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Research article

Gracilaria dura extract confers drought tolerance in wheat by modulating abscisic acid homeostasis

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ABSTRACT

Water stress severely reduces the production of wheat. Application of seaweed extracts have started to show promise in protecting plants from environmental stresses as they contain several biostimulants. However, the modes of action of these biostimulants are not clear. Here, we investigated the role of *Gracilaria dura* (GD), a red alga, in conferring stress tolerance to wheat during drought under glasshouse and agro-ecological conditions by integrating molecular studies with physiological and field investigations. GD-sap application conferred drought tolerance (as the biomass increased by up to 57% and crop yield by 70%), via facilitating physiological changes associated to maintaining higher water content. GD-sap application significantly increased ABA accumulation (2.34 and 1.46 fold at 4 and 6 days of drought, respectively) due to enhanced expression of biosynthesis genes. This followed an activation of ABA response genes and physiological processes including reduced stomatal opening, thus reducing water loss. Moreover, GD-sap application enhanced the expression of stress-protective genes specifically under water stress. Treatment with fluridone, an ABA inhibitor, further support the role of ABA in GD-sap mediated drought tolerance in wheat. The findings of this study provide insights into the functional role of GD-sap in improving drought tolerance and show the potential to commercialize GD-sap as a potent biostimulant for sustainable agriculture in regions prone to drought.

1. Introduction

Wheat (*Triticum aestivum* L) is one of the world's most widely grown crops that serve as an important staple food (Sahu et al., 2016; Sharma et al., 2018). Wheat farming represents 30% of cereal cultivated area and provides 20% of the calories to humans (Bi et al., 2017). The production of wheat is severely affected by various environmental stresses including drought. Drought has become a major constraint to plant growth and crop production as it significantly reduces the yield. The continuous change in the climate along with the reduction in rainfall is a frequent cause of the onset of drought stress (Zhang et al., 2017). Drought stress alone has significantly reduced wheat production by 20–40% during 1980–2015 across the globe (Daryanto et al., 2016; Fahad et al., 2017). Such yield losses need to be minimized to meet the growing food demands (Tilman et al., 2011). A comprehensive understanding of the physiological and genetic mechanisms that drive adaptive responses of plants (Sharma et al., 2018) is essential for improving the wheat performance under drought regimes.

Advanced agronomical practices, traditional breeding and modern biotechnological tools are being used to prevent the yield losses due to water stress. In addition, application of biostimulants or liquid fertilizers, such as seaweed extracts or saps, is currently being pursued to improve the plant performance (Abdel Latef et al., 2017; Battacharyya et al., 2015; Craigie, 2011). Being prepared from natural sources, such biofertilizer extracts are eco-friendly and safe for sustainable agriculture without producing any adverse effects on soil ecosystem. The effect of biostimulants on plant growth has been assessed on various plant species to determine their impact on seed germination (Demir et al., 2006), root development (Khan et al., 2009), leaf growth (Zheng et al., 2016), early flowering (Manna et al., 2012) and production as well as product quality (Chouliaras et al., 2009). Further, various seaweed extracts have been reported to enhance plant tolerance against wide range of abiotic stresses (Aziz et al., 2011; Battacharyya et al., 2015; Santaniello et al., 2017; Shukla et al., 2017). It is believed that

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seaweed extracts contain several bio-active molecules such as sugars, phytohormones, polysaccharides, polyphenols, minerals, and carbohydrates that function synergistically to improve plant performance under sub-optimal conditions (Battacharyya et al., 2015; González et al., 2013; Khan et al., 2009). However, the precise mechanism by which seaweed extracts improve plant stress tolerance is still elusive.

A few attempts have been made to understand the modes of action of seaweed biostimulants in protecting the plant growth under various stress conditions (Khan et al., 2011; Nair et al., 2012; Rayorath et al., 2008), yet detailed (mechanistic) insights are lacking. Reports have suggested that phytohormones are responsible for the favorable changes in plants. Phytohormones are the key regulators of various aspects of growth, development and stress adaptation. Amongst these, abscisic acid (ABA) is a stress hormone that accumulates at a very high concentration during various abiotic stresses (Kalladan et al., 2017; Osakabe et al., 2014). An increased level of ABA activates diverse array of signalling and stress responses that includes rapid closing of the stomata, reprogramming of development of root architecture, growth regulation, gene expression changes and enhanced survival of crop plants under drought stress (Lim et al., 2015; Raghavendra et al., 2010; Xiong and Zhu, 2003). This observation has been supported by several studies where plants with higher ABA levels, either due to exogenous ABA treatment or activation of endogenous ABA biosynthesis, showed better performance under dehydration stress (Waterland et al., 2010; Wei et al., 2015; Zhang et al., 2015; Zhu, 2002). However, it is not clear whether improved drought tolerance in plants treated with seaweed extracts is associated with a change in ABA homeostasis and downstream stress response.

Brown algae have often been used for improving crop production (Santaniello et al., 2017; Shukla et al., 2017; Wally et al., 2013), whereas the information on the potential of using red seaweeds as agents to improve drought tolerance in plants is limited. Seaweed extracts vary in their mode of action due to differences in their chemical composition and physiochemical properties that are heavily dependent on several factors including seaweed type (red, brown, and green), the spatiotemporal changes and the environment from where the raw material has been harvested (Goñi et al., 2016). Compositional differences of seaweed extracts may alter their functional activity and effect on plant growth (Abdel Latef et al., 2017; Goñi et al., 2016). Therefore, it is important to explore novel seaweeds for their ability to enhance plant stress tolerance.

Gracilaria dura (C. Agardh), which is a red seaweed with a potential to produce high-quality agar for biotechnological applications (Gupta et al., 2011b; Mantri et al., 2009). The sap of *Gracilaria* species has been explored in improving the agricultural production in various plant species (Layek et al., 2018, 2017; Pramanick et al., 2016, 2014; 2013; Shah et al., 2013). A detail analysis of *Gracilaria* sap showed presence of high concentrations of various bio-stimulants such as indole 3-acetic acid (IAA), zeatin, Gibberlin (GA_3), choline, glycine betaine and other micro and macro nutrients, which might be the reason for its growth stimulation effect on various plant species under unstressed condition (Basavaraja et al., 2018; Kurepin et al., 2013). However, *Gracilaria dura* (hereafter referred to as 'GD-sap') has been little explored for its potential as a source for elevating drought tolerance in plants.

To investigate the applications of GD-sap in agricultural production, we evaluated its effects on the performance of drought stressed wheat under both, controlled as well as field conditions. Our results indicate that the GD-sap treatment indeed enhances drought tolerance in wheat. We further investigated the molecular mechanism of GD-sap action for enhancing wheat performance under drought stress.

2. Materials and methods

2.1. Plant materials, growth conditions and stress treatments

The seeds of a locally adapted wheat variety, Sharbati Tukdi, which is widely grown in semi-arid and drought prone regions of Gujarat, India, were used for the experiments. For soil drying experiments, seeds were placed in the pots filled with the equal amount of soil and incubated in the dark. After two days of incubation, pots were transferred to controlled growth conditions (8 h light/16 h dark; 23–25 °C, light intensity of 100–150 μmol photons m^{-2} s^{-1}). After 7 days (d), each seedling was foliar sprayed one time with 8 ml of 1%, 5% and 10% GD-sap (which amounts to 0.08, 0.4 and 0.8 ml of GD-sap, respectively, applied to each plant) and water stress was imposed by withholding the water for next 10 days followed by re-watering. Samples were collected at different time points of drought (4, 6 and 10 d) and after 3 d of stress recovery. For physiological assays and gene expression analysis, leaf samples were collected after 6 d of drought treatment. Treatment of ABA inhibitor was given to 7 d old wheat seedlings with 50 μM fluridone (Sigma, St. Louis). Soil moisture was measured every other day starting on the 4th day of soil drying with the help of an infrared moisture analyzer MA35 (Sartorius, Germany). Water stress severity (%) was calculated by taking a percent ratio of dead and green leaves for each treatment.

To obtain the GD-sap, algal samples were collected from Simar, Gujarat (21.0128° N, 70.2728° E), a seaweed cultivation site. Samples were thoroughly washed, homogenized in a grinder, filtered and stored in −80 °C until further use. To prepare the working concentrations, GD-sap was thawed at room temperature and diluted further to appropriate amounts of 1%, 5% and 10% with only water (Pramanick et al., 2013). Ethanol or any other organic solvent was not used for diluting the sap.

2.2. Physiological assays

For total chlorophyll content, 100 mg leaf samples were grounded in liquid nitrogen, homogenized with N,N-dimethyl formamide and incubated in dark for 30 min with gentle shaking at 4 °C. After centrifugation, supernatant was used for absorbance reading at 647 and 664 nm. Total chlorophyll content was calculated as described by Inskeep and Bloom (1985). Proline content was analysed by ninhydrin assay (Bates et al., 1973; Sharma et al., 2011). The osmotic potential (Ψ_s) of plant tissues was determined using Vapor Pressure Osmometer (Wescor, Logan UT, USA).

Relative water content was calculated as (fresh weight − dry weight)/(hydrated weight − dry weight) × 100. To calculate the water loss, 7 d old wheat seedlings were treated with GD-sap for two days and fully expanded leaves were weighed over the course of 12 h to monitor water loss. The stomatal aperture was determined as described previously (Shahinnia et al., 2016). Briefly, 7 d old seedlings were treated with GD-sap and top leaf was collected after 4 d of drought. After applying the glue at the adaxial surface of top leaf, a thin layer was peeled off and immediately mounted on a glass slide and analysed with Axio Imager (Carl Zeiss AG, Germany). 5–6 biological replicates were used for each treatment for the analysis. The antioxidant activities of superoxide dismutase (SOD), glutathione reductase (GR) and peroxidase (POX) were determined as explained previously by Beyer Jr and Fridovich (1987), Smith et al. (1988) and Jebara et al. (2005), respectively.

For the analysis of the ionic composition, GD-sap was digested with a solution containing perchloric acid and nitric acid (3:1). The solution was heated, diluted with autoclaved deionized water and filtered through a 0.22 μm filter. Various ions were measured using an inductively coupled plasma optical emission spectroscopy (ICP; Optima, 2000DV, Perkin Elmer). For starch quantification, GD-sap was mixed with anthrone reagent (0.2% anthrone in ice cold 95% sulphuric acid) and absorbance was measured at 630 nm (Hansen and Møller, 1975). Different concentrations of glucose were used to prepare a standard curve. The final values were multiplied by a factor of 0.9 to convert sugar values into starch content (McCready et al., 1950). Soluble sugar was also estimated with anthrone reagent at 630 nm absorbance. For reducing sugar quantification, dinitro salicylic acid (DNS) was mixed in samples and absorbance was measured at 540 nm. The polyphenol contents were estimated following Chandler and Dodds (1983). Total amino acid contents were estimated using ninhydrin reagents and absorbance reading was measured at 570 nm against a standard curve prepared with glycine (Yemm and Willis, 1954).

2.3. ABA quantification

ABA measurement was done as described previously by Pan et al. (2010). Briefly, aerial tissues of GD-sap treated or untreated wheat seedlings were collected at 4th and 6th d of soil drying treatment. Samples were ground with liquid nitrogen and homogenized with extraction solvent (2-propanol/H_2O/concentrated HCl (2:1:0.002, vol/vol/vol) and incubated at 4 °C for 30 min with continuous shaking. For partitioning, dichloromethane was added in each tube and mixed well on ice for 30 min. The samples were centrifuged at full speed for 10 min at 4 °C and the lower phase was transferred into new tubes, concentrated using nitrogen flow and re-suspended into methanol. Standard curves ranging between 0.031 and 1 μM ABA were prepared to calculate ABA content. 0.2 μg IPA (indole-3-propionic acid) was used as an internal control. ABA quantification was carried out with high pressure liquid chromatography (SHIMADZU Corp, Japan) that was equipped with DAD detector (Patel et al., 2018).

2.4. Retrieval of genes and quantitative real time PCR analysis

Sequences of wheat *NCED* genes (*TaNCED*) were retrieved from Phytozome database (https://phytozome.jgi.doe.gov) and multiple sequence alignment was done with *Arabidopsis NCED* genes (www.ebi.ac.uk/Tools/msa/clustalo/) as ABA signalling and drought response is well characterized in *Arabidopsis*. Certain marker genes of drought response were retrieved from literature (Table S2). Wheat genes corresponding to these *Arabidopsis* genes were searched in International Wheat Genome Sequence Consortium (ftp://ftpmips.helmholtz-muenchen.de/plants/wheat/IWGSC/version2.2/), Phytozome (https://phytozome.jgi.doe.gov) and Ensemble Plants databases (http://plants.ensembl.org/index.html) and sequences were retrieved. The wheat and *Arabidopsis* genes were aligned to ascertain homology. Primer sequences of *TaZAP1*, *TaABA8'OH1*, *TaABA8'OH2, TaAREB3* and *TaMYB31* were obtained from published literature (Bi et al., 2016; Ji et al., 2011; Wang et al., 2016). Detail information of the primers is provided in Table S3.

Leaves of 7 d old wheat seedlings treated with GD-sap and drought stress for 6 d were collected for RNA isolation. Total RNA was isolated with Plant RNAse kit (Qiagen) with on-column DNAse treatment and quantified with NanoDrop™ spectrophotometer (Thermo scientific, USA). 0.5–1 μg of total RNA was reverse transcribed by using ImProm-II™ reverse transcription system (Promega, USA). For real time analysis, 10 fold diluted cDNA was used in a reaction mixture with PowerUp SYBR™ Green Master Mix (Invitrogen, USA) and run in a Real-Time iQ5 Cycler (Bio-Rad, USA). Three biological samples for each treatment were processed, and the reaction was set up in triplicates. The elongation factor gene was used as an endogenous control (Table S3). The relative fold change was determined by $2^{-\Delta\Delta Ct}$ method.

2.5. Field experiment

A field experiment was conducted during December to March 2016 at Bhavnagar, Gujarat (21.7645° N, 72.1519° E). Seeds were planted in 4 m row with 30 × 45 cm distance between plants and rows, respectively. One-month-old wheat plants were foliar sprayed with an average of 15 ml of 5% concentration of GD-sap solution (450 ml solution sprayed on 30 plants per row; 0.75 ml of GD-sap per plant) and subjected to water stress by withholding the water for 16 days. The GD-sap treatment was given only once before implementing drought. At the end of soil drying (16th d), chlorophyll content was determined and top leaf was collected for gene expression analysis. Plants were re-watered after drought treatment and the crop was harvested at maturity. Yield and other yield related parameters (Sharma et al., 2018) such as dry weight, thousand kernel weight, spike numbers and spike length were determined at end of the experiment. Spike number and lengths were determined from individual plants (30–40 plants/treatment). For dry weight measurement, average weight of 20–25 plants were taken. Plants without GD-sap treatment were taken as control.

2.6. Statistical analysis

Data analysis was performed with the help of IBM SPSS statistics 19. Treatment means were compared by Student-Newman-Keuls (SNK) test at 5% probability ($p < 0.05$). In some cases, significant differences were calculated by *t*-test at 5% probability ($p < 0.05$). Data are represented as means ± SE (standard error).

3. Results

3.1. GD-sap treatment improves wheat performance under water stress

In a preliminary analysis of the composition of GD-sap, we found various metabolites (Table S1), which could have a beneficial role in plant stress adaptation. To investigate the effect of GD-sap on wheat, seven days old wheat seedlings were foliar sprayed with 1, 5 and 10% concentrations of GD-sap and drought was imposed thereafter by withholding of water up to 10 days (Fig. 1 and Figs. S1 and S2) and seedling performance was determined. No difference was observed in GD-sap treated and untreated wheat at different time points of watered control (Fig. 1A, G). Under drought, GD-sap treatment had no effect on seedlings biomass at 4 d; however, at 6 and 10 d, GD-sap treated wheat showed significantly higher biomass (Student-Newman-Keuls (SNK) test, $p < 0.05$) as compared to untreated control (Fig. 1 B, H) at similar moisture content (Fig. 1C). GD-sap treated and untreated seedlings showed similar leaf numbers under control and drought stress (Figs. S1A and B). The leaf area of the wheat plants that were grown under drought stress was less than the leaf area of the plants grown under watered conditions; however, GD-sap treated and untreated seedlings had no difference in leaf area in either of the treatment (Fig. S1 C-H). Higher concentration of GD-sap (10%) also showed increased biomass under drought (Fig. S2). Whereas, 1% GD-sap concentration had no effect in either, control or drought conditions (SNK test, $p < 0.05$) (Fig. S2).

Plants were recovered after drought treatment and biomass was determined. The data showed that the GD-sap treatment also improved the recovery of the drought stressed seedlings (Fig. 1 D, E, I). Water stress severity, a ratio of dead and live leaves, was significantly less (43%) in 5% GD-sap treated seedlings than the controls (Fig. 1F). These observations indicate that GD-sap treatment protect the wheat seedlings under drought stress and also help them to recover from the stress injury. Based on the results of soil drying experiments, we choose 5% GD-sap concentration and 6 d drought treatment for subsequent experiments.

3.2. Drought tolerance of GD-sap treated wheat is associated with stress related physiological changes

To better understand the basis of drought tolerance of GD-sap treated plants, various physiological assays were performed on wheat seedlings treated with GD-sap (5%) and subjected to 6 d of drought stress. Leaf relative water content (RWC) was similar between GD-sap treated and non-treated seedlings under control condition; however, under water stress, the RWC was significantly higher in GD-sap treated seedlings than the non-treated (SNK test, $p < 0.05$), suggesting that the GD-sap treatment helped the plants in reducing the amount of water loss due to evaporation especially under drought stress (Fig. 2A).

Plants accumulate several osmolytes when stressed, which reduce

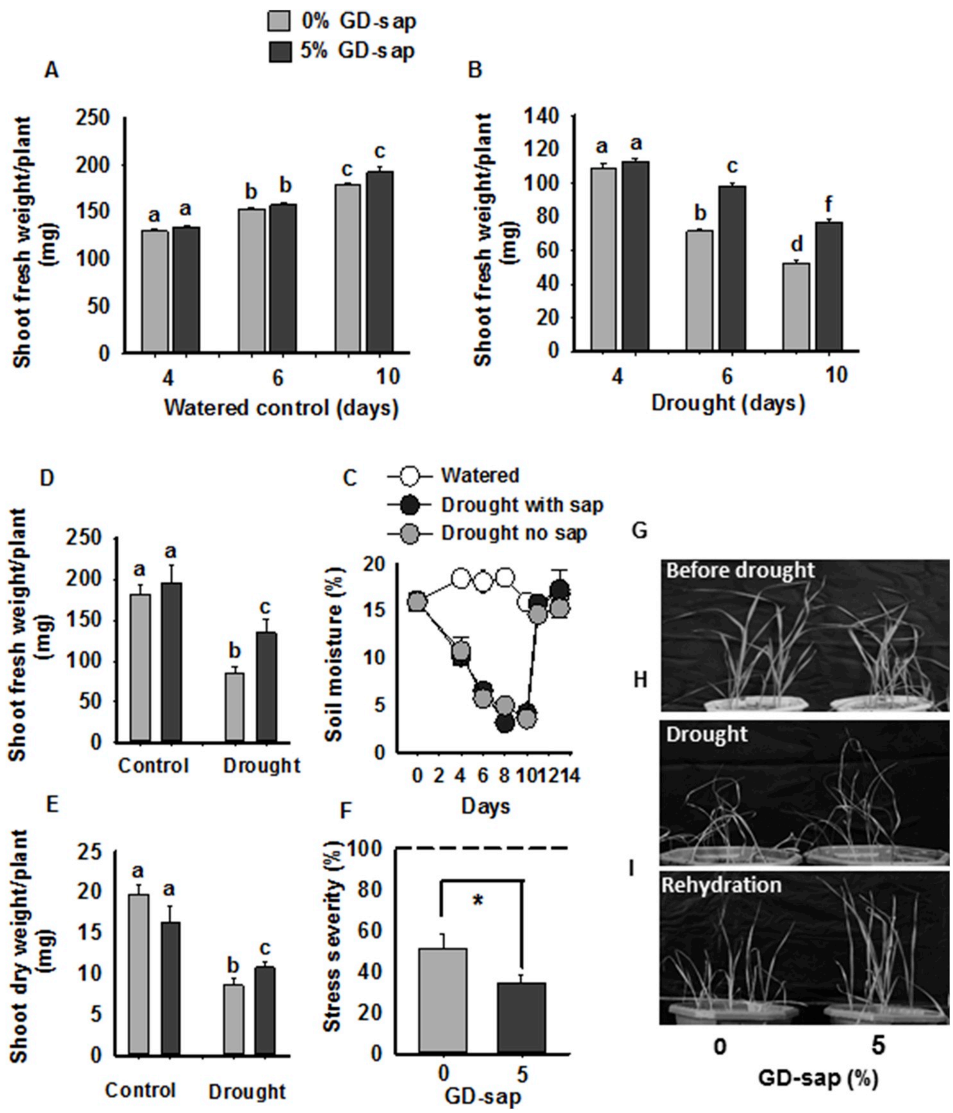

Fig. 1. Effect of GD-sap on the performance of wheat seedlings under water stress and stress recovery: 7 d old wheat seedlings were foliar sprayed with 5% GD-sap and subjected to water stress treatment for next 10 d followed by 3 d of rehydration. (A, B) seedlings biomass of GD-sap treated (5%) and non-treated seedlings at different time points of drought stress. Data are means ± SE (n = 18–20). Experiment was repeated with similar results. (C) Pot moisture at different time intervals of soil drying and re-watering experiment. (D, E) seedlings biomass after 3 d of water stress recovery. Data are means ± SE (n = 15–20) and combined from two independent experiments. (F) Water stress severity was calculated by taking ratio of dead and green leaves after 3 d of rehydration treatment. Dash line indicates water control. (G) 7 d old wheat seedlings before initiating the drought treatment, after 10 d of drought (H) and 3 d of rehydration (I). Representative seedlings picture are shown here. Different letters shows significant difference between GD-sap treated and non-treated seedlings (SNK test $p \leq 0.05$).

the osmotic potential (Ψs) to prevent the flow of water to outside of the plant cell (Bhaskara et al., 2012). Compared to untreated seedlings, GD-sap treated seedlings had lower Ψs indicating accumulation of more solutes under water stress (Fig. 2B). Proline levels generally increase under drought stress and could partially explain Ψs differences of GD-sap treated and untreated seedlings (Verslues and Sharma, 2010; Sharma et al., 2011). Proline content was significantly increased in drought-stressed seedlings than the water control and was further induced in GD-sap treated plants as compared to the untreated ones (Fig. 2C).

Similarly, GD-sap treatment improved the total chlorophyll content as compared to untreated seedlings under water stress (Fig. 2D). The activity of antioxidant enzymes were also analysed where peroxidase (POX) and glutathione reductase (GR) were decreased after GD-sap treatment under drought and watered conditions, whereas superoxide dismutase (SOD) activity was reduced specifically under drought (Fig. S3). These observations demonstrated that the GD-sap treatment produces stress-related physiological and biochemical changes that may confer tolerance to wheat seedlings under drought.

3.3. *GD-sap treatment increases endogenous ABA levels and alter the expression of ABA metabolism genes in wheat under water stress*

ABA is the major regulator of plant stress tolerance that controls various aspects of physiological and biochemical changes and helps plants to adapt better to water stress (Kalladan et al., 2017; Zhu, 2002). To determine whether drought tolerance of GD-sap treated wheat was associated with a change in the ABA metabolism, 7 d old wheat seedlings were treated with GD-sap and ABA content was measured at 4th and 6th d of drought treatment. No difference in ABA content was observed between 0 and 5% GD-sap treated seedlings under watered condition (Fig. 3A). ABA level was increased by 4.71-fold and 8.2 fold at 4th and 6th d of drought treatment, respectively as compared to unstressed condition. Interestingly, GD-sap treatment further increased the ABA level. GD-sap treated seedlings had 2.34 and 1.45-fold higher ABA accumulation at 4th and 6th d of drought than the untreated drought stressed seedlings, respectively (Fig. 3A).

To understand the molecular basis of the higher accumulation of ABA content in GD-sap treated wheat, we determined changes in the expression of several genes of ABA metabolism (synthesis and catabolism) after 6 d of drought stress (Fig. 3B–D). 9-*cis*-epoxycarotenoid dioxygenase (NCED) catalyses the conversion of epoxycarotenoids to produce xanthoxin and is considered as a rate-limiting enzyme of ABA biosynthesis (Xiong and Zhu, 2003). Phytozome database (https://phytozome.jgi.doe.gov) contains five wheat genes annotated to *NCED* gene family. The gene, Traes_5BL_4BED1CA17.1, is annotated as *TaNCED3* in the phytozome database. This gene is 72% identical to *Arabidopsis thaliana NCED3* (*AtNCED3*) (Xiong and Zhu, 2003, Fig. 3D

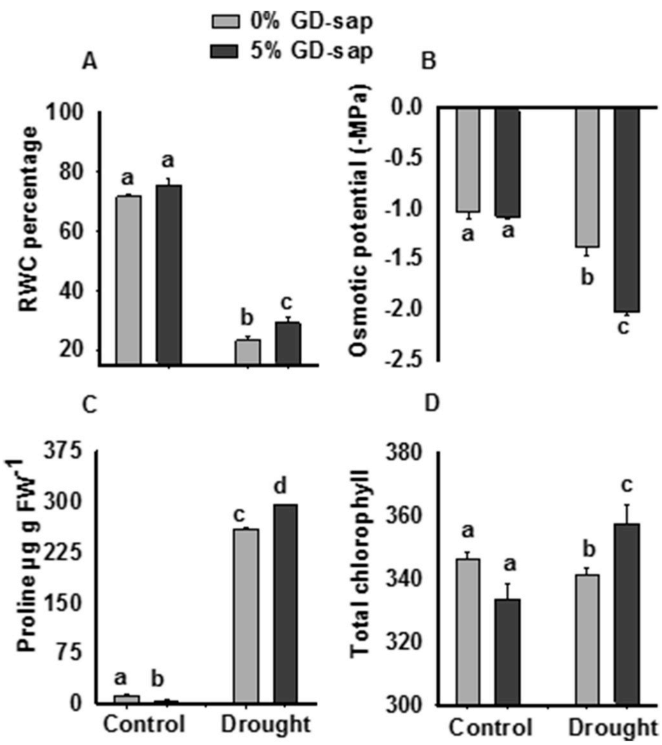

Fig. 2. Physiological changes in GD-sap treated and non-treated wheat after drought stress: 7 d old wheat seedlings were treated with 5% of GD-sap and subjected to water stress for 6 days. Leaf samples were collected and physiological assays were performed. (**A**) Relative water content (RWC), (**B**) osmotic potential, (**C**) proline levels, (**D**) total chlorophyll content were recorded. Data are means ± SE (n = 3–5). Independent experiment was repeated to show similar results. Different letter shows significant difference (SNK test $p \leq 0.05$).

and Supplemental Fig. S3). Indeed, ABA metabolism is best understood in the model plant *Arabidopsis*, where *AtNCED3* is a crucial rate limiting enzyme (Xiong and Zhu, 2003; Kalladan et al., 2017). Another gene, Traes_2AL_BDB97A5BA.1, showed 69.7% identity with Traes_5BL_4-BED1CA17.1 (Supplemental Fig. S4A). A blast analysis was performed against the *Arabidopsis* genome to identify the closest homologue of this gene. With a query coverage of > 250 amino acids, Traes_2AL_BDB97A5BA.1 showed maximum identity (70.5%) to *AtNCED3* (E value $1.7\,E^{-160}$). Therefore, we have referred Traes_5BL_4-BED1CA17.1 as *TaNCED3.1* and Traes_2AL_BDB97A5BA.1 as *TaNCED3.2* in our study. Traes_6DL_037954803.1 has already been annotated as *TaNCED4* (Colasuonno et al. 2017) and had 68.4% identity to *AtNCED4* (Fig. S4). Two genes, Traes_5DS_E58EBABFD.3 and Traes_5BS_B626C522B.1, had more than 98% identity amongst themselves. As Traes_5BS_B626C522B.1 was previously annotated as *TaNCED2* (Ji et al., 2011) and showed 62.1% identity to *AtNCED2,* it was chosen for expression analysis. The expression of another biosynthetic gene, zeaxanthin epoxidase (*TaZEP1*), and the two catabolic genes, *TaABA8'OH1* and *TaABA8'OH2*, were also analysed (Ji et al., 2011); Fig. 3D). The conversion of 8'-hydroxylation of ABA into phaseic acid is a predominant pathway for ABA catabolism, which is catalysed by the ABA 8'- hydroxylase, a member of CYP707 gene family (Ji et al., 2011; Xiong and Zhu, 2003).

As compared to watered control, Traes_5BL_4BED1CA17.1 (*TaNCED3.1*) had ~11 fold higher induction under drought in the seedlings not treated with GD-sap. However, induction was further increased in GD-sap treated wheat where *TaNCED3.1* level was 6.38 fold higher than untreated drought stressed seedlings (Fig. 3B). Under drought conditions, the other homologue, *TaNCED3.2* (Traes_2AL_BDB97A5BA.1) also had a 2.3 fold increased expression in GD-sap treated seedlings than the untreated controls (Fig. 3B). Drought induced the expression of *TaNCED2*; however, its level was similar in both, GD-sap treated and untreated seedlings. Similarly, the expression of *TaNCED4* was induced by the water stress; however, it was relatively less induced in GD-sap treated wheat than the untreated seedlings (Fig. 3B). Except *TaNCED4*, no other ABA biosynthetic genes showed differential expression between GD-sap treated or untreated plants under watered control. *TaZEP1* expression was not changed in any of the treatments. These results indicate that GD-sap increased the expression of *NCED3* genes for higher accumulation of ABA in wheat under drought stress (Fig. 3B, D).

Although, the expression of both, *TaABA8'OH1* and *TaABA8'OH2* were upregulated under drought (SNK test, $p < 0.05$), no difference in *TaABA8'OH1* levels were observed between GD-sap treated and untreated seedlings under drought stress. On the other hand, *TaABA8'OH2* expression was significantly reduced by GD-sap treatment by 1.93 fold than the drought stressed untreated seedlings (SNK test, $p < 0.05$; Fig. 3C). Interestingly, GD-sap treatment alone could significantly induce the expressions of both, *TaABA8'OH1* and *TaABA8'OH2* by 3.46 and 1.56 fold, respectively, without even water stress (Fig. 3C). These results suggested that the treatment with GD-sap could activate the ABA catabolism in wheat even under well-watered condition.

Taken together, the above results demonstrated that the GD-sap treatment altered the ABA homeostasis in wheat under drought.

3.4. GD-sap treatment reduces water loss and increases the stomatal closure

ABA is a key regulator of stomatal movement that, upon higher accumulation, induces closing of the stomata to prevent water loss and to improve avoidance of dehydration inducted by osmotic stress (Xiong and Zhu, 2003; Zhu, 2002). To address whether increased ABA accumulation after GD-sap treatment activates the ABA-related responses in wheat under osmotic stress, we performed short-term water loss assay on 7 d old seedlings that were treated or not treated with GD-sap. After 2 days, leaves were detached and weighed over time course of stress treatment up to 12 h. As compared to untreated control, GD-sap treated seedlings had reduced water loss (Fig. 4A). We also investigated the stomatal aperture in seedlings of both the treatments after 4 d of drought; stomatal apertures were significantly reduced in GD-sap treated seedlings (*t*-test $p < 0.05$; Fig. 4B). No difference in stomatal aperture was observed in watered control (Fig. 4B). These observations indicate that the GD-sap treatment activated ABA related response that decreased water loss from the stomata in wheat under water stress.

3.5. Effect of fluridone on GD-sap treated wheat under watered and drought stress

To further provide a support for the role of ABA in GD-sap mediated drought tolerance, we treated the wheat seedlings with 50 µM fluridone and its effect on leaf survival was determined after 10 days of watered control and drought stress. Fluridone is a potent inhibitor of carotenoid/ABA biosynthesis and effective to inhibit ABA biosynthesis with this concentration in crop plants (González-Villagra et al., 2018; Seiler et al., 2011). Seedlings treated with fluridone showed significantly higher number (56.7%) of dead leaves than the untreated seedlings even under watered control (SNK test $p < 0.05$) (Fig. 5A, C). This is consistent with previous observation where fluridone treatment caused severe damage to the plants by blocking the carotenoid biosynthesis (Barrero et al., 2007; Zou et al., 2018). Interestingly, GD-sap treatment with fluridone had relatively less damage than the fluridone treatment alone, which indicates that GD-sap can protect wheat seedlings from other stresses as well (Fig. 5A, C).

Under water stress, seedlings treated with fluridone showed higher stress injury than the untreated seedlings (Fig. 5B, D). GD-sap + fluridone treatment had higher survival than only the fluridone treatment; however, the treatment had significantly reduced leaf survival than the

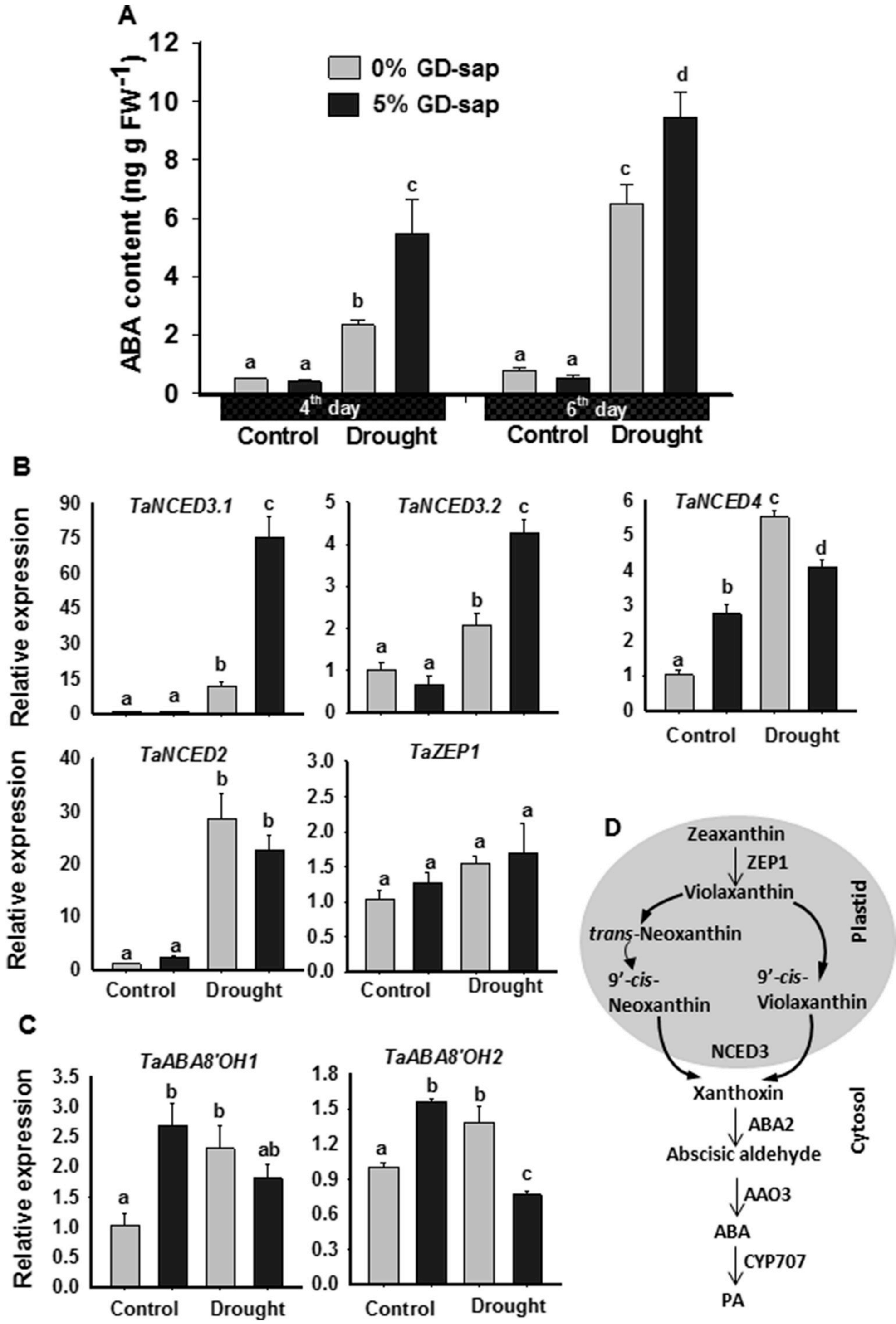

Fig. 3. GD-sap treatment alters ABA content and expression of ABA metabolism genes in wheat under drought. (**A**) GD-sap treated and non-treated 7 d old wheat seedlings were subjected to drought stress and ABA was determined after 4 and 6 d of stress treatment. Data are means ± SE (n = 6–10) from two independent experiments. (**B, C**) Gene expression analysis of ABA metabolism genes in 6 d drought stressed seedlings that were pre-treated with GD-sap. Data are means ± SE (n = 3). Different letter shows significant difference between GD-sap treated and non-treated seedlings under control and drought stress (SNK test $p \leq 0.05$). (**D**) Schematic pathway of ABA metabolism (biosynthesis and catabolism) based on the information available from research in *Arabidopsis*. Conversion of zeaxanthin to xanthoxin occurs in chloroplast, whereas the other steps follow in the cytosol. NCED catalyses critical step of ABA synthesis. *TaABA8'OH1* and *TaABA8'OH2* are the members of the *CYP707* gene family that catabolizes ABA into 8'hydroxyl ABA that subsequently converts into phasic acid (PA), an inactive form of ABA. The elongation factor gene was used to normalize real time data.

GD-sap treatment (SNK test $p < 0.05$) (Fig. 5B, D). The results clearly point out the importance of ABA in conferring drought tolerant response by GD-sap; however, GD-sap might uses alternative mechanisms, other than ABA, to protect the wheat under drought stress.

3.6. GD-sap treatment potently activates the expression of stress protective genes under drought

We further determined whether GD-sap mediated drought tolerance of wheat is also associated with the transcriptional reprogramming of stress protective genes. Such genes were selected on the basis of their well-defined functions in stress tolerance mechanisms in various plant species (Janiak et al., 2015); Table S2). The Traes_3AL_E58742B88.2 gene encodes for *Highly ABA-Induced* (*HAI1*) gene (*TaHAI1*), a clade-A member of PP2Cs family that functions as negative regulator of ABA signalling and shows elevated expression to regulate osmolyte accumulation under water stress in *Arabidopsis* (Bhaskara et al., 2012). Drought stress induced the *TaHAI1* transcripts by 5.5 fold; in GD-sap treatment, *TaHAI1* induction was 8.9 fold higher (Fig. 6). An increased expression of *HAI1* in drought stressed GD-sap treated wheat suggests that the *TaHAI1* might play important roles in drought adaptation response. However, unlike *Arabidopsis*, *TaHAI1* may not regulate proline and solute accumulation in wheat under drought stress. It is postulated that the higher levels of *HAI1* is an important mechanism that

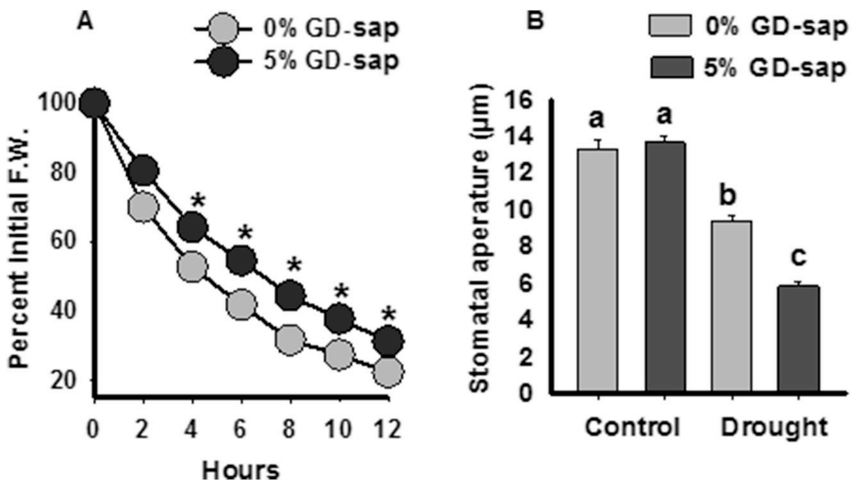

Fig. 4. GD-sap treatment affects leaf water loss and stomatal aperture. (**A**) Water loss from detached leaves after 2 d treatment of GD-sap. Data are means ± SE (n = 10–12) from 2 to 3 independent experiments. (**B**) Stomatal aperture was analysed from 7 d old GD-sap treated wheat seedlings after 4 days of drought. Watered plants were taken as control. Data are means ± SE (n = 5–6). Experiment was repeated with similar results. Asterisks shows significant difference (calculated by *t*-test at $p < 0.05$) between the 0% and 5% GD-sap treated samples at any given time.

maintains drought signalling for better adaptation of plants under water stress (Bhaskara et al., 2012). Genes, Traes_4AS_1E79E1072.1 (*TaRAP2-4*) and Traes_1Bl_BDF0801D01.2 encode the members of *DREB* subfamily A6 and A2 of ERF/AP2 transcription factor family, respectively (Supplemental Table S2). DREBs A6 and A2 are involved in ABA dependent and independent regulation of drought tolerance in various plant species (Lata and Prasad, 2011). The expressions of *TaRAP2-4* and other *DREB* homologue (Traes_1Bl_BDF0801D01.2) were significantly higher by 1.96 and 2.74 fold, respectively, in drought stressed GD-sap treated wheat than the untreated seedlings (SNK test $p < 0.05$; Fig. 6).

Dehydrins are multi-family proteins that are generally produced under cold and drought stress and are associated to crucial protective functions (Wang et al., 2014). Because of increased expression of dehydrins in response to ABA, they are also referred as RAB (Responsive to ABA) (Hanin et al., 2011). The Traes_6AL_DAE444F2F.1 gene encodes for a dehydrin, which showed 53.4 and 44.3% identity with wheat *Dhn-5* (Brini et al., 2011) and *RAB18* of *Arabidopsis*, respectively (Sharma and Verslues, 2010). As expected, *dehydrin* expression was highly induced by drought in both treatments, where the level was ~3 fold higher in GD-sap treated wheat than untreated stressed seedlings (Fig. 6). The expression of *Delta 1-Pyrroline-5-Carboxylate Synthetase* (*P5CS1*) (Traes_1Al_014F29DA6.1) was induced by drought by 3.8 fold as compared to the unstressed controls. GD-sap treatment further induced the *TaP5CS1* level by 1.86 fold over the untreated seedlings under water stress (Fig. 6). P5CS1 catalyses the conversion of glutamate into Pyrroline-5-Carboxylate and is considered as a rate-limiting enzyme for stress-induced proline synthesis in plants (Sharma et al., 2011; Sharma and Verslues, 2010). An increased expression of *TaP5CS1* could explain the proline differences in GD-sap treated and untreated seedlings under drought (Fig. 2).

Three transcription factors, zinc finger protein 2 (Traes_4DS_676CACEAC.1), *TaMYB31* (Traes_5BL_632EBAD09) and *TaAREB3* (ABA-responsive element binding protein) were also analysed due to their relevance in drought tolerance (Bi et al., 2016; Huang et al., 2011; Wang et al., 2016). All these genes had significantly higher expressions in GD-sap treated seedlings than the untreated under drought stress (Fig. 6).

We also analysed the expression of *EARLY-RESPONSIVE TO DEHYDRATION 2* (*ERD2*, a heat shock protein 70; Traes_4Bl_C8337C9F6; Supplemental Table S2) that is induced by drought stress but not by

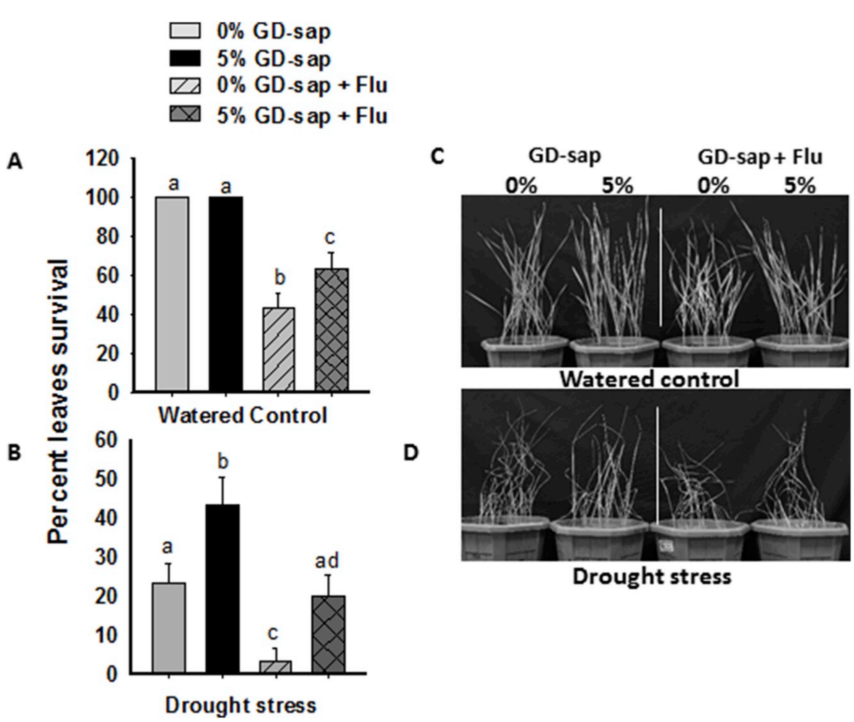

Fig. 5. Effect of fluridone on GD-sap treated and untreated wheat under watered and drought stress. (**A**) 7 d old wheat seedlings were treated with GD-sap, 50 µM fluridone and GD-sap + fluridone and, leaf survival percentage was determined after 10 days of watered control (**A**) and drought treatment (**B**). Data are means ± SE (n = 6–10). (**C, D**) Representative pictures shown the effect of fluridone on GD-sap treated and untreated seedlings under control (**C**) and drought stress (**D**).

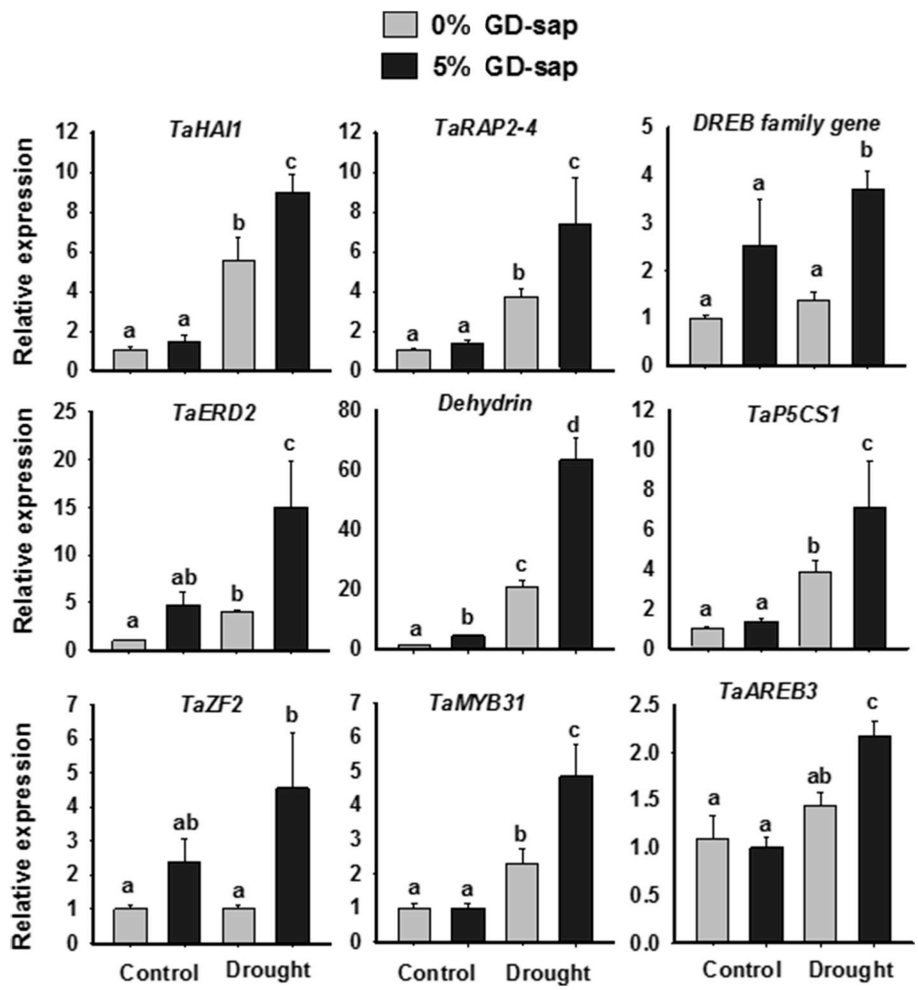

Fig. 6. Increased expression of drought responsive genes in GD-sap treated wheat seedlings under drought stress. Data are means ± SE (n = 3). Different letter shows significant difference between GD-sap treated and non-treated seedlings under control and drought stress (SNK test $p \le 0.05$). Experiment was repeated independently and show similar results. The elongation factor gene was used to normalize real time data.

ABA (Kiyosue et al., 1994) to check if GD-sap can also elevate expression of stress related genes independent of ABA accumulation. Traes_4Bl_C8337C9F6 showed 3.7 fold increased expression after GD-sap treatment than in the untreated seedlings under drought (Fig. 6). Such observation suggested that the GD-sap treatment activated a stress specific response that controls the expression of various drought responsive genes, which may contribute to enhanced drought tolerance in wheat.

3.7. GD-sap treatment enhances drought tolerance of wheat grown in field conditions

To investigate the effect of GD-sap treatment under field conditions, one-month old wheat plants were foliar sprayed with 5% of GD-sap and subjected to drought stress by withholding water for 16 d followed by re-watering of the plants. The chlorophyll content of top leaf was decreased (16%) in drought stressed plants as compared to the watered controls (Fig. 7A). In contrast, chlorophyll content in GD-sap treated drought-stressed plants was 35.4% higher than the untreated stressed plants (Fig. 7A; SNK test, $p < 0.05$). Also, there was a slight but significant increase (13.7%; SNK test, $p < 0.05$) in the total chlorophyll content of GD-sap treated wheat under watered condition (Fig. 7A). Top leaf was collected at the end of drought treatment (16 d) and expression analysis was performed on selected genes to test whether these genes respond to GD-sap treatment in a similar manner under field conditions. All the four genes, *TaNCED3.1*, *TaERD2*, *TaHAI1* and *DREB* homologue, were induced by drought treatment and their levels were further increased by GD-sap treatment, as were observed under controlled/laboratory condition (Fig. 7B).

Various yield parameters were analysed at end of the experiment. Yield was significantly reduced by drought stress for both, GD-sap treated and untreated wheat as compared to their respective watered controls. However, the growth inhibition effect of water stress was more pronounced on wheat not treated with GD-sap (Fig. 7C). Interestingly, in field conditions, GD-sap treatment was effective not only to drought stress, but also under well-watered conditions where GD-sap treated plants had significantly increased yield (13%) than the untreated control (SNK test $p < 0.05$). There was also a growth stimulatory effect on yield-associated parameters such as spike length and spike numbers, which were increased in GD-sap treated wheat under water limited condition (Fig. 7D and E). Thousand kernel weight (TKW) was not different between GD-sap treated and untreated wheat during drought or watered control (Supplemental Fig. S5).

Taken together, the results show that GD-sap treatment increased the performance and production of wheat under field grown conditions. It is safe to conclude that the results from our field study corroborates with those from green house to show the application of GD-sap in enhancing drought tolerance/stress recovery and the plausible underlying mechanism.

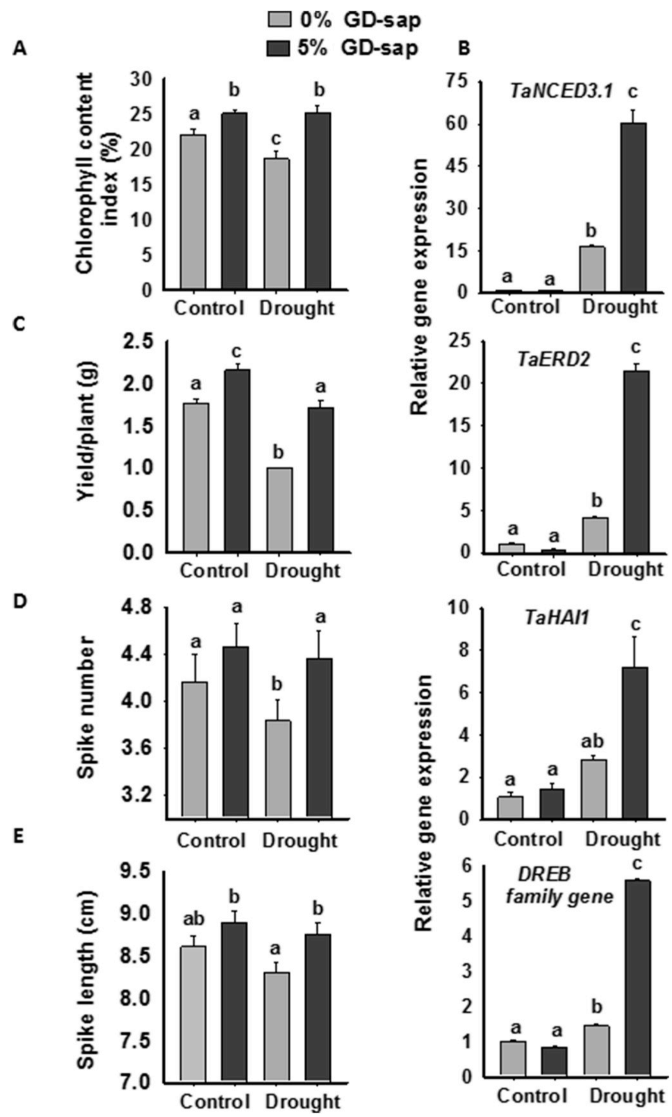

Fig. 7. GD-sap treatment improves wheat growth in field condition under drought stress. (**A**) One month old wheat plants were subjected to drought stress for 16 d after pre-treatment with GD-sap and chlorophyll content was analysed. Data are means ± SE (n = 30–35). (**B**) Top leaf samples of 16 d drought stressed plants were collected to analyze the expression of drought responsive genes. Data are means ± SE (n = 3). (**C**) Total yield and other yield-associated parameters like spike number (**D**), and spike length (**E**) were determined after harvest. Data are means ± SE (n = 100–120). Different letters shows significant difference between GD-sap treated and non-treated seedlings (SNK test p ≤ 0.05). The elongation factor gene was used to normalize real time data.

4. Discussion

In this study, we demonstrated the functional relevance and physiological role of *G. dura* sap application in improving drought tolerance in wheat. The application of GD-sap improved the performance of wheat not only under laboratory conditions, but also in field conditions. An improved drought/stress survival of GD-sap treated wheat was associated with various physiological changes (such as in chlorophyll content, RWC, osmotic potential, proline content) that could be modulated by alteration in ABA content and signalling. The enhanced ABA signalling after GD-sap treatment plausibly helps plants to maintain high water content during deficit periods. GD-sap treatments altered the ABA homeostasis, reduced water loss and increased the expression of drought responsive genes in wheat under water stress.

GD-sap altered ABA content and activated the ABA-related response in wheat under water stress. Although ABA or ABA-like compounds have been detected in some seaweed extracts (Gupta et al., 2011a; Stirk et al., 2014), the net ABA content in seaweed extracts is believed to be too low to elicit a physiological response under field condition (Wally et al., 2013). At the same time, the seaweed extracts are complex in their chemical composition (as revealed for GD-sap, Table S1), and may contain several biostimulants (Santaniello et al., 2017). Such regulatory molecules/biostimulants or ABA like compounds may complementarily induce the accumulation of ABA by activating genes in ABA biosynthesis pathway when wheat is under water stress. Our gene expression analysis supports such a model, where GD-sap treated drought-stressed wheat seedlings had increased expression of *TaNCED3.1* and *TaNCED3.2* genes. Other homologues of *NCED* gene family, *TaNCED2* and *TaNCED4*, were significantly changed under drought but showed no expression differences between GD-sap treated and untreated seedlings. This is in agreement with previous findings where *NCED3* is considered as a rate limiting gene of ABA biosynthesis and increased expression of this gene have positive correlation with ABA accumulation under osmotic stress in various plant species (Ahrazem et al., 2011; Song et al., 2016; Xiong and Zhu, 2003). In addition, GD-sap treatment reduced the transcript of *TaABA8'OH2* under drought stress. Stress induced ABA accumulation may be a result of, both, simultaneous activation of ABA biosynthesis and decrease in ABA catabolism (Xiong and Zhu, 2003; Zhu, 2002). TaABA8'OH are the key enzymes in ABA catabolism pathway. A decrease in the expression of *TaABA8'OH2*, along with increased level of *NCED* genes, may lead to higher ABA content in GD-sap treated wheat seedlings during drought stress. Overall, our results imply that the biostimulants in GD-sap regulate the expression of *TaNCED3* (1 and 2) and *TaABA8'OH2* genes for increased accumulation of endogenous ABA in wheat under water stress. In addition, transcripts of ABA catabolism genes, *TaABA8'OH1* and *TaABA8'OH2*, were increased in GD-sap treated seedlings under well-watered condition. This implies that the GD-sap treatment maintains the ABA homeostasis to avoid any growth inhibition effect that may occur due to higher ABA content under non-stressed condition.

Plants having higher ABA levels show better performance under water stress (Iuchi et al., 2001; Zhang et al., 2015). This is due to an activation of ABA signalling that rapidly closes the leaf stomata to prevent water loss from the plants. In addition, drought accumulated ABA may activate a diverse array of signalling to protect the plants from stress injury (Kalladan et al., 2017; Osakabe et al., 2014; Raghavendra et al., 2010; Shinozaki and Yamaguchi-Shinozaki, 2007; Tran et al., 2007, 2004). Our results showed that the GD-sap treatment induced higher accumulation of ABA in wheat which, in turn, activates signalling to close the stomata to prevent the water loss (Fig. 4). In addition, GD-sap upregulates the expression of several genes that might be involved in controlling the drought tolerance in wheat (Bi et al., 2016; Huang et al., 2011; Kiyosue et al., 1994; Sharma and Verslues, 2010; Shinozaki and Yamaguchi-Shinozaki, 2007; Xiong and Zhu, 2003; Zhu, 2002). Taken together, GD-sap can potentially trigger diverse stress signalling components that induce higher ABA accumulation to control the water loss and elevate gene expression to alleviate drought effect from the wheat plants. Similar observations were also recorded in field experiment where increased yield of GD-sap-treated wheat was associated with elevated expression of *NCED3.1* and other drought response genes. These findings further support the conclusion that the GD-sap regulates ABA homeostasis to confer the drought tolerance in wheat.

Seaweed extracts vary in their action due to their compositional differences. For instance, a seaweed extract from *Ascophyllum nodosum* (ANE), a brown alga, improves plant performance under drought by increasing photosynthetic rates in *Arabidopsis* (Santaniello et al., 2017). In contrast to our observation where ABA accumulation seems to play an important role in enhancing the drought tolerance, the expression of *AtNCED3* was decreased (thus, indicating a reduced ABA accumulation) in ANE-treated seedlings at the later stages of dehydration stress.

Furthermore, seaweed sap of a red alga, *K. alvarezeii*, modulated various metabolites to enhance drought tolerance of wheat (Patel et al., 2018). These observations suggest that seaweed extracts adopt different mechanisms to improve the drought tolerance in various plant species. The modes of action of seaweed extracts are mainly dependent on the nature and abundance of biostimulants that could be treatment and species specific. When we analysed the GD-sap content (Table S1), we indeed found a complex composition. It is plausible that GD-sap may influence wheat's physiology by additional mechanisms complementary to ABA signalling (Fig. 5). Enhanced performance of wheat under field conditions is a good indicator that in agro-ecological environments, plants are able to better utilize the seaweed resources. Interestingly, when tested in laboratory conditions, these changes were observed mostly in drought but not in watered controls, indicating that the GD-sap treatment specifically activates stress protective mechanisms to improve wheat performance under drought. On the other hand, field grown wheat had increased growth and production even in watered controls. We hypothesise that, such results might plausibly be due to several factors, such as due to complex natural environment that may help plants to better utilize GD-sap. The developmental stage of the plants at the time of GD-sap treatment may also influence the outcomes as was evident in maize where the growth stages of the plants significantly influenced the capacity of *Kappaphycus alvarezii* sap to improve productivity (Trivedi et al., 2017). Additionally, it is possible that the GD-sap improves the performance of plants under physiological drought conditions in fields, a condition in which the plants are not able to take up water in spite of its presence in soil. Nevertheless, our study highlights the importance of conducting 'real-world' tests of function while studying stress adaptation of plants (Pandey et al., 2008; Sharma et al., 2018). Which agronomic factors may influence GD-sap's protective functions need further investigation. Further insights in molecular mechanisms underlying GD-sap triggered response of wheat during drought stress would be elucidated by *–omics*-guided functional studies.

In conclusion, our results, to the best of our knowledge, are the first demonstration of GD-sap application as a method to improve drought resistance of wheat. The study also provides insights into the functional role of GD-sap in enhancing drought tolerance by changing ABA homeostasis and elevated gene expression. Our study paves the way for use of GD-sap as biostimulant to increase the production of wheat and possibly other crops in drought prone areas. The next step in investigation would be more extensive field tests and further experiments to understand mechanism of action of GD-sap under drought conditions.

Author's contributions

S.S. designed the research and executed most of the experiments and drafted the manuscript. S.S., K.K. and M.S.R. performed physiological assays. S.S. performed ABA measurement. C.C. and S.P.P retrieved gene sequences of wheat for expression analysis. S.S., S.P.P. and C.C. analysed the data and S.S. and S.P.P. wrote the manuscript. All authors read and approved manuscript.

Disclosures

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.01.015.

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