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Introduction

The roots and stolons of Glycyrrhiza plants (G. glabra L.) are among the most important sources of crude herbal drugs worldwide (Gibson, 1978). Triterpene saponins are high molecular weight glycosides that are composed of a sugar moiety linked to triterpene or a steroid aglycone. Precursors of saponins typically undergo various modifications prior to the addition of sugar molecules. The types and extent of the sugar molecules that comprise triterpene saponins differ greatly among saponin-containing plants (Hayashi et al., 1990, 1992; Vincken et al., 2007). The root of liquorice contains a large amount (up to 15%) of glycyrrhizin and olean-ane-type triterpene saponins, which are known to have various food, industrial, cosmetic and pharmaceutical applications (Armanini et al., 2005). Saponins are commercially used in food and industrial settings as foaming, detergent, wetting, emulsifying and sweetening agents (Hostettmann and Marston, 2005; Shibata, 2000). They are also utilised as ingredients in the cosmetics, cleansing and personal care sectors, such as shower gels, shampoos, hair conditioners, lotions, liquid soaps, baby care products, mouthwashes, and toothpastes. The pharmacological properties of glycyrrhizin and structural elucidation of triterpenes and saponins have been widely studied, revealing that these compounds have important pharmaceutical activities aside from their involvement in plant defence responses (Agrell et al., 2003; Mahato et al., 1992, 1988; Osbourn, 1996). These important properties include anti-inflammatory, anti-bacterial, molluscicidal, antiflu, insecticidal, and anti-allergic activities, as well as involvement in immune system activation (De Leo et al., 2006; Gopalsamy et al., 1990; He et al., 2001; Kuzina et al., 2009; Matsui et al., 2004; Park et al., 2004; Takahara et al., 1994). Glycyrrhizin also displays antiviral activity against several viruses, such as HIV (Ito et al., 1987, 1988) and severe acute respiratory syndrome (SARS) caused by Coronavirus (Cinatl et al., 2003).

A number of sequential enzymatic reactions are required for the biosynthesis of triterpene saponins in plants. Squalene is the immediate biological precursor of all triterpenoids. Squalene synthase
The production of plant secondary metabolites is strongly associated with the growth conditions, and stress treatments. The differences were considered to be significant if \( P < 0.01 \) based on Duncan’s multiple range test.

Results

Relative water content (RWC)

At the seedling stage, the RWC of the entire seedlings was significantly decreased \( (P < 0.01) \) throughout the experimental period (4, 8 and 24 h) under the water-stressed conditions achieved using 15% (w/v) PEG6000, reaching a minimum value at 24 h (Fig. 1).

At the adult plant stage, 10-month-old plants were selected for drought stress treatment, in which irrigation was discontinued thereafter. The stolons of these plants were collected after 2, 16 and 28 days of water stress, designated as \( S_{2d} \), \( S_{16d} \) and \( S_{28d} \), respectively. The control plants \( (C_{2d}, C_{16d} \) and \( C_{28d} ) \) were maintained under normal irrigation conditions. No significant difference was detected in the RWC between the \( S_{2d} \) and \( C_{2d} \) samples, indicating that \( S_{2d} \) was not significantly stressed. However, the RWC was significantly reduced \( (P < 0.01) \) in the \( S_{16d} \) and \( S_{28d} \) samples compared to their corresponding controls \( (C_{16d} \) and \( C_{28d} \), respectively). \( S_{16d} \) was considered as moderate stress, whereas \( S_{28d} \) displayed more severe stress. There was no significant difference in the RWC between the control samples from \( C_{2d} \), \( C_{16d} \) and \( C_{28d} \) (data not shown) (Fig. 2).

Effects of drought and osmotic stress on the gene expression levels of SQS, bAS, CAS and LUS

We assessed the gene expression levels of SQS, bAS, CAS and LUS at the seedling stage under drought stress conditions for four durations \( (0, 4, 8 \) and \( 24 \) h) and compared these expression levels...
to those of the control plants via semi-quantitative PCR using gene-specific primers. The data in Fig. 3 illustrate the levels of SQS expression in liquorice seedlings over time. The expression level of SQS under stress conditions was increased shortly after stress treatment (4 h) compared to the control conditions and remained unchanged afterwards. Similarly, the expression level of bAS gene under stress conditions (at 4, 8 and 24 h after stress treatment) was higher than that of the respective C16d and C28d controls. The mRNA expression level of bAS was higher in the S16d sample than the S28d sample (Fig. 4). On the other hand, the gene expression level of CAS was relatively constant throughout the drought stress (Fig. 4). The gene expression level of LUS was not detectable in this experiment (data not shown).

**Discussion**

The enzymes SQS, bAS, CAS and LUS are known to be involved in triterpenoid synthesis (Hayashi, 2009). SQS converts squalene to 2,3-oxidosqualene, which serves as the substrate for the synthesis of glycyrrhizin and other triterpenoids. CAS, bAS, and LUS encode OSCs, which catalyse the cyclisation of 2,3-oxidosqualene, a precursor of glycyrrhizin, betulinic acid and sitosterol (Abe et al., 1993; Haralampidis et al., 2002). Glycyrrhizin, soyasaponins and betulinic acid are located in different regions of intact liquorice plants, and the biosynthetic regulation of these constituents is also specific. It was previously demonstrated that the expression level of OSCs was the most influential regulator of glycyrrhizin biosynthesis (Hayashi, 2009). In addition, gene expression analysis revealed that the transcriptional expression level of bAS was higher in cell culture and thickened main roots and root nodules of liquorice plants (Hayashi, 2009). Choi et al. (2005) demonstrated that the gene expression of SQS was up-regulated in hairy root cultures of *Panax ginseng* by treatment with methyl jasmonate (MeJa).
In another study, up-regulation of the SQS and bAS genes via MeJa and salicylic acid (SA) was reported (Chen et al., 2007; Suzuki et al., 2005). In contrast, application of gibberellin A3 resulted in down-regulation of BAS, but the gene expression levels of LUS and CAS were unchanged. Likewise, the gene expression levels of SQS and bAS were down-regulated in G. glabra cells treated with jasmonate acid (JA) or MeJa, whereas under the same conditions, the mRNA level of the CAS gene was not altered (Hayashi et al., 2004). In addition, the LUS mRNA levels in the thickened main roots of G. glabra remained relatively constant throughout the seasons, indicating that the CAS gene displays housekeeping gene-like expression in G. glabra (Hayashi et al., 2004). In agreement with these findings, our results also revealed that the gene expression of CAS remained constant in plants grown under drought stress or control conditions. Hayashi et al. (2004) reported that the level of LUS mRNA was not detectable under various cultivation conditions, which is similar to our finding that the expression of the LUS gene was not detectable (data not shown). Chen et al. (2007) found that the transcriptional expression level of bAS in the roots of Bupleurum koi was doubled due to treatment with MeJa. Additionally, it was reported that the transcriptional expression level of bAS in significantly increased in liquorice and Medicago truncatula plants due to treatment with salicylic acid (Suzuki et al., 2005).

Pan et al. (2006) found that when Glycyrrhiza uralensis seedlings were exposed to drought stress, the antioxidant enzymes were hyper-activated. Studies using various plant systems demonstrated that environmental stress, including drought or salt stress, enhances the transcriptional expression levels and the activities of enzymes involved in oxygen radical scavenging (Gueta-Dahan et al., 1997). Drought stress generally stimulates oxidative stress (Lei et al., 2006) resulting in the formation of ROS in organelles, including chloroplasts and mitochondria (Fu and Huang, 2001). Subsequently, as a feed-back regulation mechanism, triterpenoids are produced as an antioxidant to scavenge ROS (Okubo and Yoshiki, 2000). There are many pieces of evidence indicating that glycyrrhizin and its hydrolysed metabolite 18β-glycyrrhetinic acid exert antioxidant effects (Kim and Lee, 2008). In Bupleurum spp., an incremental effect of drought stress on the levels of triterpenoids was also reported (Zhu et al., 2009). Triterpenes and α- and β-amyrin metabolites extracted from Jatropha gaumeri leaves displayed antioxidant activity (Can-Aké et al., 2004). In Hypericum brasiliense, the content of various betulinic acids was greatly higher in plants grown under drought stress than control conditions (Nacif de Abreu and Mazzafera, 2005). Nacif de Abreu and Mazzafera (2005) also reported that the total level of secondary metabolites in H. brasiliense was increased under drought stress compared to normal conditions.

Subsequently, as a feed-back regulation mechanism, triterpenoids may help to increase the glycyrrhizin content in liquorice plants. Therefore, we propose applying special irrigation regimes to increase the glycyrrhizin content in liquorice plants. The recent over-utilisation of wild Glycyrrhiza is grown in some countries, but the glycyrrhizin content extracted from these plants is often low (Hayashi and Sudo, 2009). Thus, identifying the conditions under which glycyrrhizin production is maximised is of interest. In this study, we found that drought stress increased the expression of important genes involved in the glycyrrhizin biosynthetic pathway. This result indicates that intense drought stress, as well as periodic drought stress, may help to increase the glycyrrhizin content in liquorice plants. Therefore, we propose applying special irrigation regimes to directly enhance secondary metabolite production. This is a simple and inexpensive, but further investigation is required to optimise metabolite production.

Materials and methods

Plant material and cultivation conditions

Seeds of liquorice plants (G. glabra) were provided by Pakan-Bazr Seed Production Company (Isfahan, Iran). The seeds

| Primer | Accession No. | Sequence | References |
|--------|---------------|----------|------------|
| SQS Forward | D86410 and D86409 | 5'-CCGTCGCCAAACTGGAAA-3' | Shabani et al. (2010) |
| SQS Reverse | | 5'-CGGCATCTGCACCTAAC-3' | Shabani et al. (2010) |
| bAS(1) Forward | AB037203 | 5'-AGAGCAGCATGCGGACT-3' | |
| bAS(1) Reverse | | 5'-CAGAGACATCAGTTTGAG-3' | |
| bAS(2) Forward | AB037203 | 5'-GGCGAGCAGCATGCGGACT-3' | Shabani et al. (2010) |
| bAS(2) Reverse | | 5'-CAGAGACATCAGTTTGAG-3' | |
| CAS Forward | AB025968 | 5'-GGGAGCGAGAGTGGGAC-3' | |
| CAS Reverse | | 5'-GGGGAAATGTTGGGAC-3' | |
| LUS(1) Forward | AB116228 | 5'-ATGCGGCATTCCAGCAGA-3' | |
| LUS(1) Reverse | | 5'-CTAGCGTCCACCCGAAAC-3' | |
| LUS(2) Forward | AB116228 | 5'-AAGAAGCTTCGTTGCGGCT-3' | |
| LUS(2) Reverse | | 5'-CCGGAATCCGTTGATTTC-3' | |
| 18s rRNA Forward | X02623 | 5'-CTCAACAGGCGGAAACTCT-3' | Shabani et al. (2010) |
| 18s rRNA Reverse | | 5'-AGACAATGCGTCCACGAC-3' | |
were disinfected using H$_2$SO$_4$ (98%) for 40 min and washed three times with sterile distilled water. The seeds were then allowed to germinate on sterile filter paper moistened with sterile distilled water in Petri dishes. Three-day-old seedlings were transferred to Petri dishes padded with sterilised filter paper soaked with 1 $\times$ Hoagland solution and incubated at 25 ± 2 °C for a photoperiod of 16 h of light. The drought stress experiment at seedling stage was performed by selecting 8-day-old seedlings displaying uniform growth. The seedlings were moved to filter paper soaked with distilled water containing 15% (w/v) PEG6000 to induce drought stress conditions. The entire seedlings were harvested for further analysis after 0, 4, 8 or 24 h of treatment.

The drought stress experiment at the adult plant stage was performed as follows. First, stolons of liquorice plants were obtained from Rishmac Company in Shiraz, Iran, and were grown in pots in a greenhouse. Each experiment was performed as a randomised complete block design (RCBD) with four replications. Water stress treatment was performed on 10-month-old plants by discontinuing irrigation. Subsequently, sampling was performed at 2, 16 and 28 days after drought imposition, designated as S2, S16d and S28d, respectively, while control plants (designated as C2d, C16d and C28d, respectively) were maintained under optimal irrigation conditions. The samples of stolons were collected, immediately frozen in liquid N$_2$ and stored at –70 °C for further analysis.

**Determination of the RWC**

The relative water content (RWC) of leaves and seedlings was calculated using the formula [(fresh weight–dry weight)/(saturated weight–dry weight)] $\times$ 100, as described previously (Slater, 1967). Fresh leaves from the treated and control plants were weighed immediately after harvesting. The saturated weight was measured after placing the leaves in vials containing distilled water at 4 °C for 24 h and then blotting the leaves on dry filter paper. The samples were dried in an oven for 72 h at 70 °C, and the dry weight of samples was measured. Analysis of variance was performed on the data using SPSS software (version 15), and significant differences compared to the control values were determined using Duncan's multiple range test.

**Total RNA extraction and cDNA synthesis**

Total RNA was extracted from stolons of *G. glabra* using RNX plus solution (CinnaGen, Iran) according to the manufacturer's instructions. To remove genomic DNA contamination, total RNA was treated with RNase-free DNase. The concentration of RNA was estimated via spectrophotometry. Approximately 6 µg of total RNA from each sample was subsequently subjected to first strand cDNA synthesis using random hexamer primers and a M-MuLV Reverse Transcriptase Kit (CinnaGen, Iran) according to the manufacturer's instructions.

**Semi-quantitative RT-PCR**

PCR was performed on aliquots of the cDNA templates to determine the gene expression levels of SQS, βAS, CAS and LUS using a thermal cycler (Eppendorf) under the following parameters: 94 °C for 2 min, followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 49 °C (SQS), 57 °C (βAS(1)), 56 °C (βAS(2)) or 50 °C (CAS) for 30 s and extension at 72 °C for 30 s, and a final extension step at 72 °C for 7 min. The PCR primers used in this study were synthesised by Bioneer (Seoul, Korea). The primers were designed using Primer3 software (developed by Steve Rozen, Helen J. Skaletsky, 1996, 1997) available on-line at [http://www-genome.wi.mit.edu](http://www-genome.wi.mit.edu). The SQS, βAS, CAS and LUS genes were amplified using the specific primers listed in Table 1. 18S ribosomal RNA (Accession No. X02623) was used as an internal control (Shabani et al., 2010). The PCR products (8 µl) were electrophoresed on 1% agarose gels in TBE buffer and visually quantified.

**Preparation of the stolon extracts**

Stolons were air-dried at room temperature for 4 days. To measure the glycyrrhizin content of each sample, 40 mg of dried stolon was lyophilised and subjected to glycyrrhizin extraction using 1 ml of 80% (v/v) methanol at 60 °C for 6 h. The samples then were centrifuged at 4000 rpm for 15 min at room temperature. The supernatant was transferred to a new tube and then evaporated to dryness using nitrogen (Hayashi et al., 1958). The residue extracts were used for high-performance liquid chromatography (HPLC).

**HPLC analysis**

A glycyrrhizin standard (glycyrrhizic acid ammonium salt) was purchased from Fluka (Switzerland). Preceding HPLC analysis, the residual extract of each sample was dissolved in water and filtered using a 0.4 µm filter. A 20 µl aliquot of each sample extract was analysed via HPLC at 25 °C. The HPLC system consisted of a Waters HPLC 510 pump and a Waters 2478 detector. The separation of glycyrrhizin was performed according to the method described previously (Hurst et al., 1983) as an isocratic elution using methanol–water–acetic acid (60: 34:6) at a flow rate of 1 ml/min through a RP column (3.9 × 150 mm), followed by measurement of UV absorbance at 254 nm. Analysis of variance was performed on the data using SPSS software (version 15), and significant differences compared to the control values were determined using Duncan’s multiple range test.

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