Flavonoids and Antioxidative Enzymes in Temperature-Challenged 
Roots of Scutellaria baicalensis Georgi

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The active compounds in the roots of Scutellaria baicalensis Georgi, a traditional Chinese medicinal plant, are mainly flavonoids which have anti-inflammatory, antitumour, and anti-HIV activity, respectively. The increasing annual average temperature has rendered the S. baicalensis plants grown in some ancient producing regions no longer suitable for their medicinal usage. Hydrogen peroxide plays an important role in root responses to abnormal temperature in S. baicalensis. Baicalin and baicalein and antioxidative enzymes were anticipated to detoxify H2O2 in S. baicalensis. Here, we show that abnormal temperatures (10 and 40 °C) decreased the content of flavonoids as compared with the normal temperature (30 °C), and the transcripts of UDP-glucuronate:baicalein 7-O-glucuronosyltransferase and β-glucuronidase involved in the interconversion between baicalin and baicalein were affected by the 40-°C treatment. High temperature also increased the activities of catalase and peroxidase. Reverse transcription-polymerase chain reaction (RT-PCR) analysis revealed that the transcript levels of peroxidase 2, peroxidase 3, monodehydroascorbate reductase 2, and dehydroascorbate reductase were significantly increased under high-temperature conditions. The respective genes would be candidates for improvement of the adaptation of S. baicalensis plants to abnormal temperatures and for regulation of the contents of the active compounds.

Key words: Scutellaria baicalensis Georgi, Abnormal Temperature, Active Compounds

Introduction

Scutellaria baicalensis Georgi is a traditional Chinese medicinal plant whose bioactive compounds include the flavones, or flavone glycosides, respectively, baicalin, baicalein (Fig. 1), wogonoside, wogonin, neobaicalein, visidulin I, and oroxylin A. These compounds possess anti-inflammatory, antitumour, and anti-HIV activities (Blach-Olszewska et al., 2008). Most S. baicalensis plants grow naturally in northern China, where the average temperature is about 10 °C in spring and autumn and around 30 °C in summer (http://cdc.cma.gov.cn/). Temperature is an important environmental factor that may affect the medicinal quality of S. baicalensis (Li et al., 2008). The baicalin content of S. baicalensis was previously shown to be correlated with the mean temperature in June and July (Yuan et al., 2010). The Intergovernmental Panel on Climate Change Working Group II reported that many natural systems are being affected by regional

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**Abbreviations:** APX, ascorbate peroxidase; CAT, catalase; DHAR, dehydroascorbate reductase; GPX, glutathione peroxidase; GUS, β-glucuronidase; MDAR, monodehydroascorbate reductase; POD, peroxidase; SOD, superoxide dismutase.

Fig. 1. Chemical structures of the flavonoids (A) baicalin and (B) baicalein.
climate change, particularly temperature increase (Rosenzweig et al., 2008). The increasing annual average temperature in Hubei Province, an ancient *S. baicalensis*-producing region, has made the *S. baicalensis* plants from this area unsuitable for medicinal use.

High temperature is an important abiotic factor that can affect plant growth and productivity by inhibiting photosynthesis, damaging cell membranes, and inducing senescence and cell death (Xu et al., 2006). One mechanism of injury at high temperature involves the overproduction of reactive oxygen species (ROS), including superoxide radicals (O$_2^•$), hydroxyl radicals (OH$^•$), hydrogen peroxide (H$_2$O$_2$), and singlet oxygen (O$_2$) (Mittler, 2002). To control the ROS levels in their cells, plants produce several ROS-scavenging enzymes, including superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), dehydroascorbate reductase (DHAR), and glutathione peroxidase (GPX). The activity levels of SOD and CAT in lotus seeds were shown to increase during the early phase of treatment at 100 °C and then decrease (Ding et al., 2008), while Cu/Zn SOD and APX expression in potato was upregulated by oxidative stress and high temperatures (Tang et al., 2006).

Low temperature also affects the ROS and detoxifying enzyme activity levels in plants. A temperature of 5 °C increased the O$_2^•$ content and activities of SOD and APX in tea (Vyas and Kumar, 2005). Pukacka and Ratajczak (2005) reported increased O$_2^•$ and H$_2$O$_2$ levels and changes in low-molecular weight antioxidants and enzymatic scavengers such as APX, DHAR, glutathione reductase (GR), CAT, SOD, and guaiacol peroxidase in *Fagus sylvatica* seeds following storage at 4, 20, and 30 °C. High and low temperatures lowered CAT activity and boosted SOD activity in tomato and watermelon plants (Rivero et al., 2005).

Flavonoids are major secondary plant metabolites that function as antioxidants to detoxify ROS (Morimoto et al., 1998). Flavonoid accumulation protects plants against various stressful conditions, including cold treatment (Lillo et al., 2008) and freezing (Hannah et al., 2006). In *S. baicalensis*, baicalein is involved in scavenging of H$_2$O$_2$, a peroxidase substrate (Morimoto et al., 1998). Baicalin and baicalein are synthesized via the phenylpropa-noid pathway which is initiated by phenylalanine ammonia-lyase (PAL). Baicalin is hydrolyzed to baikalid by β-glucuronidase (GUS), and is formed from baikalid by UDP-glucuronate:baicalin 7-O-glucuronosyltransferase (UGBAT). PAL expression and the anthocyanin content in tomato plants were shown to be increased in response to lower temperatures (Lovdal et al., 2009). In comparison, high temperatures decreased the concentration of phenolic compounds in apple (Devic et al., 1995), while Olsen et al. (2009) found that the initial anthocyanin flux and flavonol degradation were temperature-independent.

In this study, we analysed how high and low temperatures affect flavonoid accumulation and the transcription and activity of antioxidative enzymes in *S. baicalensis*. We also investigated the function of the flavonoids in *S. baicalensis* at 10 and 40 °C.

**Material and Methods**

**Plant materials and experimental conditions**

Seeds of *S. baicalensis* obtained from the Institute of Chinese Materia Medica, Academy of Chinese Medical Sciences (Beijing, China) were grown in pots containing 500 g of a mixture of peat and sand (2:1) in a growth chamber maintained at 30 °C under long-day conditions (light intensity, 600 μM m$^{-2}$ s$^{-1}$). The plants were irrigated every third day with 80 g of distilled water. Three-month-old *S. baicalensis* plants were exposed to temperatures of 10, 30, and 40 °C, respectively. The roots were sampled 30, 40, and 50 d after treatment.

**Flavonoid content**

Powdered root material (100 mg) was extracted in 1 mL ethanol for 1 h at room temperature. The solution was filtered through a membrane filter (0.2 μm), and the concentrations of the flavonoids were determined by HPLC using a Diamonsil C$^{18}$ column (4.6 × 250 mm, 5 μm) at a flow rate of 1.0 mL min$^{-1}$. The detection wavelength was set at 280 nm, and the column temperature was maintained at 30 °C. The mobile phase consisted of (A) acetonitrile/deionized water/methanoic acid (21:78:1, v/v/v) and (B) acetonitrile/deionized water/methanoic acid (80:19:1, v/v/v). HPLC-grade acetonitrile (Merck, Darmstadt, Germany) was used. The conditions were A:B (100:0, v/v) for 15 min, followed by linear changes to A:B (87:13, v/v) within 25 min, A:B (52:48, v/v) for 40 min, and A:B (0:100, v/v) for 60 min. Peaks were identified based on the retention time of standards.
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(0.208 mg mL\(^{-1}\) baicalin and 0.602 mg mL\(^{-1}\) baicalein) obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The injection volume was 20 μL for the sample solution, and the experiment was repeated six times. The amounts of baicalin and baicalein were calculated according to the method of Li et al. (2009).

Quantitative analysis of the total flavonoid content was performed on a sample using the aluminum chloride colorimetric assay method (Marinova et al., 2005). The total flavonoid content was calculated as baicalin equivalents. The experiment was repeated six times.

**H\(_2\)O\(_2\) concentration**

Fresh root tissue (0.1 g) was ground in liquid nitrogen and extracted with 50 mM K\(_3\)PO\(_4\) buffer (pH 7.8), and the H\(_2\)O\(_2\) concentration was measured by monitoring the titanium peroxide complex absorbance at 415 nm, as described by Patterson et al. (1984).

**Assay of enzymatic activities**

Fresh root tissue (100 mg) was ground in liquid nitrogen and extracted with 50 mM Na\(_3\)PO\(_4\) buffer [pH 7.0 for CAT and pH 7.8 for SOD and POD (peroxidase)] containing 1% (w/v) polyvinyl pyrrolidone and 0.1 mM Na\(_2\)EDTA. The homogenate was filtered through four layers of cheesecloth and centrifuged at 15,000 x g for 20 min. The supernatant was used to determine the enzyme activities and protein concentration. The extractions and enzyme activity assays were done at 4 and 25 °C, respectively.

The SOD activity was measured spectrophotometrically as described by Beyer and Fridovich (1987) with 1 unit of SOD being defined as the amount of SOD required to inhibit the photoreduction of nitroblue tetrazolium by 50%. The CAT activity was assayed according to Clairborne (1985) with the decomposition of H\(_2\)O\(_2\) followed by the decline in absorbance at 240 nm for 2 min. One unit of CAT converts 1 mmol of H\(_2\)O\(_2\) min\(^{-1}\). The POD activity was determined as described by Chance and Maehly (1955) using guaiacol as an electron donor.

The PAL activity was determined as described by Ke and Saltveit (1986). The sample was ground in liquid nitrogen and extracted with 0.1 M Na\(_2\)B\(_4\)O\(_7\) (pH 8.8). The change in absorbance at 290 nm was monitored in 1-cm light path cells at 10- to 15-min intervals for 30 min at 30 °C. Under these conditions, a change in absorbance of 0.01 was found to be equivalent to the production of 1 μg mL\(^{-1}\) cinnamic acid.

A quantitative analysis of the GUS activity was done by the MUG assay in root extracts (Wang et al., 2002). The production of 4-methylumbelliferone (4-MU) was measured using a fluorometer (CytoFluor; Applied Biosystems, Foster City, CA, USA). The amount of 4-MU was determined from a standard curve. The protein concentration in the extracts was measured by the method of Bradford (1976).

**Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis**

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA), quantified by spectrophotometry, and reverse-transcribed using a PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) following the manufacturer’s instructions (the concentration of RNA in the reaction tube was 100 μg mL\(^{-1}\)). Gene expression was investigated using the primers listed in Table I. The RT-PCR conditions were 94 °C for 3 min, followed by 31 cycles at 94 °C for 30 s, annealing temperature according to Table I for 40 s, and 72 °C for 40 s, with a final hold at 72 °C for 10 min. The amplified fragments were separated on 1.5% agarose gels. Expression of 18S rRNA was used as an internal control to normalize for sample-to-sample variation in the amount of total RNA. The experiments were repeated at least three times using independently isolated RNA samples.

**Statistical analyses**

The effects of high- and low-temperature treatment were analysed with two-way analysis of variance (ANOVA). Asterisks shown above a column in the figures indicate significant differences between high- and low-temperature-treated and control (30 °C) roots, respectively. In addition, significant differences (\(P < 0.05\)) among the different temperatures were calculated with Tukey’s multiple comparison test.
Results

Effects of high and low temperatures on flavonoid content

The total flavonoid content and levels of baikalin and baicalein did not change over time at 30 °C. In comparison, exposure to 10 °C decreased the total flavonoid content and baikalin levels after 40 days. At 40 °C, the baikalein content decreased after 30 days and the plants were dead after 50 days at 40 °C (Fig. 2).

To better understand how temperature changes affect flavonoid accumulation, we analysed the transcript levels of three flavonoid biosynthetic genes, i.e. PAL, GUS, and UBGAT, under various conditions. We found that high temperature reduced the level of GUS and increased that of UBGAT transcript, respectively, whereas low temperature did not affect the levels of these transcripts compared with their levels at 30 °C (Fig. 3). At 40 °C, the expression of UBGAT after 40-days exposure was significantly higher than that after 30 days. The expression of all genes did not change during the exposure time under 30 °C.

Effect of temperature on antioxidative enzyme activities

Because baikalin is an important antioxidant that functions to eliminate H$_2$O$_2$ in *S. baicalensis* (Morimoto *et al*., 1998), we analysed the H$_2$O$_2$ content at different temperatures (Fig. 4). The H$_2$O$_2$ content was unaffected by a temperature change.

We further analysed the activities of four enzymes involved in ROS elimination. At high temperatures, the POD activity was increased after 40 days and the CAT activity after 30 and 40 days. Low temperature did not affect the antioxidative enzyme activities.

Effect of temperature on the expression of genes encoding antioxidative enzymes

Two full-length clones encoding SOD were found from a full-length cDNA library constructed in our laboratory, and the levels of the respec-
tive transcripts at different temperatures were analysed by RT-PCR. Our results indicate that the expression of both SOD genes was unaffected by a change in temperature.

A BLAST analysis using the sequences of known Arabidopsis POD genes against those in GenBank identified three POD genes in S. baicalensis. We also identified S. baicalensis APX, DHAR, and MDAR genes. We further analysed the expression levels of these genes at different temperatures by RT-PCR. Our results indicate that the expression levels of POD2 and POD3 increased at high temperatures compared with the levels at 30 °C. The expression of POD1, MDHAR1, MDHAR3, and APX was not affected by high or low temperatures (Fig. 5). DHAR expression was increased at high and low temperatures after 40 days of exposure compared with the level at 30 °C. Only MDHAR2 expression was increased at high temperatures after 40 days (Fig. 5).

**Discussion**

It is generally believed that temperature affects flavonoid production in plants. For example, high temperatures led to a lower level of the flavonoid kaempferol in broccoli (Mrkic et al., 2006). Pan et al. (2004) reported a progressive reduction in the anthocyanin content in strawberry fruits at

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**Fig. 2.** Temperature effects on the content of flavonoid compounds. The asterisks above the columns indicate significant differences between control and high- and low-temperature-treated roots according to Tukey's multiple test ($P < 0.05$). (A) Total flavonoid content; (B) baicalin content; (C) baicalein content.

**Fig. 3.** Temperature effects on the expression level (transcript abundance/18S RNA) of flavonoid biosynthetic genes. The asterisks above the columns indicate significant differences between control and high- and low-temperature-treated roots according to Tukey's multiple test ($P < 0.05$).
temperatures ranging from 30 to 45 °C, and they suggested that this reduction is responsible for the onset of oxidative damage. Low temperatures also affect the flavonoid content in plants. For example, Akhtar et al. (2010) reported flavone accumulation in *Lemna gibba* upon exposure to low temperatures. Anthocyanins also show markedly increased levels in tomato plants when the growth temperature is lowered from 24 to 18 or 12 °C. The medicinal quality of the roots of *S. baikalensis* is closely related to the flavonoid content. We found that a low temperature decreased the total flavonoid content and the level of the active compound baicalin, while high temperature decreased the baicalein content.

Further work was done to determine whether the change in the rate of flavonoid biosynthesis caused the decrease in these active compounds. *PAL* expression was increased in response to lower temperatures, which is in agreement with a corresponding increase in the flavonoid content in the leaves of tomato (Lovdal et al., 2009). The transcription of the chalcone synthase (*CHS*) gene and other biosynthesis genes in the isoflavone calycosin-7-O-beta-D-glucoside pathway was up-regulated in *Astragalus* transferred from 2 to
25 °C (Pan et al., 2008). In the roots of *S. baicalensis*, the transcript levels of *PAL* did not change after high- and low-temperature treatment, indicating that the changes of active compounds were not due to the alterations in the rate of flavonoid biosynthesis. Nevertheless, higher expres-
 UBGAT and lower expression of GUS in response to high-temperature treatment were observed, indicating that high temperature promotes the synthesis of baicalin from baicalein.

Both low and high temperature decreased the baicalein content, however, no change in flavonoid biosynthetic gene expression was found, indicating that the change of flavonoid levels might be related to the degradation pathway. Flavonoids protect plants against various biotic and abiotic stresses, and many of the biological roles of flavonoids are attributed to their potential cytotoxicity and antioxidant abilities (Pourcel et al., 2007).

Flavonoids are important antioxidants that function to eliminate ROS after abnormal temperature exposure. A similar change in flavonoid content and flavonoid scavenging of $O_2^\cdot$ and $OH^\cdot$ observed in soybean seedlings suggests that the flavonoids enhance the degradation of ROS (Peng and Zhou, 2009). In the roots of *S. baicalensis*, high temperatures did not affect the $H_2O_2$ content, but they increased the activity levels of POD and CAT. Low temperature did not affect the $H_2O_2$ content and the activities of APX, CAT, SOD, and POD.

Transcript levels of ROS-eliminating enzymes varied at different temperatures. To determine the transcript of which gene was related to the flavonoid content, the transcript levels of two SOD, one APX, one DHAR, three MDHAR, and three POD genes at 10, 30, and 40 °C were analysed. Among these genes, POD2, POD3, and MDHAR2 responded strongly to high temperature only, whereas DHAR responded strongly to both low and high temperatures. The increase in the transcript level of *S. baicalensis* DHAR was significantly correlated with the content of baicalein (Fig. 6), indicating that high DHAR expression might lead to a high level of antioxidant recycling in the ascorbate-glutathione cycle, then promoting the conversion from baicalein to baicalin. Kim et al. (2010) reported that peroxidase contributes to ROS production during *Arabidopsis* root response to potassium deficiency. Cotton POD may be responsible for the high level of ROS production that occurs during cotton fiber elongation (Mei and Qin, 2009). These genes are good candidates for the adaptation of *S. baicalensis* to high and low temperatures and in the regulation of the flavonoid content in *S. baicalensis*.

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