P5CS expression level and proline accumulation in the sensitive and tolerant wheat cultivars under control and drought stress conditions in the presence/absence of silicon and salicylic acid

Kobra Maghsoudia, Yahya Emanb, Ali Niazi, Mohammad Pessaraklic and Mohammad Javad Arvind

aDepartment of Crop Production and Plant Breeding, College of Agriculture, Shiraz University, Shiraz, Iran; bInstitute of Biotechnology, Shiraz University, Shiraz, Iran; cSchool of Plant Sciences, The University of Arizona, Tucson, AZ, USA; dDepartment of Horticulture, College of Agriculture, Shahid Bahonar University, Kerman, Iran

ABSTRACT
The effects of silicon (Si) and salicylic acid (SA) applications on proline content and expression of Δ1-pyrroline-5-carboxylate synthetase (P5CS) were examined under different drought levels and different drought exposure times. Two wheat cultivars, a drought tolerant and a drought sensitive were used. The experiment was a factorial based on completely randomized design with three replicates. Expression analysis by the quantitative real time PCR showed that the tolerant cultivar had significantly higher P5CS expressions compared to the sensitive one under drought stress. In sampling time points, the maximum level of mRNA was observed at 48 h after stress was applied. At 48 h after stress induction, the expression of P5CS was almost 3.1 fold higher in the tolerant cultivar compared to the sensitive one. In both cultivars, gene expression decreased from 48 to 72 h. The stressed plants treated with Si + SA showed a higher expression. Proline content started to increase by Si and SA treatments and the maximum proline content was obtained at simultaneous application of Si + SA. Drought stress significantly reduced chlorophyll content, relative water content and leaf water potential of both cultivars, while increased electrolyte leakage (EL) of the leaves. In contrast, foliar-applied Si and SA significantly increased these parameters and reduced EL, and the effect of simultaneous application of Si and SA was greater. The results suggest that the P5CS is a stress inducible gene. This gene has the potential to be used for improvement of drought stress tolerance in wheat. Network analysis highlighted positive interaction of osmotic stress, drought and cold stress on P5CS1 and the regulatory role of MYB2, ERF-1, and EIN3 transcription factors. In conclusion, alleviation of drought stress by application of Si and SA was associated partially with enhanced expression of P5CS gene and following proline accumulation.

INTRODUCTION
Wheat (Triticum aestivum L.) is an important food crop grown all over the world. Wheat productivity is hampered due to a variety of abiotic stresses such as drought, salinity, and heat (Costa et al. 2011). Under semi-arid climatic conditions, wheat is usually exposed to drought stress periods during the growing season (Dhanda et al. 2004). Therefore, research to develop wheat cultivars resistant to drought is crucial to expand the range of wheat growth to arid or semi-arid areas (Lobato et al. 2009).

A major component of drought tolerance is the production and accumulation of osmotically active substances, known as osmoregulation (Reddy et al. 2004; Zhu et al. 2005). Osmoregulation, which involves maintenance of cell turgor or volume by accumulation of solutes, is a significant adaptation mechanism under water stress conditions (Hummel et al. 2010). Several plants modify their metabolism under water deficit by accumulating proline, soluble carbohydrates, organic acids, and amino acids (Lobato et al. 2009). Previous studies have suggested that proline accumulation contributes to increase osmotic stress tolerance (Yamada et al. 2005; Silva Ortega et al. 2008; Verbruggen and Hermans 2008; Soltan Shahattary and Mansournia 2017; Sourour et al. 2017; Ghodke et al. 2018). However, the significance of proline accumulation is still controversial, and it is unclear whether proline accumulation in plant tissues confers some adaptive advantages to the plant under osmotic stress or it is a consequence of stress induced changes in metabolism (Yamada et al. 2005). In plants, proline biosynthesis occurs via two pathways: glutamate or ornithine (Hu et al. 1992). In the first pathway, glutamate is converted to proline by two successive reductions, catalyzed by pyrroline-5-carboxylate (PSC) synthetase and P5C reductase, respectively. PSC synthetase is a bifunctional enzyme, which initially catalyzes the activation of glutamate by phosphorylation followed by reduction of the labile intermediate γ-glutamyl phosphate into glutamate semialdehyde (GSA), which is in equilibrium with the PSC form (Hu et al. 1992). A strong correlation between P5CS expression and the accumulation of proline has been shown in rice and Arabidopsis thaliana (Hein et al. 2003; Szekely et al. 2008). Ornithine, the alternate precursor can be transaminated to P5C by ornithine-γ-amino-transferase (OAT), a mitochondrial enzyme (Roosens et al. 1998). In stressed plants, proline is preferably synthesized directly from glutamate via D1-pyrroline-5-carboxylate...
synthetase (Delauney et al. 1993). Transgenic plants over-expressing P5CS accumulate more proline than the control plants and are tolerant to osmotic stress (Kavi Kishor et al. 1995). Expression of proline metabolizing enzymes and proline levels have been reported for transgenic plants (De Ronde et al. 2001; De Ronde et al. 2004; Molinari et al. 2004; Su and Wu 2004), seedlings (Phutela et al. 2003), and for plants exposed to shock treatments (Igarashi et al. 1997; Soltan Shahattary and Mansourifar 2017; Sourour et al. 2017; Ghodke et al. 2018).

Proline acts as osmoprotectant; it can also function as a protein stabilizer, and a hydroxyl radical scavenger, stabilizes cell membranes by interacting with phospholipids, and serves as a source of carbon and nitrogen (Kavi Kishor et al. 2005). P5CS gene has been isolated from several plant species. P5CS gene was first cloned from Vigna aconitifolia (Hu et al. 1992). In some species, two closely related P5CS genes have been identified, but they apparently have no unified functions. For example, in Arabidopsis thaliana, AtP5CS1 is inducible by drought, salt and ABA, whereas AtP5CS2 is apparently a housekeeping gene active in dividing tissues (Strizhov et al. 1997). The expression patterns of two P5CS orthologues were also different under NaCl stress in tomato (Fujita et al. 1998). The transcript of tomPRO2 increased more than 3-fold, whereas the transcript of tomPRO1 was undetectable (Fujita et al. 1998). In Brassica napus, however, both BnP5SCS1 and BnP5SCS2 were inducible by ABA, NaCl and PEG (Xue et al. 2009). In Oryza sativa and Phaseolus vulgaris, OsP5CS1 and OsP5CS2, PvP5CS1 and PvP5CS2 were also up-regulated by various stresses (Chen et al. 2009). OsP5CS1 is ubiquitously expressed contrasting with OsP5CS2, which is mainly expressed in mature organs. Prior studies demonstrated the up-regulation of the expression levels of P5CS and the accumulation of proline had a cause and effect relationship, except in tomato. Overexpression of P5CS also increased stress tolerance of transgenic potato, rice and wheat as a result of the increased proline content (Vendruscolo et al. 2007).

Silicon (Si) is the second most prevalent element within the soil and a non-essential nutrient element for the majority of plants (Agostinho et al. 2017). Si uptake by plants provides many benefits such as improved abiotic and biotic stresses, such as drought stress (Gong et al. 2008; Shen et al. 2010), salt stress (Ashraf et al. 2010) and freezing stress (Liang et al. 2008). In general, species of Poaceae family accumulate much more Si than other species (Gong et al. 2008). Different mechanisms for the Si-mediated stress alleviation have been proposed (Shen et al. 2010). Ming et al. (2012) suggested that cereal crops supplied with Si could sustain a higher leaf water potential ($\psi_w$) than crops grown without Si application under water deficit conditions. Also, Si can increase the anti-oxidative defense systems, both enzymatic and non-enzymatic, and thereby alleviate ROS damage induced by stresses (Ashraf and Harris 2004; Gong et al. 2008; Xu et al. 2008; Ashraf 2009). Furthermore, under drought stress conditions, exogenous application of plant growth regulators (PGR) may overcome much of the internal PGR deficiency and mitigate drought-induced inhibitory effects. Like other known plant growth regulators, salicylic acid (SA) is believed to play a major role in defense mechanisms against drought stress (Ashraf and Foolad 2007). Foliar application of SA modulates activities of key intracellular antioxidant enzymes and consequently increases plant tolerance to environmental stresses (Ashraf and Foolad 2007; Nikolaeva et al. 2010). These investigators (Ashraf and Foolad 2007; Nikolaeva et al. 2010) also showed that SA foliar application alleviated the adverse effects of stresses, which were mainly ascribed to the enhanced accumulation of free proline and soluble proteins. Previous studies have demonstrated that SA plays an important role in determining the tolerance of crops to drought stress (Hussein et al. 2007; Ashraf et al. 2010).

The objectives of this study were to determine the influence of Si and SA applications on expression of P5CS gene under drought stress in wheat cultivars and to establish a relationship between the proline accumulation and the expression of P5CS gene. The results contribute to a better understanding of the mechanisms of Si and SA-induced improvement in drought tolerance of wheat cultivars.

Materials and methods

Plant materials and growth conditions

The experiment was carried out at the greenhouse of the College of Agriculture, Shiraz University, Iran, in 2014 growing season using two wheat cultivars (Sirvan and Shiraz). Minimum and maximum temperatures in the greenhouse were 14 and 28 °C, respectively, where relative humidity varied between 55-60%. The wheat plants were exposed to a 14 h photoperiod. All seeds were surface-sterilized in 1% sodium hypochlorite solution for 10 min, rinsed thoroughly with distilled water. The seeds were germinated on a moist filter paper placed in Petri dishes for 48 h. The 10 days old seedlings were transplanted into 5 L size plastic pots (10 seedlings per pot).

Experimental design and treatments

The experiment was a factorial based on randomized complete block design with three replicates. There were eight treatments for each wheat cultivar: no drought stress and no foliar application (wet, Si- SA-), no drought stress with silicon (Si) application (wet, Si+), no drought stress with salicylic acid (SA) application (wet, SA+), no drought stress with Si + SA application (wet, Si+ SA+), drought stress without foliar application (dry, Si- SA-), drought stress with Si application (dry, Si+), drought stress with SA application (dry, SA+), and drought stress with Si + SA application (dry, Si+ SA+). The pots were watered regularly to maintain soil moisture at field capacity (F.C.) before starting irrigation treatments. Drought stress treatment (40% F.C.) was imposed 30 days after sowing and continued for a period of 20 days. The control pots were regularly watered to maintain 100% F.C. Foliar application of silicon (6 mM) and SA (1 mM) were carried out at 25 days after sowing. Si and SA were applied (with a hand sprayer until the solution began to drip off leaves) at sunset. To assure Si and SA uptake by the leaves, they were applied on four consecutive days. The pots not receiving Si or SA were treated similarly with equivalent amount of distilled water. Leaf samples were collected at 0, 48, and 72 h after the initiation of the drought treatment. The samples were immediately frozen in liquid nitrogen and then stored at −80°C for determination of total RNA, proline, and chlorophyll content.
Primer design

Primer design was carried out using Allele ID 7 software for the internal control and P5CS (A-1-pyrrolin-5-carboxylate synthetase) gene. The accession number of the P5CS gene used to design the primers was JQ063082 in wheat. In this research, the wheat elongation factor (EF) gene was used as the internal control for data normalization. The EF gene was chosen based on the literature (Caldana et al. 2007; Shim et al. 2016). Expression of elongation factor gene was not influenced by drought stress (Caldana et al. 2007; Shim et al. 2016).

Two primer pairs were designed for P5CS (forward: 5'-ACAGAGATAAAGTACAGACAG-3' and reverse: 5'-AGACCTTCAACCACGAGC3'-3') and elongation factor gene (forward: 5'-GGTTAAGATGATTCCCACCAAGCC-3' and reverse: 5'-GACAACACCAAAGCAACGTCTG-3').

RNA extraction and cDNA synthesis

Total RNA was extracted using RNX-Plus buffer. Briefly, about 100 mg of tissue was ground in liquid nitrogen. The ground powder was transferred to 1 ml of RNX-Plus buffer in an RNase-free microtube, mixed thoroughly, and left at room temperature for 5 min. Then, 0.2 ml of chloroform was added to the slurry and mixed gently. The mixture was centrifuged at 13,000 g at 4 °C for 15 min. The supernatant was transferred to a new tube and precipitated with an equal volume of isopropanol for 15 min on ice. The RNA pellet was washed using 75% v/v ethanol and briefly dried and re-suspended in 50 µl of RNase-free water (Figure 1). The purified total RNA was quantified by Nano-Drop ND 1000 Spectrophotometer (Wilmington, USA) and gel electrophoresis. Then, DNase treatment was carried out using Fermentas (Fermentas, Hanover, MD) DNase Kit according to the manufacturer’s instructions. Then, 5 μg of DNase-treated RNA was used for the first strand cDNA synthesis, using 100 pmol oligo-dT (18 mer), 15 pmol dNTPs, 20 U RNase inhibitor, and 200 U M-Mulv reverse transcriptase (all from Fermentas) in a 20 µl final volume.

Quantitative real-Time PCR analysis

Quantitative real-time PCR was performed in a 20 µl volume containing 1 µl cDNA, 19 Syber Green buffer, and 4 pmol of each primer. The amplification reactions were carried out in a line-gene K thermal cycler (Bioer, China) with initial denaturing of 94 °C for 2 min, followed by 40 cycles of 94 °C for 10 s, annealing temperature (Ta) of each of the primer pairs for 15 and 30 s of extension at 54 °C. After 40 cycles, the specificity of the amplifications was checked based on melting curves resulted by heating the amplicons from 50 to 95 °C. All amplification reactions were repeated twice under identical conditions, in addition to a negative control and five standard samples. To ensure that the PCR products were generated from cDNA and not genomic DNA, proper control reactions were carried out without reverse transcriptase treatment. For quantitative real-time PCR data, relative expression for P5CS was calculated based on the threshold cycle (CT) method. The CT for each sample was calculated using the Line-gene K software and Larionov et al. (2005) method. Accordingly, the fold expressions of target mRNAs over the reference values were calculated by the equation 2-DDCT, where DCT was determined by subtracting the corresponding internal control CT value from the specific CT of the targets (P5CS), and DDCT was obtained by subtracting the DCT of each experimental sample from that of the control sample.

Network construction

To construct P5CS interaction network, Pathway Studio package 10 (Elsevier) was used. Pathway Studio employs the RESNET Plant database. This database is a comprehensive molecular interaction database in plants, based on Arabidopsis genome as a model plant (Hosseinpour et al. 2012). The package collects information by text-mining via MedScan tool and process under taken data by natural language processing (NLP) and interprets the mined text to logical concepts as functional relationships between proteins, small molecules, and cellular processes (Nikitin et al. 2003).

Determination of proline

Free proline was extracted and colorimetrically estimated by acid-ninhydrin method from frozen tissues (Bates et al. 1973) collected at the time points as described above. Samples of 0.5 g frozen leaves were homogenized with 10 ml 3% sulphosalicylic acid in boiling water for 10 min. The extract was filtered, and the filtrate was mixed with equal volumes of glacial acetic acid and acid-ninhydrin reagent (1.25 g ninhydrin, 30 ml of glacial acetic acid, and 20 ml of 6 M H₃PO₄) and incubated for 40 min in boiling water. The reaction was stopped by placing the test tubes in cold water. After 3 ml of toluene were added, the solution was rigorously mixed. The light absorbance of toluene phase was estimated at 520 nm on a UV-V spectrophotometer, and then, the proline concentration was determined using a standard curve prepared by using proline.

Chlorophyll content

Chlorophyll a was determined according to Lichtenthaler and Wellburn (1983) method. One-hundred mg of fresh leaf materials were taken from the youngest fully expanded leaves and extracted with 99% methanol. Absorption was read using a spectrophotometer at 653 and 666 nm wavelengths, for chlorophyll a and b, respectively. The chlorophyll content was calculated by using the following equations and expressed as mg per gram fresh weight.

$$Chl\ a = (12.25A_{663} - 2.79A_{646})$$
$$Chl\ b = (21.21A_{646} - 5.1A_{662})$$

Measurement of leaf water potential and relative water content

Leaf water potential was measured using a pressure chamber technique (PMS instrument company, ALBANY Oregon 97322) at 0, 48, and 72 h after the initiation of the drought treatments. Furthermore, in these times, relative water content (RWC) was estimated according to the method of Castillo (1996). Samples were saturated in 100 ml distilled water for 24 h at 4 °C in the dark and their turgid weights were recorded. Then, they were oven-dried at 65 °C for 48 h and their dry weights were recorded. Relative water content of a plant tissue is expressed as percentage and calculated...
by using the following equation. Where, FM, DM, and TM were the fresh, dry, and turgid masses, respectively.

\[
RWC = \left( \frac{FM}{TM} - DM \right) \times 100
\]  (3)

Electrolyte leakage

The method of Sullivan and Ross (1979) was used to measure electrolyte leakage (EL) at 0, 48, and 72 h after the initiation of the drought treatments. To remove solutes from leaf surface and damaged areas in leaf samples (0.2 g), they were washed for three times with distilled water. The samples were placed in Pyrex test tubes and incubated with 15 ml distilled water at 23°C for 24 h in the dark. The test tubes were kept at 25°C. After a strong hand shaking of the test tubes, the electrical conductivity (EC) of the electrolytes was measured by a conductivity meter. Following the EC measurements, all samples were autoclaved for 15 min at 60°C. The samples were then returned to 25°C and EC was measured again. EL was calculated by using the following equation: Where, \( C_1 \) and \( C_2 \) refer to the initial and final EC, respectively.

\[
EL = \frac{C_1}{C_2}.
\]  (4)

Data analysis

Means, standard deviation (SD), standard error (SE), and results of the expression analysis (qPCR results) were calculated using SAS package. All collected data were subjected to analysis of variance (ANOVA) and the mean differences were compared using Duncan Multiple Range test (\( P \leq 0.05 \)).

Results

Proline content

The proline content in the leaves of both cultivars was significantly higher in the stressed plants than in the control plants. There was a significant difference between the two wheat cultivars in proline content, so that, cv. Sirvan showed significantly more proline content than Shiraz under drought stress conditions (Figure 1(a)). In sampling time points, the maximum proline content was recorded in Sirvan cultivar at 72 h after exposure to stress (Figure 1(b)). Silicon (Si) and salicylic acid (SA) application increased the proline content of water-stressed plants. Furthermore, the effect of Si + SA on proline content was greater compared to the application of Si or SA separately (Figure 2).

Quantitative real-Time PCR analysis

The quantitative expression patterns for P5CS gene under different drought levels, different time courses of drought treatments and application of Si and SA in two wheat cultivars are shown in Figures 3 and 4, respectively. Furthermore, Analysis of variance showed significant differences between P5CS gene expression in wheat cultivars, under different drought levels. Sirvan (drought tolerant cultivar) had significantly higher expressions of P5CS compared to Shiraz (drought sensitive cultivar), under both drought stress levels (Figure 3(a)). Generally, Sirvan and Shiraz cultivars had significantly higher expressions of P5CS in 100% F.C. compared to 40% F.C. conditions (Figure 3(a)).

Analysis of variance for investigation of differences in the P5CS gene expression profile under different times after drought treatment, in each wheat cultivar, showed that there is a sudden increase in P5CS transcripts at 48 h after drought treatment in Sirvan and Shiraz cultivars. Indeed, in both cultivars, gene expression decreased from 48 to 72 h (Figure 3(c)). In sampling time points, the maximum level of mRNA, accumulated at 48 h after stress applied in both tolerant and sensitive cultivars, while the expression of transcript in tolerant cultivar was almost 3.1 fold higher compared with sensitive cultivar (Figure 3(b)). Measurement of the relationships between the P5CS expression levels in different drought levels showed a significant correlation between gene expression profiles at 100% F.C. and 50% F.C in both cultivars. This relationship is highly significant in Sirvan cultivar. As shown in Figure 4, P5CS expression was affected by Si and SA. The transcript level of P5CS gradually increased by...
application of Si and SA. The gene expression was significantly higher in the stressed plants and treated by Si + SA than in the stressed plants without application of Si and SA (Figure 4). In comparison to P5CS expression, proline content started to increase by Si and SA treatment and the maximum proline content was obtained at simultaneous application of Si + SA (Figure 2).

P5CS network
The predicted network and its relationships are presented in Table 1. The network showed a positive interaction of osmotic stress, drought, and cold stress on P5CS and the regulatory role of MYB2, ERF-1, and EIN3 transcription factors. P5CS is involved in RNA splicing, proline content, response to dehydration, leaf morphogenesis and negative control of root growth.

Chlorophyll content
Drought stress reduced chlorophyll a and chlorophyll b in wheat cultivars. There was a significant difference between wheat cultivars in chlorophyll concentration. The cv. Sirvan (drought tolerant cultivar) showed significantly more chlorophyll a and chlorophyll b than Shiraz (drought sensitive cultivar) under drought stress conditions (Figure 5(a) and Figure 6(a)). However, exogenous application of Si and SA improved concentrations of chlorophyll pigments in both cultivars under drought stress conditions. Indeed, the effect of Si + SA on chlorophyll a and b content was greater compared to application of Si or SA separately (Figure 7). In sampling time points, the minimum chlorophyll a and chlorophyll b contents were found at 72 h after stress applied without application of Si or SA in both cultivars, while the chlorophyll content in resistant cultivar was significantly higher compared with susceptible cultivar (Figure 5(b) and Figure 6(b)).

Leaf water potential ($\Psi_w$), relative water content (RWC), and electrolyte leakage (EL)
Drought stress reduced the $\Psi_w$ and RWC in wheat cultivars. However, a significant difference between the two cultivars was observed. Sirvan cultivar had higher $\Psi_w$ and RWC compared to Shiraz cultivar under drought stress conditions.
In contrast, EL in wheat cultivars, especially in Shiraz cultivar, increased under drought stress conditions (Figure 8(c)).

In sampling time points, the minimum $\Psi_w$ and RWC were recorded at 72 h after stress was applied (Figure 9(a) and figure 9(b)). Relative to their corresponding control, drought stress, 72 h after stress increased EL in both wheat cultivars (Figure 9(c)). Si and SA treatment increased the $\Psi_w$ under water deficit conditions (Figure 10(a)). Application of Si and SA significantly improved the RWC of drought-stressed plants. However, the silicon-applied plants could maintain a better water status under drought stress conditions. This indicates that the Si and SA application could improve the water status of wheat cultivars under drought stress conditions (Figure 10(b)). Furthermore, the effect of Si + SA on RWC and $\Psi_w$ was...
greater compared to application of Si or SA separately (Figure 10(a) and Figure 10(b)). In contrast, Si, SA, and Si + SA application significantly decreased EL under drought stress conditions, while the effect of SA application was significantly higher compared with Si or Si + SA application (Figure 10(c)).

Discussion

Drought stress conditions are believed to affect the physiological and molecular responses of almost all crops, including cereals (Reddy et al. 2004; Soltan Shahattary and Mansourifar 2017). In this study, it was found that proline content significantly increased in wheat leaves under drought stress and it was more pronounced in Sirvan as drought tolerant cultivar compared to Shiraz as drought sensitive cultivar (Figure 2). This increase in proline content is proved to be essential for stress tolerance due to active role of proline in osmotic adjustment, protection of enzyme structure, stabilization of membranes and defense against hydroxyl radicals (Nayyar and Walia 2003; de-Lacerda et al. 2003; Sourour et al. 2017; Ghodke et al. 2018). Proline has also been shown to act as a molecular chaperone involved in protection of protein integrity and enhancement of the activities of different key enzymes (Ashraf and Foolad 2007; Szabados and Savoure 2009; Sourour et al. 2017; Ghodke et al. 2018). The positive role of proline in osmoregulation has also been reported by other researchers (Chaves et al. 2002; Sonobe et al. 2011; Mure et al. 2017). Szabados and Savoure (2009) suggested that the accumulation of proline in leaves might be involved in one or more of the above processes and contribute to drought tolerance. The rate-limiting step in proline synthesis is controlled by a bifunctional P5CS enzyme, which is encoded by two highly homologous genes in Arabidopsis and many other plants (Strizhov et al. 1997; Fujita et al. 1998). P5CS exhibits both γ-glutamyl kinase (γ-GK) and glutamic-γ-semialdehyde dehydrogenase (GSA-DH) activities which correspond to the ProB and ProA proteins of E. coli,
respectively. The \( P5CS \) gene in higher plants was first isolated from \( V. \) aconitifolia by a functional complementation technique. Va\( P5CS \) encoded a complete \( P5CS \) enzyme, which plays a key role in proline synthesis in \( V. \) aconitifolia (Hu et al. 1992).

In this research, \( P5CS \) expressions level in Sirvan (tolerant cultivar) was significantly higher compared to Shiraz (sensitive cultivar), under drought stress conditions. In sampling time points, the maximum level of mRNA was observed at 48 h after stress was applied, while the expression of gene in the tolerant cultivar was almost 3.1 fold higher compared to the sensitive one. It has been suggested that proline synthesis is regulated not only at the level of enzyme activity, but also is influenced by the level of \( P5CS \) gene expression (Kavi Kishor et al. 2005; Wang et al. 2017). In the present study, we noticed that the induction of higher \( P5CS \) expression preceded the accumulation of proline under drought stress. Similar findings occurred in Arabidopsis during drought stress (Yoshiba et al. 1995) and in common bean during salt and drought stress (Chen et al. 2009).
relatively higher expression of \( P5CS \) was observed in the leaves of cv. Sirvan compared to cv. Shiraz, presenting evidence for higher rate of synthesis, thereby, leading to the increased accumulation of proline in cv. Sirvan. Similar increase in \( PSC \) reductase in relation to proline accumulation has been reported (Ramanjulu and Sudhakar 2000; Kumar et al. 2003; Wang et al. 2017). In Arabidopsis and rice, \( P5CS \) was over-expressed under high salinity and dehydration and a simultaneous accumulation of proline was also observed (Hein et al. 2003). In Arabidopsis, there are two \( P5CS \) isoforms playing specific roles in the control of proline biosynthesis (Szekely et al. 2008) and in few other cases, \( P5CS \) is reported to be encoded by two genes (Fujita et al. 1998). Between the two closely related \( P5CS \) genes recently identified in \textit{Arabidopsis thaliana}, \( P5CSI \) is the major one functioning under stress and its expression is usually controlled by different signaling pathways (Szekely et al. 2008). The results of this research also showed that foliar application of silicon (Si) and salicylic acid (SA) significantly increased proline concentration under water deficit conditions (Figure 3). The SA-induced increase in proline content under stress conditions has been reported in barely (El-Tayeb 2005) and wheat (Nayyar and Walia 2003). Singh and Gautam (2013) believed that the SA-induced enhanced synthesis of proline improves plant tolerance against stresses. Furthermore, a significant positive correlation has been reported between the enhanced concentration of intracellular proline and the ability of plants to survive under abiotic stress conditions (Ashraf and Foolad 2007; Murmu et al. 2017; Sourour et al. 2017; Ghodke et al. 2018).

In the present study, RT–PCR analysis indicated that increased expression of \( P5CS \) was induced by Si and SA treatments (Figure 5). Indeed, the effect of simultaneous application of Si and SA was greater compared to application of Si or SA separately. We presumed that the increased accumulation of proline in wheat under drought and Si and SA treatment might be associated with the increased expression of \( P5CS \). SA is a plant regulator that appears to be involved in the regulation of proline metabolism. Several recent studies reported a promoting effect of SA on proline accumulation even in the absence of stresses (El-Tayeb 2005; Misra and Saxena 2009). Treatment with SA elevated the proline content in the shoots of lentil seedlings, possibly by enhancing \( PSC \) reductase activity and decreasing the activity of \( PDH \) (Pyruvate dehydrogenase complex) (Misra and Saxena 2009). The impact of SA on proline accumulation was further increased under high salinity conditions, leading to the assumption that the stress-protective effect of SA might partially be achieved via control of proline metabolism (Misra and Saxena 2009). Since SA was also reported to increase photosynthetic rate in plants (Khan et al. 2003; Khodary 2004), these data suggest that SA-mediated signals might regulate proline metabolism in photosynthetically active tissues. Furthermore, SA was demonstrated to be a regulator of proline synthesis, associated with reproductive development as well as stress responses (Khodary 2004; Misra and Saxena 2009). SA signaling is also involved in the induction of the hypersensitive response and \( P5CS2 \) expression after infection with avirulent \textit{Pseudomonas syringae} trains in Arabidopsis (Fabro et al. 2004). Plants with low SA levels due to mutation of \( EDS5 \) or overexpression of a bacterial salicylate hydroxylase (NahG) did not show enhanced \( P5CS2 \) expression or proline accumulation in response to pathogen attack (Fabro et al. 2004). Many researchers have already shown that Si could increase the tolerance of plants exposed to stressful environments (Liang et al. 2008; Chen et al. 2011; Gong and Chen 2012; Agostinho et al. 2017). However, comprehensive studies have not been carried out yet to uncover the possible mechanisms for Si-enhanced tolerance of plants to abiotic stresses (Chen et al. 2011). SA has an effective role in protecting plants against abiotic stresses (Khodary 2004; El-Tayeb 2005).

The roles of Si and SA in water uptake and osmoregulation in plants under water-deficit stress are yet not been fully understood. The osmolyte activity (proline) of the plants treated with Si and SA under water deficit improved significantly (Figure 3). Thus, Si and SA treatment could improve the efficiency of osmotic adjustment of wheat plants under drought stress, which could explain the higher water content and leaf water potential in plants. Isa et al. (2010) reported that application of Si under water deficit enhances the strength and rigidity of cell walls and then it might help to reduce the leakage of soluble solutes and stabilize the plasma membrane structure. In the present study, drought stress significantly decreased chlorophyll pigment concentrations, RWC, and \( \Psi_w \) in both wheat cultivars. Furthermore, Si and SA improved all these parameters under water deficit conditions. Chlorophyll content is considered as an important indicator of plant productivity, because it is directly related to the photosynthetic rate of plants for biomass production (Wang and Huang, 2004). Reduction in chlorophyll content in the stressed plants could be ascribed to enhanced activity of chlorophyllase, a chlorophyll-degrading enzyme (Chen et al. 2011). Furthermore, Si application resulted in an increase in chlorophyll content and an improvement in the antioxidant system in plants exposed to salt stress (Aghabary et al. 2004). Feng et al. (2010) investigated the interaction between Si and nitrogen and reported an increase in the levels of chlorophyll \( a \) in rice plants. Similar to the results of this research, increase in chlorophyll content has been reported with SA application in plants (Khodary 2004). Maintenance of higher \( \Psi_w \) under stress is one of the remarkable features of Si application (Shen et al. 2010). Ma (2004) suggested that Si can contribute to higher resistance of xylem vessels, which are structures responsible for water transport into the plant. Therefore, plants with firmer xylem vessel walls can potentially avoid problems in these structures during drought, besides increasing water volume assimilated by plants. In the leaves of the stressed plants, we also observed that \( \Psi_w \) was greater in Si + SA application (Figure 10). Application of Si is reported to enhance \( \Psi_w \) under drought stress conditions (Gong et al. 2005). The beneficial effects of silicon and SA application have been previously observed on a variety of species under biotic and abiotic stresses by many researchers (Ma 2004; Gong et al. 2005; Szabados and Savoure 2009; Singh and Gautam 2013). However, there is a little evidence on the beneficial effects of application of Si and SA simultaneously. Data reported here showed the beneficial effects of concomitant application of Si and SA on chlorophyll content and proline content and maintenance of \( \Psi_w \) and RWC in wheat plants under drought stress conditions, which suggests enhanced drought tolerance in wheat plants.

**Conclusion**

In short, the results of this study highlighted the \( P5CS \) gene expression was significantly higher in the stressed plants,
especially, when treated by Si + SA. The results suggest that the PSCS is a stress inducible gene. This gene has the potential to be used for improvement of drought stress tolerance in wheat. In comparison with PSCS expression, proline content started to increase by Si and SA treatment and the maximum proline content was obtained at simultaneous application of Si + SA. It was concluded that alleviation of drought stress by application of Si and SA was associated partly with enhanced expression of PSCS gene and as a result proline accumulation. This proline accumulation might function as an osmoregulator for the intracellular osmotic adjustment and could play a critical role in maintaining RWC and Ψw in wheat plants under drought stress conditions. Overall, Si and SA applications proved to be of a great potential in promoting drought tolerant in wheat under drought-prone areas.

Disclosure statement
No potential conflict of interest was reported by the authors.

References
Agostinho FB, Tubana BS, Martins MS, Datnoff LE. 2017. Effect of different silicon sources on yield and silicon uptake of rice grown under varying phosphorus rates. Plants. 6:35–52. doi:10.3390/plants6030035.

Al-aghabary K, Zhu Z, Shi Q. 2004. Influence of silicon supply on chlorophyll content, chlorophyll fluorescence, and antioxidative enzyme activities in tomato plants under salt stress. J Plant Nutr. 27:2101–2115. doi:10.1080/01904160490500461.

Ashraf M. 2009. Biotechnological approach of improving plant salt tolerance using antioxidators as markers. Biotechnol Adv. 27:84–93. doi:10.1016/j.biotechadv.2008.09.003.

Ashraf M, Akram NA, Arteca RN, Foolad MR. 2010. The physiological, biochemical and molecular roles of brassinosteroids and salicylic acid in plant processes and salt tolerance. Crit Rev Plant Sci. 29:162–190. doi:10.1080/07352680903512336.

Ashraf M, Foolad MR. 2007. Roles of glycine betaine and proline in wheat. In comparison with P5CS gene expression in plants6030035.

Chen W, Yao X, Cai K, Chen J. 2011. Silicon alleviates drought stress of rice plants by improving plant water status, photosynthesis and mineral nutrient absorption. Biol Trace Elem Res. 142:67–76. doi:10.1007/s12011-010-8742-x.

Costa RCL, Lobato AKS, Silveira JAG, Laughinghouse IV. 2011. ABA-mediated proline synthesis in cowpea leaves exposed to water deficit and dehydration. Turk J Agric For. 35:309–317.

de-Lacerda CF, Cambraia J, Oliva MA, Ruíz HA, Frisotti JT. 2003. Solute accumulation and distribution during shoot and leaf development in two sorghum genotypes under salt stress. Environ Exp Bot. 49:107–120. doi:10.1016/S0098-8472(02)00064-3.

Delaney AJ, Hu CA, Kavi Kishor PB, Verma DPS. 1993. Cloning of ornithin delta-aminotransferase cDNA from vigna aconitifolia by trans-complementation in escherichia coli and regulation of proline biosynthesis. J Biol Chem. 268:18673–18678.

De Ronde JA, Cress WA, Kruger GHJ, Strasser RJ, van Staden J. 2004. Photosynthetic response of transgenic soybean plants, containing an arabidopsis P5CR gene, during heat and drought stress. J Plant Physiol. 161:1211–1224. doi:10.1016/j.jplph.2004.01.014.

De Ronde JA, Strasser RJ, van Staden J. 2001. Interaction of osmotic and temperature stress on transgenic soybean. Afr J Bot. 67:655–660. doi:10.1016/S0252-6299(15)31196-0.

Dhanda SS, Sethi GS, Behl RK. 2004. Indices of drought tolerance in wheat genotypes at early stages of plant growth. J Agron Crop Sci. 190:6–12. doi:10.1111/j.1439-037X.2004.00592.x.

El-Tayeb MA. 2005. Response of barley grains to the interactive effect of salinity and salicylic acid. Plant Growth Regul. 45:215–224. doi:10.1007/s10725-005-4928-1.

Fabro G, Kovacs I, Pavet V, Szabados L, Alvarez ME. 2004. Proline accumulation and ATP5CS2 gene activation are induced by plant-pathogen incompatible interactions in arabidopsis. Mol Plant Microbe Interact. 17:343–350. doi:10.1094/MPMI.2004.17.4.343.

Feng JP, Shi QH, Wang XF, Wei M, Yang FJ, Xu HN. 2010. Silicon supplementation ameliorated the inhibition of photosynthesis and nitrate metabolism by cadmium (Cd) toxicity in cucumis sativus L. Sci Hortic. 123:521–530. doi:10.1016/j.scienta.2009.10.013.

Fujita T, Maggio A, García-Ríos M, Bressan RA, Coonka L. 2013. Comparative analysis of the regulation of expression and structures of two evolutionarily divergent genes for Δ1-pyrroline-5-carboxylate synthetase from tomato. Plant Physiol. 118:661–674. doi:10.1111/j.1110-1641.pp.1182.661.

Ghodke PH, Andhale PS, Gijare UM, Thangasamy A, Khade YP, Mahajan V, Singh M. 2018. Physiological and biochemical responses in onion crop to drought stress. Int J Curr Microbiol App Sci. 7:2054–2068. doi:10.20546/ijcmas.2018.701.247.

Gong H, Chen K. 2012. The regulatory role of silicon on water relations, photosynthetic gas exchange, and carboxylation activities of wheat leaves in field drought conditions. Acta Physiol Plant. 34:1589–1594. doi:10.1007/s11738-012-0954-6.

Gong H, Chen KM, Zhao ZG, Chen GC, Zhou WJ. 2008. Effects of silicon on defense of wheat against oxidative stress under drought at different developmental stages. Bio Plant. 52:592–596. doi:10.1007/s10535-008-0118-0.

Gong H, Zhu X, Chen K, Wang S, Zhang C. 2005. Silicon alleviates oxidative damage of wheat plants in pots under drought. Plant Sci. 169:313–321. doi:10.1016/j.plantsci.2005.02.023.

Hein DT, Jacobs M, Anengon G, Herrmans C, Thu TT, Son LV, Roosens NH. 2003. Proline accumulation and Δ1-pyrroline-5-carboxylate synthetase gene properties in three rice cultivars differing in salinity and drought tolerance. Plant Sci. 165:1059–1068. doi:10.1016/S0168-9452(03)00301-7.

Hosseinpour B, Haji Hoseini V, Kashi R, Ebrahimie M, Hemmatzadeh F. 2010. Protein interaction network of arabidopsis thaliana female gametophyte development identifies novel proteins and relations. Plos ONE. 7(12):e09931. doi:10.1371/journal.pone.009931.

Hu CA, Delaunay AJ, Verma DPS. 1992. A bifunctional enzyme (1-pyrroline-5-carboxylate synthetase) catalyzes the first two steps in proline biosynthesis in plants. Proc Natl Acad Sci USA. 89:9354–9358. doi:10.1073/pnas.89.19.9354.

Hummel I, Pantin F, Sulpice R, Piques M, Rolland G, Dauzat M, Christophe M, A, Pervent M, Bouteille M, Stitt M, et al. 2010. Arabidopsis plants acclimate to water deficit at low cost through changes of carbon usage: an integrated perspective using growth, metabolism, enzyme, and gene expression analysis. American Soc Plant Biol. 154:357–372.

Hussein MM, Balbba IK, Gaballah MS. 2007. Salicylic acid and salinity effects on growth of maize plants. J Agric Biol Sci. 3:321–328.

Igarashi Y, Yoshida T, Sanada Y, Yamaguchi-Shinozaki K, Wada K, Shinozaki K. 1997. Characterization of the gene for delta-1-pyrroline-5-carboxylate synthase and correlation between the expression of the gene and SALT tolerance in oryza sativa. Plant Mol Biol. 33:857–865. doi:10.1023/A:1005702048601.

Iwata M, Bai S, Yokoyama T, Ma JP, Ishibashi Y, Yuasa T, et al. 2010. Silicon enhances growth independent of silica deposition in a low-silica rice mutant. Plant Soil. 331:361–375. doi:10.1007/s11104-009-0258-9.

KaviKishor PB, Hong Z, Miao GH, Hu CAA, Verma DPS. 1995. Overexpression of D1-pyrroline-5-carboxylate synthase increases
proline production and confers osmotolerance in transgenic plants. Plant Physiol. 108:1387–1394. doi:10.1104/pp.108.14.1387.
Kavi Kishor PB, Sangam S, Amrutha RN, Sri Laxmi P, Naidu KR, Rao KRSS, Rao S, Reddy JK, Theriappan P, Sreenivasulu N. 2005. Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: Its implications in plant growth and abiotic stress tolerance. Curr Sci. 88(3):424–438.
Khan W, Pritihiwar B, Smith D. 2003. Photosynthetic responses of corn and soybean to foliar application of salicylates. J Plant Physiol. 160:485–492. doi:10.1016/S0176-1617(03)00865-8.
Khodary SEA. 2004. Effect of salicylic acid on growth, photosynthesis and carbohydrate metabolism in salt-stressed maize plants. Int J Agric Biol. 6:3–8.
Kumar SG, Matta Reddy A, Sudhakar C. 2003. NaCl effects on proline metabolism in two high yielding genotypes of mulberry (morus alba L) with contrasting salt tolerance. Plant Sci. 165:1245–1251. doi:10.1016/S0168-9452(03)00332-7.
Larionov A, Krause A, Miller W. 2005. A standard curve based method for relative real time PCR data processing. BMC Bioinform. 6(2):1–16.
Liang YC, Zhu J, Li ZJ. 2008. Role of silicon in enhancing resistance to Lobato AKS, Luz LM, Costa Santos RCL, Filho BG, Meirelles ACS, Oliveira MB, Neto CF, Laughinghouse HD, Neto MAM, Alves GAR, Lopes MJS, Xie Z. 2017. Exogenous proline and confers osmotolerance in transgenic plants. J Plant Physiol. 184:492–498. doi:10.1016/j.jplphysiol.2017.06.005.
Shen X, Zhou Y, Duan L, Li Z, Eneji AE, Li J. 2010. Si effects on photosynthesis and antioxidant parameters of soybean seedlings under drought and ultraviolet-B radiation. J Plant Physiol. 167:1248–1252. doi:10.1016/j.jplph.2010.04.011.
Shim J, Shim E, Kim GH, Han JW, Zucarello GC. 2016. Keeping house: evaluation of housekeeping genes for real-time PCR in the red alga, bostycyta moritiziana (florideophyceae). Algae. 31:167–174. doi:10.490/algae.2016.31.5.25.
Silva-Ortega CO, Ochoa-Alfaro AE, Reyes-Aguero JA, Aguado-Santacruz GA, Jimenez-Bremont JF. 2008. Salt stress increases the expression of PSC5 gene and induces proline accumulation in cactus pear. Plant Physiol Biochem. 46:82–92. doi:10.1016/j.plaphy.2007.10.011.
Singh PK, Gautam S. 2013. Role of salicylic acid on physiological and biochemical mechanism of salinity stress tolerance in plants. Acta Physiol Plant. 35:38–22.
Soltan Shahattary F, Mansourifar C. 2017. The effect of drought stress on morphological and physiological traits and essence percentage of medicinal plant, nigella sativa. Biosci Biotech Res Comm. 1:298–305.
Sonobe K, Hattori T, An P, Tsui W, Eneji AE, Kobayashi S, Kawamura Y, Tanaka K, Inanaga S. 2011. Effect of silicon application on sorghum root responses to water stress. J Plant Nutr. 34:71–82. doi:10.1080/019041611.2011.53136.
Sourour A, Afel O, Mounir M, Mong BY. 2017. A review: morphological, physiological, biochemical and molecular plant responses to water deficit stress. Inter J Engin. Sci. 61–4. doi:10.1016/j.ijjesc.2010-061010104.
Strizhov N, Abraham E, Okresz L, Blishing L, Zilberstein A, Schell J, Concz K, Szabados L. 1997. Differential expression of two PSC5 genes controlling proline accumulation during salt-stress requires ABA and is regulated by ABA1, ABI1 and AXR2 in arabidopsis. Plant J. 12:557–569. doi:10.1042/bst0110591.
Su J, Wu R. 2004. Stress-inducible synthesis of proline in transgenic rice confers faster growth under stress conditions than that with constitutive synthesis. Plant Cell. 166:941–948. doi:10.1016/j.plantsci.2003.12.004.
Sullivan CY, Ross WM. 1979. Selecting for drought and heat resistance in grain sorghum. In Stress Physiology in Crop Plants. (Ed.), Mussel H, Staples RC., pp. 263–238. John Wiley and Sons. New York.
Xu PL, Guo YK, Bai JG, Shang L, Wang XJ. 2008. The effect of salicylic acid on growth, photosynthesis and antioxidative carbon metabolism in barley seedlings under water deficit. J Agron Crop Sci. 198:14–26. doi:10.1111/j.1439-0432.2008.00486.x.
Xu Y, Tanaka K, Inanaga S. 2011. Correlation between the induction of a gene for delta 1-pyrroline-5-carboxylate synthase (P5CS) and salt stress in arabidopsis thaliana. J Plant Physiol. 168:895–902. doi:10.1016/j.jplph.2010.04.011.
Y, Tanaka K, Inanaga S. 2011. Regulation of proline biosynthesis and degradation in contrast wheat genotypes as a cing osmotic adjustment. J Agron Crop Sci. 198:14–26. doi:10.1111/j.1439-0432.2008.00486.x.
Y, Tanaka K, Inanaga S. 2011. Correlation between the induction of a gene for delta 1-pyrroline-5-carboxylate synthase (P5CS) and salt stress in arabidopsis thaliana. J Plant Physiol. 168:895–902. doi:10.1016/j.jplph.2010.04.011.
Y, Tanaka K, Inanaga S. 2011. Regulation of proline biosynthesis and degradation in contrast wheat genotypes as a cing osmotic adjustment. J Agron Crop Sci. 198:14–26. doi:10.1111/j.1439-0432.2008.00486.x.
Y, Tanaka K, Inanaga S. 2011. Correlation between the induction of a gene for delta 1-pyrroline-5-carboxylate synthase (P5CS) and salt stress in arabidopsis thaliana. J Plant Physiol. 168:895–902. doi:10.1016/j.jplph.2010.04.011.
Yoshida Y, Kiyosue T, Katagiri T, Ueda H, Mizoguchi Y, Yamaguchi-Shinozaki K, Wada K, Harada Y, Shinozaki K. 1995. Correlation between the induction of a gene for delta 1-pyrroline-5-carboxylate synthase and the accumulation of proline in arabidopsis thaliana under osmotic stress. Plant J. 7:751–760. doi:10.1046/j.1365-313X.1995.7005751.x.
Zhu X, Gong H, Chen G, Wang S, Zhang C. 2005. Different solute levels in two spring wheat cultivars induced by progressive field water stress at different developmental stages. J Arid Environ. 62 14. doi:10.1016/j.jaridenv.2004.10.010.
Zhu X, Gong H, Chen G, Wang S, Zhang C. 2005. Different solute levels in two spring wheat cultivars induced by progressive field water stress at different developmental stages. J Arid Environ. 62:1–4. doi:10.1016/j.jaridenv.2004.10.010.