acid (ABA) accumulation was significantly increased in LRA-5166 while reduced in NBRI-67 under water deficit, which was accompanied by relatively less reduced 6-benzylaminopurine (6-BAP) level in LRA-5166 than NBRI-67.Thus, improved ABA/6-BAP ratio was observed in both the varieties of cotton. Critically, LRA-5166 has reduced stomatal index, aperture size and significantly higher A and intrinsic water use efficiency (WUE_i), thus higher drought tolerance than NBRI-67. Furthermore, we found that endogenous ABA predominantly maintains the stomatal behaviour and regulates its physiology either by antagonizing 6-BAP or alone to coordinate with water deficit signals. Overall, our findings describe a new insight as to how drought modulates endogenous ABA and 6-BAP homeostasis in cotton leaf and the mechanism of stomatal regulation by ABA and 6-BAP in cotton.

Keywords: 6-Benzylaminopurine, Abiotic stress, Abscisic acid, Carbon isotope discrimination, *Gossypium hirsutum*, Stomatal conductance, Water deficit

Water scarcity is a major constraint that adversely affects plant development and growth performance in crops across the globe[1]. Drought stress conditions negatively alter leaf gas exchange attributes including decline in rate of photosynthesis, transpiration and stomatal conductance[2-5]. To combat environmental stress plants improve the conservation of water by regulating their stomatal behaviour. The mutual interaction between the stomatal number and aperture is crucial for the prevention of transpirational water loss and enhanced CO_2 uptake capacity, which improves plants ability to balance water use[6-8].

Plants reduce water loss by modulating their stomata as a survival mechanism in drought[9]. Various study suggests that the regulation of ABA and cytokinin (CK) is required by plants to cope up with drought stress[10-12]. In response to drought stimuli, ABA optimizes stomatal aperture movement and development that contributes to the balance between CO_2 influx and transpiration in plants[13,14]. Several studies have exhibited that ABA negatively regulates stomatal opening and development[8,15]. Although, cytokinin promotes stomatal development and rate of water loss[16]. Together, these studies suggested that the relationship between endogenous ABA and CK strongly regulates stomatal behaviour to increase the plants survival in response to drought stress however, the mechanism is still not well established[11,17].

The analysis of carbon isotope discrimination (CID) provides an accurate, reliable, sensitive method for assessing water use capacity, as it reduces the artefact of fluctuating environmental conditions and is negatively correlated with water use efficiency (WUE)[18,19]. The CID method has been based on the

*Correspondence:
Phone: +91 522-2297928; Fax: +91 522 2205847
E-mail: pashirke@nbri.res.in
ORCID ID: 0000-0002-1077-3344
# Equally contributing authors
** Council of Scientific and Industrial Research

ability of C_3 plants to discriminate ^{13}C (carbon isotope) against ^{12}C either by CO_2 assimilating enzymes or stomatal physical responses during photosynthesis. Although, the exact working mechanism of stomata remains unclear so far[19-21].

Cotton is one of the natural cellulose fibre and oilseed crop and its growth and yield is severely affected by water deprived condition[22-24]. Drought tolerant cotton plants have better ability to adjust to several physiological features including gas exchange parameters, as well as *WUE*[3,5] and biosynthesis of ABA than drought sensitive plants under low water availability conditions[22].

In the present study, we investigated the impacts of drought on the endogenous level of ABA and 6-BAP, a cytokinin, stomatal behaviour, leaf gas exchange and the CID in two cotton varieties (*Gossypium hirsutum*), the drought tolerant variety (LRA-5166) largely grown in different states of India[22,25] and the drought susceptible variety (NBRI-67).

Materials and Methods

Plant growth condition and drought treatment

All experiments were conducted on two contrasting varieties of cotton (*Gossypium hirsutum* L.) *viz*., a drought tolerant variety (LRA-5166) and a drought sensitive variety (NBRI-67). Seeds were sown in 5L plastic pots filled with 5/1(v/v) of loamy soil (pH approx. 7.7) and black manure (vermicompost), respectively. 20 cotton plants were grown in a polyhouse under ambient conditions with, CO_2 approximately 400 ppm, with maximum photosynthetic photon flux density of approximately 1200 µmol m^{-2} s^{-1} during the day hours; RH varied between 37–55% and temperatures ranged between 28–42 °C. The plants were grown in pots (5L capacity) for each variety under well-watered condition (WW) treated with 500 mL water daily[2,26]. About 51 days after sowing (DAS), all the plants were split into two groups, one group was used as well-watered control samples and treated with 500 mL water daily and another group was subjected to water deficit (WD) for 15 days and watered with 100 mL of water on alternate days. During the water deficit period, all the pots were covered with aluminium foil to curtail water loss from soil[2]. The potted plants position was rotated inside the polyhouse every 5 days to reduce the effect of position on the plants if any[27].

Measurement of relative water content (RWC) and stem elongation pattern

The changes in water loss during water treatments were measured by assessing the relative water content. Fourth fully expanded leaf from the top was excised and immediately fresh weight (FW) was recorded on day 0, 5, 10 and 15 of drought, along with leaves from the well watered plants. The turgid weight (TW) was recorded after 8h of imbibing the leaf with distilled water; later the leaves were dried at 70 °C for 72h and the dry weight (DW) was recorded.

RWC was calculated as[28]: RWC (%) = [(FW-DW)/ (TW-DW)] ×100

For plant growth analysis, the height of cotton plants (stem elongation) was measured using measuring tape at the initiation of drought; day 0 and at the end of 15 days of drought, respectively.

Measurement of physiological variables

The stomatal conductance (g_s), net CO_2 assimilation rate (A) and transpiration rate (E) were measured on the fourth fully expanded leaf from the top of the plant, that had emerged and developed during well-watered and water deficit condition after 15 days of water treatment. For each water treatment, measurements were made in four plants per variety. Measurements were made using portable photosynthesis system (Li-6800, LI-COR, Lincoln, NE, Nebraska, and USA). The photosynthetic photon flux density was maintained at 1000 µmol $m^{-2}s^{-1}$, level of CO_2 inside the leaf cuvette was maintained at 400 µmol CO_2 mol^{-1}, vapour pressure deficit level was less than 3 KPa and the leaf temperature was at 30 °C. All the gas exchange measurements were measured between 8:00 and 11:00 h to avoid midday depression[2,29]. Both intrinsic (WUE_i) and instantaneous (WUE_{inst}) water use efficiency were assessed, where former was calculated as the ratio of A/g_s and later as the ratio of A/E, respectively.

Measurement of leaf growth

Leaf area and petiole length were measured in fully matured leaf, developed during post-water treatments (both well-watered and water deficit) in each variety of cotton. The leaf area was calculated by tracing the leaf on paper. While the petiole length was measured in cm using a ruler.

Measurement of stomatal aperture and index

For the analysis of stomatal aperture, after 15 days of each treatment, the fourth fully expanded leaf of cotton was sampled from each variety i.e. three plants per water treatment group. Excised leaves were

placed in a stomata opening solution containing 10 mM MES-KOH buffer, pH 6.15[15]. The samples were incubated at 25 ℃ for 4h, under 300 µmol $m^{-2}s^{-1}$ light and 50% RH, and then epidermal peels were removed for microscopic studies. The stomatal opening was analysed using a bright field microscope (LEICA DM2500, Germany) attached to a computer system. To calculate the stomatal aperture index as the degree of stomatal openness (aperture size), at least 150 stomatal aperture lengths and width were measured in leaves from three individual plants for each treatment of each variety using a digital ruler. Stomatal aperture index (SAI) was calculated as the ratio of aperture width/aperture length.

At least 12 images from 4 plants per water treatment group for each variety (3 images per leaf from 4 plants) were analysed. Canada balsam was used to develop the leaf impression and then impression was peeled out using transparent tape. The impression peel was placed on a clean and transparent glass slide. Stomatal and epidermal cell numbers were counted on a surface area of 0.072 mm^2. The stomatal index was calculated as[30]: (SI; %) = stomatal number/(stomatal number + epidermal cell number) ×100.

Carbon isotope discrimination analysis

Whole plant carbon isotope discrimination analysis was performed in both the varieties of cotton at the end of water stress treatment experiments. Five plants were pooled per water treatment group for each variety. Leaf, stem and root samples from each variety of cotton were dried for 72 h at 70 ℃ in an oven and grounded to powder in a mortar and pestle, the fine powder was obtained by using a sieve. The samples were weighed (90 to 150 µg) and placed in tin capsules and combusted in an Isotope ratio mass spectrometer (IRMS, Thermo Electron Corporation, Waltham, Massachusetts, USA) and the ratio of ^{13}C to ^{12}C (carbon isotope; $\delta^{13}C$) was measured. The $\delta^{13}C$ of plant organs (root, stem, and leaf; $\delta^{13}C$) values were used for calculation of carbon isotope discrimination by using the formula as given by Farquhar & Richards[18] and Franks *et al*.[31]:

$$\Delta\ (‰) = (\delta^{13}C_A - \delta^{13}C_P) / (1+\delta^{13}C_P/1000)$$

Where, C_P is the $\delta^{13}C$ of dry plant organs and C_A is the $\delta^{13}C$ of ambient air CO_2 (−8‰).

Quantification of Plant hormones

Concentrations of endogenous abscisic acid (ABA) and 6-BAP (a member of Cytokinin family) were determined in the fully developed leaf. At the end of 15 days of water treatments, fourth fully expanded leaf from the apex of the stem was sampled from two plants per treatment group for each variety of cotton. The leaf sample was frozen in liquid nitrogen immediately on excision for the extraction of endogenous plant hormones. About 500 mg of fresh tissues of the fourth leaf that developed in both water treatments were used. The different endogenous plant hormones were extracted according to the method proposed by Pan *et al*.[32] with minor modifications, and chromatographic detection and quantification of the different hormones using high-performance liquid chromatography system (HPLC Model: Prominence, Shimadzu, Kyoto, Japan) equipped with dual Shimadzu LC-10 ATVP reciprocating pumps, SPD-M20 PDA detector, and SIL 20 AC HT auto sampler, with minor modifications according to Kelen *et al*.[33].

Statistical analysis

All graphs were prepared using Graph Pad Prism 5. One-way analysis of variance (ANOVA) and Pearson correlation were determined using SPSS16.0 software, and *t*-test was conducted using Microsoft office excel software.

Results

Changes in leaf water status and plant growth

After 15 days of water deficit, the two varieties of cotton showed a significant reduction in plant relative water content (RWC), this decrement was significantly lower in LRA-5166 than in the variety NBRI-67 (Fig. 1A). Similarly both the tolerant and the sensitive varieties showed significantly reduced plant height after 15 days of drought as compared to the well watered plants. However, the decrease was significantly more in the sensitive variety, NBRI-67 in comparison to LRA-5166, the tolerant variety (Fig. 1B). Similarly, the leaf area too showed a significant decrease in both the varieties in comparison to the well-watered plants (Fig. 1C). Coupled with this the petiole also showed a reduction in their length in both the varieties under water deficit condition as compared to their respective well watered plants, and this reduction was more affected in NBRI-67 (Fig. 1D).

Water deficit abates gas exchange and enhances water use efficiency

When leaf gas exchange was analyzed under water deficit, a significant reduction in photosynthesis rate (*A*), transpiration rate (*E*), and stomatal conductance (g_s) was observed in both the varieties of cotton plants than well-watered condition (Fig. 2 A-C). Net photosynthesis was

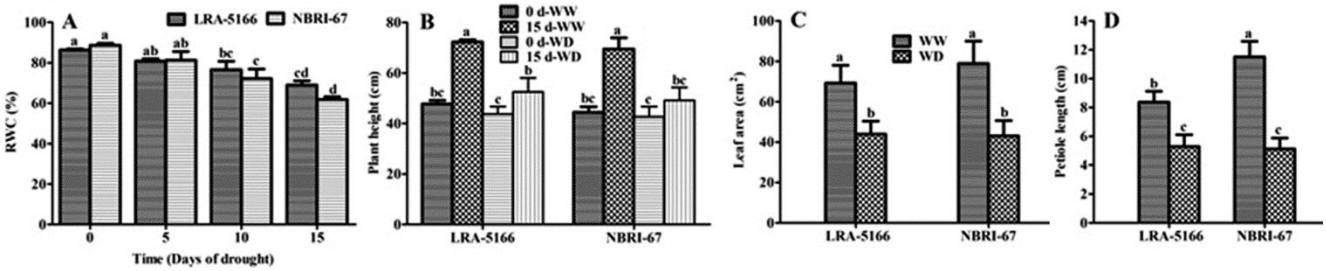

Fig. 1 — Water deficit changes relative water content (RWC) and growth in cotton varieties, LRA-5166 and NBRI-67. (A) RWC on day 0, 5, 10, and 15 of drought. Values are means ± standard deviations (n= 3); (B) Stem elongation in developing plants from initiation of drought; day 0 to end of 15 days of drought; (C) Leaf area and (D) Petiole length on day 15 of water deficit (WD) and well-watered (WW) treatments. [One-way ANOVA was conducted, the letters above the bars indicate significant variation between the two varieties under two water treatments at P <0.05 level. Values are means ± SD (n=4)]

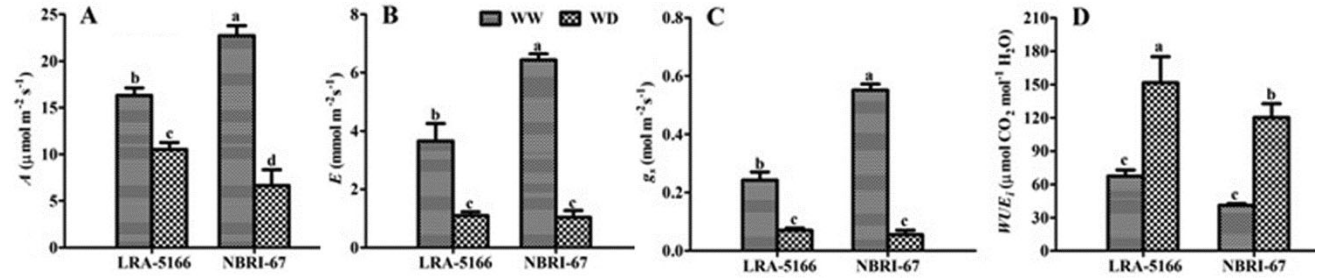

Fig. 2 — Water deficit changes photosynthesis, transpiration and stomatal conductance as well as water use efficiency (*WUE*) in cotton varieties, LRA-5166 and NBRI-67. Variation in leaf (A) Photosynthesis rate (A); (B) Transpiration rate (E); (C) Stomatal conductance (g_s); and (D) WUE_i (A/g_s) at 15 days of water deficit (WD) and well-watered (WW) treatments. [One-way ANOVA was conducted, the letters above the bars show significant variation between two varieties under two water treatments at P <0.05 level. Values are means ± SD (n=4)]

less reduced in LRA-5166 than in NBRI-67 in water deficit conditions (Fig. 2A). Under water deficit the value of E was significantly more decreased in NBRI-67 than in LRA-5166 (Fig. 2B). Similar reduction pattern was observed in g_s in both the varieties during water deficit treatment (Fig. 2C). By contrast, both WUE_i (Fig. 2D) and WUE_{inst} (Suppl. Fig. S1. *All supplementary data are available only online along with the respective paper at NOPR repository at http://nopr.res.in*) were significantly increased in each variety of cotton with a higher value in LRA-5166 than NBRI-67 during water deficit condition as compared to their respective well-watered plants.

Drought decreases carbon isotope discrimination in different plant organs

To validate the impacts of drought on WUE_i, carbon isotope discrimination (CID; $\Delta^{13}C$) of various plant organs were analysed. In water deficit, the CID of leaf was significantly reduced by 5% and 3% of well-watered plants for LRA-5166 and NBRI-67, respectively, (Fig. 3). In stem, the CID of LRA-5166 and NBRI-67 was significantly decreased by 6% and 5% of well-watered plants, respectively, (Suppl. Fig. S2A). Water stress significantly reduced CID of root

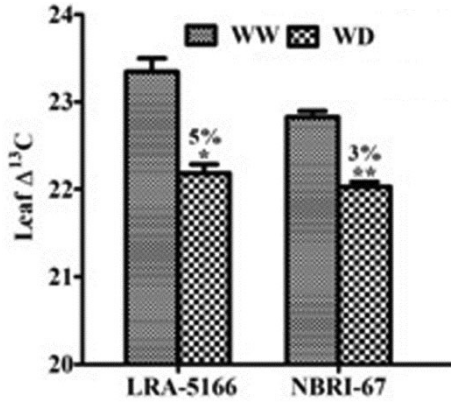

Fig. 3 — Water deficit (WD) changes leaf carbon isotope discrimination (CID; $\Delta^{13}C$) in cotton varieties, LRA-5166 and NBRI-67, against the well-watered (WW) treatment. [Leaf CID ($\Delta^{13}C$), values are means ± SD (n=2 from pool sample of 5 different plants). Significant variation at *P <0.05, **P <0.01 level was according to t-test]

by 8% and 3% in LRA-5166 and NBRI-67 compared to well-watered plants, respectively, (Suppl. Fig. S2B).

Water deficit results in an altered stomatal index and opening

Stomatal index of LRA-5166 was significantly reduced between the two varieties during water deficit, while SI of NBRI-67 showed less significant change (Fig. 4A). The epidermal cell density was less

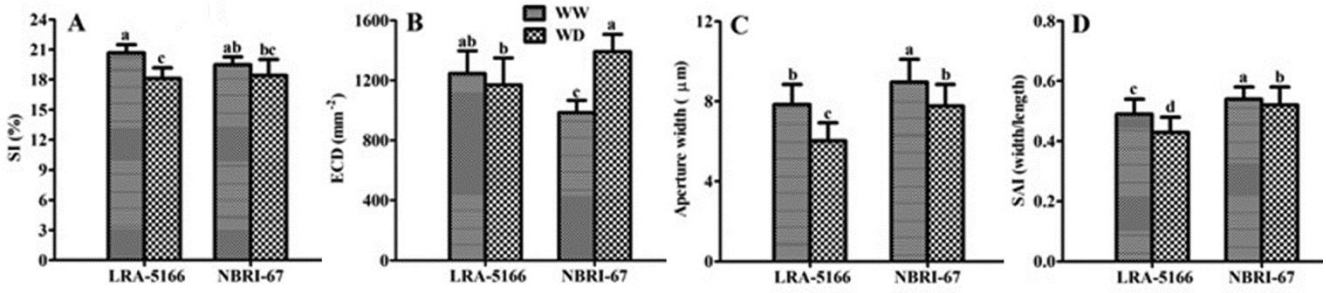

Fig. 4 — Effects of water deficit on stomatal behaviour (stomatal index and aperture) in leaves of cotton varieties, LRA-5166 and NBRI-67. Changes in, (A) Stomatal index (SI); (B) Epidermal cell density (ECD); (C) Aperture width; and (D) Stomatal aperture index (SAI). [Values for (A-B) are means ± SD (n=12 leaf impression images from four different leaves). One-way ANOVA was conducted. The letters above the bars determine significant variation between two varieties under two water treatments, well-watered (WW) and water deficit (WD), at P <0.05 level. Values for (C-D) are means ± SD (n=150 stomata from three different leaf for each treatment; one leaf/plant)]

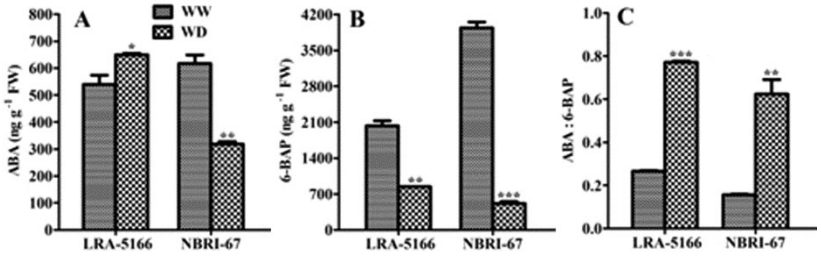

Fig. 5 — Effects of water deficit (WD) on endogenous phytohormones content (ng g^{-1} FW) in leaves of cotton varieties, LRA-5166 and NBRI-67 in comparison to well-watered (WW) plants. Variation in (A) Abscisic acid (ABA); (B) 6-Benzylaminopurine (6-BAP); and (C) ABA and 6-BAP ratio (ABA:6-BAP). [Values are means ± SD of two replicates tested for significance using Student's t-test at *P <0.05; **P <0.01, ***P <0.001 level]

significantly reduced in LRA-5166, whereas it was significantly increased in NBRI-67 during water deficit (Fig. 4B). Besides, the apparent images of leaf peel showed that epidermal cell size was similar in LRA-5166, whereas it was decreased in NBRI-67 in water deficit condition as compared to well watered treatment (Suppl. Fig. S 3A-D). A significant reduction in stomatal aperture width was observed in both cotton varieties during water deficit than well watered leaf sample with a significant decrease in LRA-5166 than NBRI-67 (Fig. 4C and Suppl. Fig. S4A-D). Similarly reduction pattern was observed in the stomatal aperture index in the two varieties, LRA-5166 and NBRI-67 during water deficit (Fig. 4D).

Drought alters the accumulation of ABA and 6-BAP

The above described results showed variation in *WUEi* and stomatal behaviour in the two varieties during water deficit than well-watered conditions. These studies prompted us to speculate whether there was fine-adjustment of ABA and 6-BAP linked with variation in stomatal behaviour and water conservation. To analyse the roles of endogenous hormones in the regulation of stomata and its gas exchange. We quantified ABA and 6-BAP in the two cotton varieties subjected to two treatments, water deficit and well-watered. Water stress significantly increased the amount of ABA in LRA-5166 by 20%, while it was significantly decreased in NBRI-67 by 48% under water deficit conditions (Fig. 5A). However, under water deficit conditions, 6-BAP content was significantly decreased by 58% and 87% in LRA-5166 and NBRI-67 than its well-watered plants, respectively (Fig. 5B). As a result of reduction in 6-BAP content and increased ABA accumulation, ratio of ABA to 6-BAP (ABA/6-BAP) was calculated to be significantly higher in LRA-5166 during water deficit than well watered samples (Fig. 5C). On the other hand, under water deficit conditions, a more decrease in 6-BAP and comparatively less decrease in ABA content attributed to significantly increased ABA/6-BAP ratio in NBRI-67 as compared to well watered plants (Fig. 5C).

Relationship between ABA:6-BAP and stomatal morpho-physiological features

The relationship between regulation of ABA and 6-BAP (i.e. ABA/6-BAP ratio) and stomatal conductance (g_s) as well as WUE_i and stomatal behaviour is depicted in Table 1. A negative

Table 1 — Pearson correlation between endogenous phytohormones (ABA/6-BAP; ABBAP) in two contrasting varieties of cotton during water deficit and well-watered treatments with stomatal behaviour and its physiology

	ABBAP	g_s	WUE_i	SI	SAI
ABBAP	1	00.886	0.997**	00.83	00.694
g_s		1	00.899	0.524	0.623
WUE_i			1	00.789	00.742
SI				1	0.335
SAI					1

[SI, stomatal index; and SAI, stomatal aperture index; g_s, stomatal conductance; and WUE_i, Intrinsic water use efficiency. **significant correlation (P <0.01)]

correlation is observed between ABA:6-BAP, stomatal conductance, SI, and SAI. While, a significant positive relationship was observed between ABA/6-BAP and WUE_i (P <0.01) in both the varieties of cotton leaf. Stomatal conductance was negatively linked with WUE_i, and was positively associated with SI and SAI. The WUE_i was negatively correlated with SI and SAI. Besides, the stomatal index was positively associated with SAI (Table 1).

Discussion

Earlier studies showed that ABA acts antagonistically to cytokinin, which modulates plant growth and adaptation by regulating stomatal responsiveness during drought stress[12,34,35]. However, the detail machinery of endogenous ABA and 6-BAP interaction mediated regulation of stomatal behaviour and their gas exchange features during water stress is yet to be studied. Markedly, a small decrease in RWC values and more increase in plant height was observed in LRA-5166 than NBRI-67 after 15 days of water deficit (Fig. 1 A & B). Besides, we observed that the leaf area and petiole length was less reduced in LRA-5166 during drought compared to the well watered plants than in NBRI-67 (Fig. 1 C & D). It was previously reported that the higher concentration of ABA is attributed to the reduced stomatal formation and cell expansion in leaf[36]. More recently, Skalák *et al*.[37] showed that cytokinin exhibited principal role in the regulation of leaf development. In consistence with these reports, our studies demonstrate that the up-regulation of ABA against 6-BAP predominantly controls stomatal development, epidermal cell division, and enlargement, which together regulates leaf size in both the two contrasting cotton varieties during drought. We found that both WUE_i (A/g_s) and WUE_{inst} (A/E) were strongly increased in LRA-5166 than NBRI-67 (Fig. 2D and Suppl. Fig. S1), which was due to comparatively small decrease in A to larger reductions in g_s and E during water deficit treatment (Fig. 2 A-C) as previously reported in mutants of *Arabidopsis* and barley, respectively[31,38]. Furthermore, we observed a high A in LRA-5166 than in NBRI-67 under water deficit (Fig. 2A). Thus, LRA-5166 exhibited better drought tolerance than NBRI-67 as it was previously reported by Singh *et al.*[5] and Abdelmoghny *et al*.[22].

Several studies have suggested that the reduction in CID values enabled the prediction of productivity and selection of drought-tolerant crop varieties[20,39,40]. Present study shows drought largely reduces leaf, stem, and root CID values in both tolerant and sensitive varieties, but the reduction in values being more in drought tolerant, LRA-5166 than the sensitive, NBRI-67 (Fig. 3 and Suppl. Fig. S2 A & B). Therefore, CID analysis more clearly indicates that LRA-5166 had higher water use efficiency than NBRI-67. The CID values of the leaf were reduced due to decreased CO_2 fixation by altering the development of stomata and epidermal cells as well as affecting stomatal opening in cotton in water deficit treatments (Fig. 4 A-D). These data suggest that drought induces carbon starvation in source tissues (leaf) and also exerted similar impacts on sink tissues (root and shoot) in cotton plants.

There was more reduced stomatal index in LRA-5166 than NBRI-67 during water deficit stress as compared to well watered conditions (Fig. 4A). We observed both epidermal cell density and size were unaltered in LRA-5166, however, NBRI-67 had increased epidermal cell density and decreased size under water deficit (Fig. 4B and Suppl. Fig. S3 A-D). Thus, our study suggest that the spacing between stomata on leaf surface are regulated either through epidermal cells specially larger size of epidermal cells as observed in LRA-5166 or by increasing number of epidermal cells as indicated in NBRI-67 (Fig. 4B and Suppl. Fig. S3 A-D), which could inhibit the malfunctioning of stomatal aperture during water deficit stress. This observation was consistent with previous study[41]. Furthermore, our study showed that drought stress significantly reduces stomatal aperture width and index in LRA-5166 as compared to NBRI-67 (Fig. 4 C & D and Supplementary Fig. S4 A-D). Additionally, our results show larger size of epidermal cells can have beneficial impact on control of stomatal water loss due to small reduction in RWC in tolerant variety than sensitive variety (Fig. 1A) during water deficit conditions. Our results

demonstrate that the tolerant variety with lower stomatal index and aperture size exhibits a significantly high WUE_i and WUE_{inst} than sensitive variety.

Interestingly, a distinct trend in ABA and 6-BAP accumulation was observed in the two contrasting varieties of cotton under water deficit conditions (Fig. 5 A & B). We found that under water deficit conditions, LRA-5166 had significantly increased content of ABA, whereas it was strongly reduced in the drought sensitive NBRI-67 (Fig. 5A). However, both the two variety significantly reduced 6-BAP during water deficit treatment (Fig. 5B). These variable results are collectively attributed to an increase in ABA/6-BAP ratio in both LRA-5166 and in NBRI-67 (Fig. 5C). These results reveal that the cotton plants regulate homeostasis between endogenous ABA and 6-BAP level by controlling ABA/6-BAP ratio to drought as reported by Yan *et al.*[35] in maize. This could also explain how alteration in stomatal properties *viz*. reduced stomatal numbers and openness modulates efficient stomatal CO_2 assimilation and water use during drought stress by ABA and 6-BAP (a cytokinin) crosstalk mediated signalling in cotton plants, as previously described in various reports[12,34,35,42]. Thus the level of drought tolerance in LRA-5166 can be attributed to increased ABA content[22] and WUE_i, as well as reduced stomatal index and aperture, accompanied with increased plant height and reduced water loss to water deficit stress. Furthermore, we also suggest that drought-induced elevation in ABA concentration significantly alters stomatal behaviour in cotton, as also previously reported in *Arabidopsis*[8,15,43].

Our results showed that endogenous ABA accumulation to 6-BAP content ratio was negatively linked with stomatal conductance and stomatal behaviour (SI and SAI) and was positively associated with WUE_i in cotton under water deficit conditions (Table 1). As hypothesized that regulation of stomatal behaviour had positive impacts on WUE_i, which is required for coordination between drought signals and endogenous phytohormones including ABA and 6-BAP contents and their functions in cotton. Thus, our study explains that the higher ABA accumulation and relatively larger reductions in 6-BAP content counteracts each other and strongly improves physiological performance by reducing stomatal index and aperture size in the drought tolerant variety LRA-5166 than the drought sensitive NBRI-67 variety to water deficit.

Conclusion

This study explores the beneficial effects of endogenous phytohormones ABA and 6-BAP on stomatal behaviour and their physiological implications during drought in cotton plants. We demonstrate dual regulatory mechanisms working together to modulate stomatal responsiveness, which efficiently regulates A and WUE_i, and which enhances drought tolerance in LRA-5166 in comparison to NBRI-67. The existence of negative interaction between ABA and 6-BAP regulates stomatal behaviour, which helps to cope plant survival during water deficit by significantly reducing stomatal index and opening in cotton. These findings add strength for the function of ABA and 6-BAP in stomatal development and aperture responses to drought stress. The mechanism that control stomatal behaviour in drought is intricate and further study is required.

Acknowledgment

Authors RD, SN, and SSD thank the University Grant Commission (UGC), New Delhi for financial support. This manuscript bears CSIR-NBRI Communication Number CSIR-NBRI_MS/2021/05/02.

Conflict of interest

Authors declare no competing interests.

References

1 Nakashima K, Yamaguchi-Shinozaki K & Shinozaki K, The transcriptional regulatory network in the drought response and its crosstalk in abiotic stress responses including drought, cold, and heat. *Front Plant Sci*, 5 (2014) 170.

2 Singh R, Naskar J, Pathre UV & Shirke PA, Reflectance and cyclic electron flow as an indicator of drought stress in cotton (*Gossypium hirsutum*). *Photochem Photobiol*, 90 (2014) 544.

3 Han J, Lei Z, Zhang Y, Yi X, Zhang W & Zhang Y, Drought-introduced variability of mesophyll conductance in *Gossypium* and its relationship with leaf anatomy. *Physiol Plant*, 166 (2019) 873.

4 Pooja NA, Nandwal AS, Chand M, Singh K, Mishra AK, Kumar A, Kumari A & Rani B, Varietal variation in physiological and biochemical attributes of sugarcane varieties under different soil moisture regimes. *Indian J Exp Biol*, 52 (2019) 721.

5 Singh C, Rajkumar BK & Kumar V, Water-deficit stress-Induced physio-biochemical changes in cotton (*Gossypium hirsutum* L.) Cultivars. *Indian J Biochem Biophys*, 58 (2021) 83.

6 Blatt MR, Brodribb TJ & Torii KU, Small pores with a big impact. *Plant Physiol*, 174 (2017) 467.

7 Haworth M, Scutt CP, Douthe C, Marino G, Gomes MTG, Loreto F, Flexas J & Centritto M, Allocation of the epidermis to stomata relates to stomatal physiological control: stomatal factors involved in the evolutionary diversification of the angiosperms and development of amphistomaty. *Environ Exp Bot*, 151 (2018) 55.

8 Zhang L, Shi X, Zhang Y, Wang J, Yang J, Ishida T, Jiang W, Han X, Kang J, Wang X & Pan L, CLE9 peptide-induced stomatal closure is mediated by abscisic acid, hydrogen peroxide, and nitric oxide in *Arabidopsis thaliana*. *Plant Cell Environ*, 42 (2019) 1033.

9 Chater C, Gray JE & Beerling DJ, Early evolutionary acquisition of stomatal control and development gene signalling networks. *Curr Opin Plant Biol*, 16 (2013) 638.

10 Zwack PJ & Rashotte AM, Interactions between cytokinin signalling and abiotic stress responses. *J Exp Bot*, 66 (2015) 4863.

11 Li YJ, Wang B, Dong RR & Hou BK, AtUGT76C2, an *Arabidopsis* cytokinin glycosyltransferase is involved in drought stress adaptation. *Plant Sci*, 236 (2015) 157.

12 Huang X, Hou L, Meng J, You H, Li Z, Gong Z, Yang S & Shi Y, The antagonistic action of abscisic acid and cytokinin signaling mediates drought stress response in *Arabidopsis*. *Molecular Plant*, 11 (2018) 970.

13 Chater CCC, Oliver J, Casson S & Gray JE, Putting the brakes on: abscisic acid as a central environmental regulator of stomatal development. *New Phytol*, 202 (2014) 376.

14 Wang Z, Wang F, Hong Y, Yao J, Ren Z, Shi H & Zhu JK, The flowering repressor SVP confers drought resistance in *Arabidopsis* by regulating abscisic acid catabolism. *Mol Plant*, 11 (2018) 1184.

15 Chater C, Peng K, Movahedi M, Dunn JA, Walker HJ, Liang YK, McLachlan DH, Casson S, Isner JC, Wilson I & Neill SJ, Elevated CO_2 induced responses in stomata require ABA and ABA signaling. *Curr Biol*, 25 (2015) 2709.

16 Farber M, Attia Z & Weiss D, Cytokinin activity increases stomatal density and transpiration rate in tomato. *J Exp Bot*, 67 (2016) 6351.

17 Zhao Y, Chan Z, Gao J, Xing L, Cao M, Yu C, Hu Y, You J, Shi H, Zhu Y & Gong Y, ABA receptor PYL9 promotes drought resistance and leaf senescence. *Proc Natl Acad Sci USA*, 113 (2016) 1949.

18 Farquhar GD & Richards RA, Isotopic composition of plant carbon correlates with water-use efficiency of wheat genotypes. *Funct Plant Biol*, 11 (1984) 539.

19 Rebetzke GJ, Condon AG, Richards RA & Farquhar GD, Selection for reduced carbon isotope discrimination increases aerial biomass and grain yield of rainfed bread wheat. *Crop Sci*, 42 (2002) 739.

20 Brito GG, Suassuna ND, Diola V, Sofiatti V, Ducatti C, Silva ETD & Morello CDL, Carbon isotope fractionation for cotton genotype selection. *Pesqui Agropecu Bras*, 49 (2014) 673.

21 Gresset S, Westermeier P, Rademacher S, Ouzunova M, Presterl T, Westhoff P & Schön CC, Stable carbon isotope discrimination is under genetic control in the C4 species maize with several genomic regions influencing trait expression. *Plant Physiol*, 164 (2014) 131.

22 Abdelmoghny AM, Raghavendra KP, Sheeba JA, Santosh HB, Meshram JH, Singh SB, Kranthi KR & Waghmare VN, Morpho-physiological and molecular characterization of drought tolerance traits in *Gossypium hirsutum* genotypes under drought stress. *Physiol Mol Biol Plants*, 26 (2020) 2339.

23 Abdelraheem A, Adams N & Zhang J, Effects of drought on agronomic and fiber quality in an introgressed backcross inbred line population of Upland cotton under field conditions. *Field Crops Res*, 254 (2020) 107850.

24 Mubarik MS, Ma C, Majeed S, Du X & Azhar MT, Revamping of Cotton Breeding Programs for Efficient Use of Genetic Resources under Changing Climate. *Agronomy*, 10 (2020) 1190.

25 Sankaranarayanan K & Nalayini P, Performance and behaviour of Bt cotton hybrids under sub-optimal rainfall situation. *Arch Agron Soil Sci*, 61 (2015) 1179.

26 Singh R, Pandey N, Naskar J & Shirke PA, Physiological performance and differential expression profiling of genes associated with drought tolerance in contrasting varieties of two *Gossypium* species. *Protoplasma*, 252 (2015) 423.

27 Claverie E, Meunier F, Javaux M & Sadok W, Increased contribution of wheat nocturnal transpiration to daily water use under drought. *Physiol Plant*, 162 (2018) 290.

28 Barrs HD & Weatherley PE, A re-examination of the relative turgidity technique for estimating water deficits in leaves. *Aust J Biol Sci*, 15 (1962) 413.

29 Upreti P, Narayan S, Khan F, Tewari LM & Shirke PA, Drought-induced responses on physiological performance in cluster bean [*Cyamopsis tetragonoloba* (L.) Taub.]. *Plant Physiol Rep*, 26 (2021) 49.

30 Wilson PB, Estavillo GM, Field KJ, Pornsiriwong W, Carroll AJ, Howell KA, Woo NS, Lake JA, Smith SM, Harvey Millar A & Von Caemmerer S, The nucleotidase/phosphatase SAL1 is a negative regulator of drought tolerance in Arabidopsis. *Plant J*, 58 (2009) 299.

31 Franks PJ, W Doheny-Adams T, Britton-Harper ZJ & Gray JE, Increasing water-use efficiency directly through genetic manipulation of stomatal density. *New Phytol*, 207 (2015) 188.

32 Pan X, Welti R & Wang X, Quantitative analysis of major plant hormones in crude plant extracts by high-performance liquid chromatography–mass spectrometry. *Nat Protoc*, 5 (2010) 986.

33 Kelen M, Demiralay EC, ŞEN S & ALSANCAK GÖ, Separation of abscisic acid, indole-3-acetic acid, gibberellic acid in 99 R (*Vitis berlandieri* × *Vitis rupestris*) and rose oil (*Rosa damascena* Mill.) by reversed phase liquid chromatography. *Turk J Chem*, 28 (2004) 603.

34 Nguyen KH, Van Ha C, Nishiyama R, Watanabe Y, Leyva-González MA, Fujita Y, Tran UT, Li W, Tanaka M, Seki M & Schaller GE, *Arabidopsis* type B cytokinin response regulators ARR1, ARR10, and ARR12 negatively regulate plant responses to drought. *Proc Natl Acad Sci USA*, 113 (2016) 3090.

35 Yan H, Wu L, Filardo F, Yang X, Zhao X & Fu D, Chemical and hydraulic signals regulate stomatal behavior and photosynthetic activity in maize during progressive drought. *Acta Physiol Plant*, 39 (2017) 125.

36 Tanaka Y, Nose T, Jikumaru Y & Kamiya Y, ABA inhibits entry into stomatal-lineage development in *Arabidopsis* leaves. *Plant J*, 74 (2013) 448.

37 Skalák J, Vercruyssen L, ClaeysH, Hradilová J, Černý M, Novák O, Plačková L, Saiz-Fernández I, Skaláková P, Coppens F & Dhondt S, Multifaceted activity of cytokinin in leaf development shapes its size and structure in *Arabidopsis*. *Plant J*, 97 (2019) 805.

38 Hughes J, Hepworth C, Dutton C, Dunn JA, Hunt L, Stephens J, Waugh R, Cameron DD & Gray JE, Reducing stomatal density in barley improves drought tolerance without impacting on yield. *Plant Physiol*, 174 (2017) 776.

39 Centritto M, Lauteri M, Monteverdi MC & Serraj R, Leaf gas exchange, carbon isotope discrimination, and grain yield in contrasting rice genotypes subjected to water deficits during the reproductive stage. *J Exp Bot*, 60 (2009) 2325.

40 Wang Y, Zhang X, Liu X, Zhang X, Shao L, Sun H & Chen S, The effects of nitrogen supply and water regime on instantaneous WUE, time-integrated WUE and carbon isotope discrimination in winter wheat. *Field Crops Res*, 144 (2013) 236.

41 Papanatsiou M, Amtmann A & Blatt MR, Stomatal spacing safeguards stomatal dynamics by facilitating guard cell ion transport independent of the epidermal solute reservoir. *Plant Physiol*, 172 (2016) 254.

42 Wei H, Jing Y, Zhang L & Kong D, Phytohormones and their crosstalk in regulating stomatal development and patterning. *J Exp Bot*, 72 (2021) 2356.

43 Jalakas P, Merilo E, Kollist H & Brosché M, ABA-mediated regulation of stomatal density is OST 1-independent. *Plant Direct*, 2 (2018) e00082.