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ORIGINAL PAPER

Abscisic acid induces heat tolerance in chickpea (*Cicer arietinum* L.) seedlings by facilitated accumulation of osmoprotectants

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Abstract The gradual rise of global temperature is of major concern for growth and development of crops. Chickpea (*Cicer arietinum* L.) is a heat-sensitive crop and hence experiences damage at its vegetative and reproductive stages. Abscisic acid (ABA), a stress-related hormone, is reported to confer heat tolerance, but its mechanism is not fully known, especially whether it involves osmolytes (such as proline, glycine betaine and trehalose) in its action or not. Osmolytes too have a vital role in saving the plants from injurious effects of heat stress by multiple mechanisms. In the present study, we examined the interactive effects of ABA and osmolytes in chickpea plants grown hydroponically at varying temperatures of 30/25°C (control), 35/30, 40/35 and 45/40°C (as day/night (12 h/12 h)): (a) in the absence of ABA; (b) with ABA; and (c) in the presence of its biosynthetic inhibitor fluridone (FLU). The findings indicated severe growth inhibition at 45/40°C that was associated with drastic reduction in endogenous ABA and osmolytes compared to the unstressed plants suggesting a possible relationship between them. Exogenous application of ABA (2.5 μM) significantly mitigated the seedling growth at 40/35 and 45/40°C, while FLU application intensified the inhibition. The increase in growth by ABA at stressful temperature was associated with enhancement of endogenous levels of ABA and osmolytes, while this was suppressed by FLU. ABA-treated plants experienced much less oxidative damage measured as malondialdehyde and hydrogen peroxide contents. Exogenous application of proline, glycine betaine and trehalose (10 μM) also promoted the growth in heat-stressed plants and their action was not significantly affected with FLU application, suggesting that these osmolytes function downstream of ABA, mediating partially the protective effect of this hormone.

Keywords Abscisic acid · High temperature · Chickpea · Osmolytes

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Introduction

The gradual increase in global temperature is being experienced as heat stress by the plants, which has consequently proven to be detrimental especially for the crops (Halford 2009). Heat stress may impair all the vital processes such as photosynthesis, respiration, membrane functioning, respiration and water relations and affect the functioning of enzymes, proteins and hormones (Wahid et al. 2007; Kumar et al. 2011). High temperatures induce oxidative stress by producing reactive oxygen species (ROS) and hence further aggravate the damage to membranes, proteins and nucleic acids (Suzuki and Mittler 2006). Plants defend themselves from heat stress by producing heat stress proteins (HSP's), antioxidants, secondary metabolites and osmolytes and by altering the hormone levels. The interactive functioning among these molecules in response to

heat stress is least understood. The ability of the various plant species to guard themselves against heat stress involving these molecules may vary due to genetic reasons that govern their tolerance and survival under such situation.

Among the plant hormones, abscisic acid (ABA) appears to have a pivotal role in governing and mediating the responses of plants to diverse environmental stresses such as drought, cold and flooding, as well as heat (Mongrand et al. 2003; Ding et al. 2010). The rise in endogenous ABA during heat stress has been indicated as a vital component of thermotolerance, suggesting its involvement in induction of biochemical pathways essential for survival under heat-induced desiccation stress (Maestri et al. 2002). ABA may confer thermotolerance by induction of several HSPs (e.g., HSP 70) (Pareek et al. 1998) and heat shock transcription factors (Rojas et al. 1999). Earlier reports indicate that exogenous ABA application also confers heat tolerance in maize (Gong et al. 1998) and *Phragmites communis* (Ding et al. 2010). There are relatively less reports about metabolic mechanisms through which ABA acts in inducing heat tolerance. While Gong et al. (1998) reported ABA's action involves calcium, recently, Song et al. (2008) suggested that ABA may impart thermotolerance by raising the levels of nitric oxide. Its involvement with osmolytes in this regard is not known in heat-stressed plants.

Osmoprotectants such as proline (Song et al. 2005), glycine betaine (Shirasawa et al. 2006) and trehalose (Kaplan et al. 2004) are reported to be elevated in response to heat stress. These molecules are believed to have diverse roles such as stress signaling, protection of enzymes from denaturation, stabilization of membranes and photosynthetic pigments acting as antioxidants and maintenance of osmotic homeostasis to sustain the turgor of stressed cells (Verbruggen and Hermans 2008; Chen and Murata 2008; Fernandez et al. 2010). Their interactive involvement with ABA during response to heat stress is not reported to the best of our knowledge; this constituted the basis of our study. In the present study, we wanted to test whether ABA functions independently or involves osmolytes in conferring protection from heat stress. We conducted these studies on chickpea (*Cicer arietinum* L.), which is a heat-sensitive crop and experiences damage to its growth due to elevated temperatures (Wang et al. 2006) making it a good model for our experiments.

Materials and methods

The seeds of chickpea (cv. GPF2) were treated with 0.1% mercuric chloride and grown hydroponically under normal temperature conditions (30/25°C) in dark for 2 days and subsequently for 2 days in light/dark [(12 h/12 h); light intensity: 250 μmol m^{-2} s^{-1}] until the emergence of shoots and roots on the 4th day. After that, the seedlings were subjected to heat stress for 10 days at 30/25, 35/30, 40/35 and 45/40°C as day/night [(12 h/12 h) (light intensity: 250 μmol m^{-2} s^{-1}] temperatures in a growth chamber and observed for growth of shoot and roots, as well as percent survival on the 10th day. In a subsequent experiment, the 4-day-old seedlings (as above) were raised in the presence of: (a) ABA (2.5 μM); (b) fluridone (1 μM; inhibitor of ABA biosynthesis); or (c) osmolytes (10 μM) at the above-stated temperatures to evaluate the effectiveness of these molecules in growth under heat stress. The concentrations of ABA and fluridone were chosen from a range of 1–10 and 0.25–1 μM, respectively, which were tested in preliminary growth experiments. The concentration of 2.5 μM for ABA was observed to be the best for stimulation of growth under heat stress conditions, while 1 μM of fluridone had the most inhibitory effect. For osmolytes, a range of 5–20 μM was explored for their beneficial effects on growth of plants under heat stress; 10 μM was found to be the best one that was used subsequently. Plants growing at 30/20°C were treated as controls. The growth of shoots and roots were observed after exposure to heat stress for 10 days. The shoots were subjected to analysis of the following parameters on the 10th day:

Measurement of endogenous ABA and osmolytes

The ABA content was measured according to Wang et al. (2002) as elaborated previously (Nayyar et al. 2005). The proline and glycine betaine contents were estimated as per the methods of Bates et al. (1973) and Grieve and Grattan (1983), respectively, as explained earlier (Nayyar 2003). The trehalose content was measured as per the methods of Trevelyan and Harrison (1956) and Brin (1956) as follows. The plant tissue was homogenized in hot ethanol (80%) and centrifuged at 5,000 rpm for 10 min. To 0.1 ml of the supernatant, 2 ml of TCA was mixed to prepare the reaction mixture. One ml of the above reaction mixture was taken out and 4 ml of anthrone reagent (0.2 g anthrone in 100 ml cold 95% sulfuric acid) was added. Appearance of yellow green color indicates the presence of trehalose. The absorbance of the above solution was read at 620 nm against blank containing 80% ethanol. The concentration was worked out from a standard curve of trehalose.

Stress injury

Stress injury was measured as electrolyte leakage (Premchandra et al. (1990), total chlorophyll (Arnon 1949) and

TTC reduction ability (Steponkus and Lanphear 1967), which have been described previously (Nayyar and Gupta 2006).

Oxidative stress

The oxidative stress was measured as malondialdehyde (MDA) and hydrogen peroxide content. For MDA estimation, which indicates the lipid peroxidation, the leaf samples (1 g) were homogenized in 10 ml of 0.1% trichloroacetic acid. The homogenate was centrifuged at 15,000*g* for 5 min. Four ml of 0.5% thiobarbituric acid in 20% trichloroacetic acid was added to a 1-ml aliquot of the supernatant. Thereafter, the mixture was heated at 95°C for 30 min and then quickly cooled in an ice bath. After centrifugation at 10,000*g* for 10 min, the absorbance was recorded at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The content was calculated using its absorption coefficient of 0.156 μmol^{-1} cm^{-1} and expressed as nmol g^{-1} dry weight (Heath and Packer 1968). The hydrogen peroxide (H_2O_2) content was measured using titanium reagent Mukherjee and Choudhuri (1983). On a hot plate, 1 g of titanium dioxide and 10 g of potassium sulfate were mixed and digested with 150 ml of concentrated sulfuric acid for 2 h. The digested mixture was cooled and diluted to make 15 ml with distilled water and used as a titanium reagent. Sample preparation and H_2O_2 estimation were done as described earlier (Nayyar and Gupta 2006). The observations were replicated thrice and analyzed statistically for Tukey's test with SPSS software.

Results

Effect of ABA and fluridone (FLU) on shoot length, survival rate and endogenous ABA in heat-stressed seedlings

At 40/35°C, the length of the shoots decreased by 32%, while at 45/40°C, a reduction of 64% was observed over control (Table 1). The survival rate (Table 1) of the plants was reduced to 63% at 40/35°C and 20% at 45/40°C besides having extremely reduced length compared to the preceding temperatures. The application of ABA did not significantly affect the length at 35/30°C, but application of fluridone (FLU) slightly inhibited it (Table 1). At 40/35 and 45/40°C, the length was improved significantly with ABA, but inhibited greatly with FLU application that was associated with significant reduction in endogenous ABA content. The survival rate also decreased significantly with ABA, but accentuated with FLU application at these temperatures.

Table 1 Effect of exogenous application of ABA (2.5 μM) or its biosynthetic inhibitor fluridone (1.0 μM; FLU) on length, survival rate and endogenous ABA content

Treatment	Shoot length (cm)	Survival rate (%)	Endogenous ABA (ng g^{-1} fw)
30/25°C	5.3 ± 0.16e	100 ± 2.3e	1.21 ± 0.11c
35/30°C	5.6 ± 0.18e	100 ± 2.8e	1.78 ± 0.13d
40/35°C	3.8 ± 0.13c	63.4 ± 2.9d	3.01 ± 0.11e
45/40°C	2.3 ± 0.15b	20.3 ± 2.8b	1.20 ± 0.14c
30/25°C + ABA	4.9 ± 0.13d	100 ± 2.6e	7.21 ± 0.16g
35/30°C + ABA	5.1 ± 0.15e	100 ± 2.3e	7.68 ± 0.13g
40/35°C + ABA	4.8 ± 0.14d	100 ± 2.7e	7.19 ± 0.14g
45/40°C + ABA	3.4 ± 0.13c	63.1 ± 2.5d	6.12 ± 0.16f
30/25°C + FLU	5.1 ± 0.16e	100 ± 2.5e	1.24 ± 0.15c
35/30°C + FLU	5.2 ± 0.17e	100 ± 2.6e	1.02 ± 0.16b
40/35°C + FLU	2.5 ± 0.16b	53.6 ± 2.9c	1.25 ± 0.14c
45/40°C + FLU	1.3 ± 0.12a	7.4 ± 2.8a	0.94 ± 0.16a

Values with same letters in the same column are not different significantly at $P < 0.05$ (Tukey's LSD test)

The endogenous ABA (Table 1) was substantiated with its exogenous application that was slightly detrimental for length at 30/25 and 35/30°C. On the other hand, in FLU-treated plants, the ABA content was observed to be much lower. At 45/40°C, the exogenous ABA application considerably improved the length of shoots (47%), while FLU severely inhibited it (42%) and further decreased the survival rate to a great extent that corroborated the requirement of ABA in defending against heat stress. Thus, these findings indicated that ABA partially alleviated the negative impact of stress, while more severe effect of the stress on growth inhibition was observed after FLU application.

Effect of ABA and fluridone (FLU) on endogenous content of osmolytes in heat-stressed seedlings

Plants experienced increase in the level of all the osmolytes as the temperature increased (Table 2). Among all the osmolytes, proline showed the greatest increase at 40/35°C (3.6-fold over control) followed by trehalose (1.8-fold over control) and glycine betaine (1.5-fold over control) indicating a larger role of proline in heat stress. At 45/40°C, these osmolytes dropped to their lowest levels and reached even levels below controls, suggesting a positive association with growth. Proline showed the largest reduction at this temperature compared to the preceding temperatures.

With ABA application, the endogenous level of all the osmolytes increased appreciably, especially in plants growing at 40/35 and 45/40°C (Table 2). The extent of increase at the highest temperature was 2.7-fold for proline and 1.7-fold for glycine betaine and trehalose. The rise in the level of osmolytes was inhibited considerably with

Table 2 Effect of exogenous application of ABA (2.5 μM) or its biosynthetic inhibitor fluridone (1.0 μM; FLU) on endogenous proline, glycine betaine and trehalose content

Treatment	Proline (μg g^{-1} fw)	Glycine betaine (μg g^{-1} fw)	Trehalose (μg g^{-1} fw)
30/25°C	1,248 ± 8.5b	3.17 ± 0.08	323 ± 5.1
35/30°C	1,728 ± 7.6d	3.49 ± 0.11	407 ± 4.6
40/35°C	4,572 ± 5.9g	4.81 ± 0.12	634 ± 5.3
45/40°C	1,120 ± 10.3b	1.26 ± 0.16b	221 ± 4.6
30/25°C + ABA	1,760 ± 10.1d	4.12 ± 0.11	350 ± 4.8
35/30°C + ABA	1,950 ± 9.5d	4.18 ± 0.13	421 ± 5.2
40/35°C + ABA	5,560 ± 8.9h	5.97 ± 0.19	696 ± 5.3
45/40°C + ABA	3,024 ± 8.6f	2.14 ± 0.17c	375 ± 4.8
30/25°C + FLU	1,200 ± 6.9b	3.16 ± 0.16	331 ± 5.4
35/30°C + FLU	1,610 ± 7.6c	3.81 ± 0.18	402 ± 5.6
40/35°C + FLU	2,890 ± 8.5e	3.34 ± 0.16	415 ± 4.6
45/40°C + FLU	760 ± 7.2a	0.98 ± 0.15a	141 ± 5.1

Values with same letters in the same column are not different significantly at $P < 0.05$ (Tukey's LSD test)

FLU application to the plants growing at 40/35 and 45/40°C. Proline showed greater diminution in this regard, suggesting its higher association with ABA.

Effect of ABA and fluridone on membranes, cellular oxidizing ability and chlorophyll content

With rise in temperature, the membrane damaged increased as indicated by rise in electrolyte leakage (EL) at 40/35 and 45/40°C (Table 3). The ABA-treated plants showed decrease in EL, while FLU application intensified it at these temperatures. The cellular oxidizing ability (as 2,3,5 triphenyl tetrazoilum chloride (TTC) reduction assay) increased at 40/35°C, but came down sharply at 45/40°C indicating respiratory dysfunction. It was improved significantly in ABA-treated plants at high temperatures, while FLU treatment was inhibitory. The chlorophyll content was affected mildly at 40/35°C, but decreased by 41% at 45/40°C. The ABA-applied plants were able to prevent loss of chlorophyll significantly at stressful temperatures, while FLU-treated plants showed further reduction in its content, especially at 40/35°C.

Effect of ABA and fluridone on oxidative damage

The oxidative stress was measured as malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) (Table 4). In heat-stressed plants, the oxidative damage increased and was highest at 45/40°C that was possibly a significant factor contributing toward stress injury and reduction in growth at this temperature (Table 4). The ABA-treated plants experienced increase in levels of these molecules at 35/30 and 40/35°C, but had significant decrease at 45/40°C. The FLU treatment intensified the oxidative stress at all the temperatures, especially at 45/40°C that further supported the role of ABA in heat protection response.

Effect of exogenous application of fluridone and osmolytes on seedling length and survival rate

The heat-stressed plants showed significant improvement in their seedling length in the presence of exogenously added osmolytes (proline, glycine betaine and trehalose) with varied results (Table 5). The response was greater at 45/40°C especially with application of proline and trehalose, while glycine betaine was less effective at its tested concentrations. It was noted that simultaneous FLU application along with osmolytes did not significantly alter the stimulatory effect of the latter on growth, indicating that osmolytes act downstream of ABA.

Table 3 Effect of exogenous application of ABA (2.5 μM) or its biosynthetic inhibitor fluridone (1.0 μM; FLU on electrolyte leakage, 2,3,5-triphenyl tetrazolium chloride (TTC) assay and chlorophyll content

Treatment	Electrolyte leakage (%)	TTC ($A_{530/g}$)	Chlorophyll (mg g^{-1} fw)
30/25°C	11.4 ± 1.8a	0.34 ± 0.11b, c	4.87 ± 0.03e
35/30°C	14.5 ± 1.6b	0.37 ± 0.08c	4.91 ± 0.05e
40/35°C	21.3 ± 1.8e	0.51 ± 0.12e	4.14 ± 0.04b
45/40°C	31.3 ± 1.7g	0.31 ± 0.09b	2.86 ± 0.08a
30/25°C + ABA	12.7 ± 1.6a	0.32 ± 0.06b	4.79 ± 0.11d
35/30°C + ABA	16.2 ± 1.8c	0.41 ± 0.07c, d	4.86 ± 0.15e
40/35°C + ABA	18.3 ± 1.9d	0.46 ± 0.08d	4.26 ± 0.18b, c
45/40°C + ABA	26.3 ± 1.6f	0.42 ± 0.06c, d	3.18 ± 0.16b
30/25°C + FLU	12.3 ± 1.5a	0.36 ± 0.07c	4.79 ± 0.17d
35/30°C + FLU	13.9 ± 1.9a, b	0.40 ± 0.06c	3.81 ± 0.13b
40/35°C + FLU	33.4 ± 1.8h	0.28 ± 0.08b	3.21 ± 0.16b
45/40°C + FLU	41.3 ± 1.6i	0.23 ± 0.09a	2.11 ± 0.14a

Values with same letters in the same column are not different significantly at $P < 0.05$ (Tukey's LSD test)

Table 4 Effect of exogenous application of ABA (2.5 μM) or its biosynthetic inhibitor fluridone (1.0 μM; FLU on malondialdehyde and hydrogen peroxide contents

Treatment	Malondialdehyde (nmoles g^{-1} dw)	Hydrogen peroxide (μmoles g^{-1}dw)
30/25°C	8.34 ± 0.76a	1.6 ± 0.24a
35/30°C	11.2 ± 0.85b	1.9 ± 0.19b
40/35°C	18.2 ± 0.79d	3.1 ± 0.13d
45/40°C	25.4 ± 0.58e	4.3 ± 0.15e
30/25°C + ABA	10.3 ± 0.64b	1.9 ± 0.16b
35/30°C + ABA	14.2 ± 0.72c	2.1 ± 0.18c
40/35°C + ABA	13.1 ± 0.68c	2.5 ± 0.15c
45/40°C + ABA	17.2 ± 0.81d	3.5 ± 0.15d
30/25°C + FLU	10.3 ± 0.86b	1.5 ± 0.13a
35/30°C + FLU	13.2 ± 0.87c	2.2 ± 0.11c
40/35°C + FLU	29.3 ± 0.94f	4.9 ± 0.15e
45/40°C + FLU	32.6 ± 0.84g	6.1 ± 0.16f

Values with same letters in the same column are not different significantly at $P < 0.05$ (Tukey's LSD test)

Discussion

In our studies, the inhibition of growth of chickpea plants due to heat stress and reduction in their survival rate are in accordance with previous studies on other plant species such as sorghum, *Pennisetum* (Howarth 1989) and maize (Karim et al. 2000), experiencing supra-optimal temperature conditions. The decrease in growth was associated with increase in membrane damage, loss of chlorophyll and cellular viability. Membranes get damaged due to direct effects of high temperature or indirectly because of the oxidative stress caused by reactive oxygen species (ROS) generated by the heat-stressed cells, which is in agreement with observations on rice (Sohn and Back 2007) and wheat (Almeselmani et al. 2009). The loss of chlorophyll caused by high temperature is attributable to impairment of chlorophyll synthesis or its degradation (Guo et al. 2006; Takahashi et al. 2008) that might limit the photosynthetic ability to reduce growth and is similar to the findings in tomato (Camejo and Torres 2001) and rice (Sohn and Back

Table 5 Effect of exogenous application of osmolytes (10 μM) or its ABA's biosynthetic inhibitor fluridone (1.0 μM FLU) on growth and survival rate

Treatment	Shoot length (cm)	Root length (cm)	Survival rate (%)
30/25°C	5.1 ± 0.14f	6.6 ± 0.16d, e	100 ± 3.1h
35/30°C	5.5 ± 0.18f	6.4 ± 0.19d, e	100 ± 2.8h
40/35°C	3.1 ± 0.13c	5.0 ± 0.11c	66.6 ± 2.4e
45/40°C	1.7 ± 0.19a	2.3 ± 0.15a	20.9 ± 1.3a
30/25°C + Pro	5.1 ± 0.16f	6.7 ± 0.16d, e	100 ± 1.6h
30/25°C + Pro + FLU	5.3 ± 0.11f	6.8 ± 0.11d, e	100 ± 1.8h
35/30°C + Pro	5.9 ± 0.13g	6.7 ± 0.11d, e	100 ± 1.6h
35/30°C + Pro + FLU	5.6 ± 0.17g	6.5 ± 0.11d, e	100 ± 2.5h
40/35°C + Pro	4.0 ± 0.16d, e	6.2 ± 0.13d	83.4 ± 2.4g
40/35°C + Pro + FLU	3.8 ± 0.17d	5.8 ± 0.14d	79.3 ± 1.8f, g
45/40°C + Pro	2.6 ± 0.14b, c	3.2 ± 0.10b	45.8 ± 1.6c, d
45/40°C + Pro + FLU	2.5 ± 0.15b, c	2.8 ± 0.12b	41.3 ± 2.1b, c
30/25°C + GB	5.4 ± 0.13f	6.6 ± 0.15d, e	100 ± 2.6h
30/25°C + GB + FLU	5.1 ± 0.15f	6.5 ± 0.13d, e	100 ± 2.4h
35/30°C + GB	5.3 ± 0.14f	6.8 ± 0.14d, e	100 ± 2.1h
35/30°C + GB +FLU	5.4 ± 0.13f	6.6 ± 0.16d, e	100 ± 2.9h
40/35°C + GB	3.6 ± 0.15d	6.0 ± 0.14d	73.1 ± 2.8e, f
40/35°C + GB + FLU	3.4 ± 0.16c	5.7 ± 0.16d	70.1 ± 2.6e
45/40°C + GB	2.3 ± 0.15b	3.1 ± 0.13b	36.1 ± 3.6a, b
45/40°C + GB + FLU	2.2 ± 0.14b	2.8 ± 0.17b	32.4 ± 3.4a, b
30/25°C + Tre	5.6 ± 0.13g	6.8 ± 0.15d, e	100 ± 3.7h
30/25°C + Tre + FLU	5.7 ± 0.15g	6.7 ± 0.13d, e	100 ± 3.6h
35/30°C + Tre	5.5 ± 0.16f	6.8 ± 0.14d, e	100 ± 2.8h
35/30°C + Tre + FLU	5.3 ± 0.14f	6.7 ± 0.16d, e	100 ± 2.9h
40/35°C + Tre	3.8 ± 0.16d	6.2 ± 0.13d, e	76.4 ± 2.2e, f
40/35°C + Tre + FLU	3.6 ± 0.13d	6.0 ± 0.12d	72.4 ± 2.6e
45/40°C + Tre	2.4 ± 0.17b	3.3 ± 0.19b	41.3 ± 2.4b, c
45/40°C + Tre + FLU	2.2 ± 0.15b	3.0 ± 0.14b	38.2 ± 2.5b

Values with same letters in the same column are not different significantly at $P < 0.05$ (Tukey's LSD test)

2007). Cellular viability that reflects the ability to carry on respiratory reactions increased at 40/35°C, but dropped considerably at 45/40°C suggesting inhibition in the process possibly because of denaturation of respiratory enzymes by heat stress (Salvucci and Crafts-Brandner 2004). In this regard, our observations are similar to the ones reported in wheat (Wang and Nguyen 1989) and potato (Coria et al. 1998) plants subjected to heat stress. The increase in oxidative stress due to stressful temperatures in our studies might also have resulted in damage to membranes, chlorophyll and overall metabolic dysfunction (Liu and Huang 2000) to eventually induce growth inhibition.

The involvement of ABA in response to heat stress was indicated by its substantial elevation in plants growing at 40/35°C relative to the lower temperatures that possibly contributed toward sustained plant growth at these temperatures. The damage to growth intensified at 45/40°C that was accompanied by a marked reduction in ABA level implying its probable link with diminution of growth under heat stress. It was also associated with damage to membranes, chlorophyll and cellular oxidizing ability in the stressed plants. It appeared that heat stress impaired the synthesis of ABA that possibly restricted the growth and induced other injury symptoms. We tested this assumption with exogenous application of ABA, which significantly improved growth and reduced stress injury to membranes and chlorophyll. On the other hand, treatment with fluridone (a biosynthetic inhibitor of ABA) intensified the inhibitory effect of heat stress that further corroborated the role of ABA in this context. Previous studies report that ABA's application to the cold-stressed plants of chickpea was able to reduce the damage to membranes and chlorophyll (Kumar et al. 2008). We also found oxidative damage (as malondialdehyde and hydrogen peroxide contents) due to heat stress decreased in ABA-treated plants, while it was aggravated with fluridone application. Earlier reports about the protective effect of exogenously applied ABA to heat-stressed plants have also indicated reduction in oxidative stress (Gong et al. 1998; Larkindale and Knight 2002). Thus, our observations indicated that ABA partly conferred heat tolerance to chickpea plants, which are in line with its similar effects reported in other heat-stressed plant species such as grapes (*Vitis* sp.; Abass and Rajashekar 1993), maize (*Zea mays*; Gong et al. 1998) and reed (*Phragmites communis;* Song et al. 2008; Ding et al. 2010). That ABA may protect from heat stress by inducing HSPs (e.g., HSP 70) (Pareek et al. 1998) and heat shock transcription factors (Rojas et al. 1999) needs to be probed in our studies. It is also speculated that exogenous ABA may prevent partial dehydration of leaves due to heat stress by improving the leaf water status because of reduction in stomatal conductance, thus preventing the cellular damage, as observed previously by Waterland et al. (2010) in drought-stressed chrysanthemums. A similar possibility might exist in our study that needs to be examined.

A vital objective of our study was to find out whether ABA's action in imparting heat tolerance involved osmolytes or not. To test this hypothesis, we first investigated the endogenous status of proline, glycine betaine and trehalose in heat-stressed plants that followed almost a similar rising pattern like that of ABA, as the temperatures elevated to 40/35°C and declined along with ABA at 45/40°C, though expressional variations existed among them at different temperatures. Proline appeared to have a larger role in heat tolerance as indicated by its greatest variation among the osmolytes. Previous studies have implicated the involvement of proline (Song et al. 2005) and glycine betaine (Shirasawa et al. 2006) and trehalose (Kaplan et al. 2004) in response to heat stress in different plant species. The concurrent rise in these osmolytes and attenuation alongside ABA levels implied a probable relationship between them that was tested by using ABA and its inhibitor.

We observed that ABA-treated plants possessed greater levels of all the osmoprotectants, especially those growing at 45/40°C, indicating a positive connection between them. The application of FLU resulted in very low endogenous contents of these osmolytes that possibly reduced growth, implicating them in mediation of ABA's protective effect. Previous studies report that ABA application can induce increase in proline (Nayyar and Walia 2003) and glycine betaine (Gao et al. 2004) in response to other abiotic stresses, while there are no such reports available on trehalose. The mechanism by which ABA up-regulates the levels of these osmolytes under heat stress remains to be investigated and will form a part of our future study.

We also tested the effect of exogenously applied osmoprotectants against heat stress and found significant improvement in growth of the plants with all of them. It appears that externally added osmolytes probably supplemented their endogenous levels and thus protected the plants from heat stress. Our findings about the protective effects of osmolytes are in accordance with some previous ones on proline (Kaushal et al. 2011), glycine betaine (Allakhverdiev et al. 2007; Li et al. 2011) and trehalose (Luo et al. 2010). Lack of significant effect of FLU on osmolytes' function indicates that osmolytes act downstream of ABA, mediating partially ABA's protective effect. A recent report (Song et al. 2008) indicated that ABA's effect on heat-stressed plants is mediated by nitric oxide. We report here for the first time that ABA can also act through up-regulation of osmoprotectants in conferring partial protection against heat stress, adding new dimension to ABA's role in defense from this stress.

Author contribution Sanjeev Kumar and Neeru Kaushal-analytical work, Harsh Nayyar-concept development and statistical analysis, P Gaur- concept development, analysis and germplasm.

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