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Research article

Expression of flavonoid biosynthesis genes and accumulation of flavonoid in wheat leaves in response to drought stress

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ABSTRACT

Flavonoids are the low molecular weight polyphenolic secondary metabolic compounds, and have various functions in growth, development, reproduction, and stress defense. However, little is known about the roles of the key enzymes in the flavonoids biosynthesis pathway in response to drought stress in winter wheat. Here, we investigated the expression pattern of flavonoids biosynthesis genes and accumulation of flavonoids in wheat leaves under drought stress. Quantitative real-time PCR analysis showed that there were a rapid increase in expression levels of *TaCHS*, *TaCHI*, *TaF3H*, *TaFNS*, *TaFLS*, *TaDFR*, and *TaANS* under drought stress in two wheat cultivars Aikang 58 (AK) and Chinese Spring (CS). The cultivar CS exhibited higher genes expression levels of *TaCHS*, *TaCHI*, *TaF3H*, *TaFLS*, *TaDFR*, and *TaANS*, and the cultivar AK showed a higher expression level of *TaFNS* gene during drought treatment. The increase rates of genes expression were superior in AK compared to CS. Total phenolics content, total flavonoids content, anthocyanin content, and schaftoside content in wheat leaves were enhanced during drought treatment and cultivar CS had a relative higher accumulation. These results suggest that the flavonoids pathway genes expression and accumulation of flavonoids compounds may be closely related to drought tolerant in wheat. Further, flavonoids response mechanism may be different between wheat cultivars.

1. Introduction

Flavonoids, a class of low-weight phenolics compounds, play important roles in plants, such as UV protection, as defense against pathogens and pests, as signaling with microorganisms, as auxin transport regulation, and pigmentation (Winkel-Shirley, 2001). Flavonoids are a group of phenolics that consist of two aromatic rings linked by three carbons. At present, more than 9000 different flavonoids have been discovered due to varieties of modification reactions in flavonoids biosynthesis (Ververidis et al., 2007). Despite their diversity of functions and structures, all flavonoids are derived from the general phenylpropanoid pathway, one of the best-known pathways in plant secondary metabolism. Chalcone synthase (CHS) was the entry point of the flavonoids pathway, which catalyzed 4-Coumaroy-CoA and Malonlyl-CoA to chalcone, leading phenylpropanoids pathway to flavonoids biosynthesis (Fig. 1) (Shih et al., 2008). The most enzymes involved in the pathways leading to major flavonoids classes have been determined, e.g. chalcone isomerase (CHI), flavanone 3- hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), flavonol synthase (FLS), flavone synthase (FNS) and anthocyanidin synthase (ANS).

Recently, much attention has focused on flavonoids functions in response to environmental stresses. Warren et al. (2003) found that the levels of flavonoids (mainly kaempferol and quercetin) in *Populus trichocarpa* leaves increased in response to UV-B irradiation. Markham et al. (1998) compared C-glycosylflavones content of different rice cultivar under UV-B light. The results showed that C-glycosylflavones were enhanced by UV-B light in a UV-tolerant rice cultivar but absent in a susceptible cultivar. In addition to the long-reported functions as UV light protective, flavonoids biosynthesis are also up-regulated in response to a wide range of other abiotic stresses, such as cold, salinity and drought. Castellarin et al. (2007) reported that anthocyanin biosynthesis in ripening fruit was strongly up-regulated by the drought treatment. Water deficit can also enhance the flavonoids production in cell suspension culture of *Glycyrrhiza inflata Batal* (Yang et al., 2007). The results of Walid and Ghazanfar (2006) indicated that the presence of phenolics, anthocyanins and flavones are related to increased salt tolerance of

Abbreviations: AK, Aikang 58; ANS, anthocyanidin synthhase; CHI, chalcone isomerase; CHS, chalcone synthase; CS, Chinese spring; DFR, dihydroflavonol-4-reductase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; FNS, flavone synthase; HPLC, high performance liquid chromatography; qRT-PCR, Quantitative real-time PCR; ROS, reactive oxygen species.

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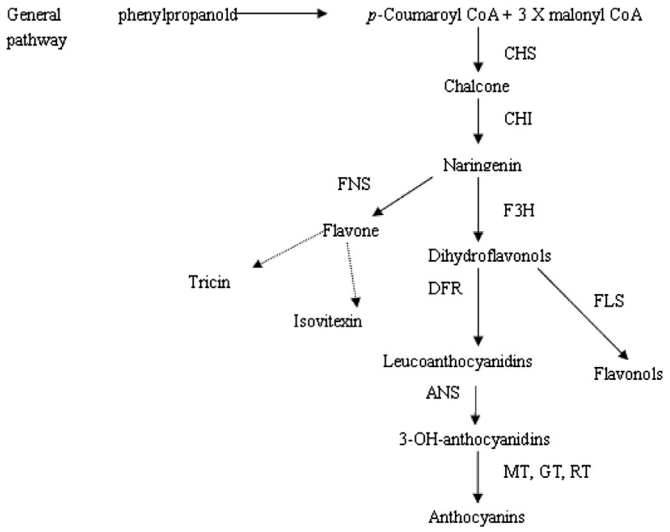

Fig. 1. Overview of the biosynthesis pathway of flavonoids in plant. CHS, chalcone synthase; CHI, chalcone isomerase, F3H, flavanone 3-hydroxylase; FNS, flavone synthase; FLS, flavonol synthase; DFR, dihydroflavonol-4-reductase; ANS, anthocyanidin synthhase. Broken arrows indicated that the biosynthetic steps are not very clear.

sugarcane. Flavonoids have been suggested as antioxidant in stressed plants. The position of their hydroxyl groups, double carbon bonds and modifications like glycosylation, prenylation and methylation determined their antioxidant properties (Rice-Evans et al., 1997).

In recent years, several studies have focused on the molecular mechanism of secondary metabolism in response to abiotic stresses. Yuan et al. (2012) reported that water deficit increased the expression of several flavonoids biosynthesis genes in *Scutellaria baicalensis* Georigi roots. Rice flavonoids pathway genes, *OsDfr* and *OsAns*, are induced by dehydration, high salt and ABA (Ithal and Reddy, 2004). Liu et al. (2013) characterized an *RsF3H* gene and suggested that the combination of increases in *RsF3H* gene expression, RsF3H enzyme activity and corresponding flavonoids production is one explanation of the stress-tolerance of *Reaumuria soongorica*.

Drought is an important environmental stress that adversely affects wheat growth and causes a reduction in wheat yield. Understanding the molecular basis of flavonoids function in improving stress, as well as the types and amounts of flavonoids synthesized under stress are a high priority for research with eye to engineering enhanced stress tolerance in crop plants (Winkel-Shirley, 2002). However, little is known about the role of flavonoids biosynthesis pathways in wheat in response to drought stress. Here, we investigated the expression profiling of flavonoids biosynthesis genes (*CHS*, *CHI*, *F3H*, *FLS*, *DFR*, *ANS*, and *FNS*) in response to drought stress and studied the accumulation of phenolics and flavonoids in wheat leaves under drought stress.

2. Materials and methods

2.1. Plant materials and treatment

Two common wheat (*Triticum aestivum* L.) genotypes were used –'Aikang 58' (AK) and 'Chinese Spring' (CS). Seeds were surface-sterilized in 70% alcohol for 5 min, treated with 0.1% HgCl for 15 min, and washed six times (2 min each) in distilled water. They were then germinated and cultured with water in Petri dishes placed in a temperature-controlled chamber at 25 °C and under a 14 h photoperiod. Plantlets were watered daily with appropriate volumes of tap water. Artificial water stress was induced with polyethyleneglycol (PEG) 6000 solutions to achieve an osmotic potential of −0.49 MPa (PEG 20%). At the three-leaf stage, drought treatment was applied by culturing seedlings in this PEG solution for 0, 12, 24, and 48 h. For the well-watered control, other seedlings were treated only with tap water over the same time span. Afterward, leaves were harvested from both stressed and control plants, and samples were immediately frozen in liquid nitrogen and stored at −80 °C prior to further analysis.

2.2. Determination of total phenolics content

The total phenolics content of the samples were extracted and determined using a modified method based on Cai et al. (2004). The 0.3 ml extracts were mixed with 2 ml Folin–Ciocalteu reagent, and the reaction was neutralized with the addition of 1.6 ml saturated sodium carbonate (75 g/l). The mixture was incubated at ambient temperature for 2 h. The absorbance at 765 nm was measured with a spectrophotometer.

2.3. Determination of total flavonoids content

Total flavonoids content was determined using a colorimetric method (Bao et al., 2005) with minor modifications. Aliquots of 0.5 ml of appropriately diluted extracts or standard solutions were pipetted into 15-ml polypropylene conical tubes containing 2 ml double distilled H_2O and mixed with 0.15 ml 5% $NaNO_2$. After 5 min, 0.15 ml 10% $AlCl_3 \cdot 6H_2O$ solution was added, and the mixture was allowed to stand for an additional 5 min, followed by the addition of 1 ml 1 M NaOH. The reaction solution was mixed well and incubated for 15 min, and the absorbance was then determined at 415 nm.

2.4. Determination of anthocyanin content

Anthocyanin content was estimated using the method of Rabio and Marcelo (Rabino and Mancinelli, 1986; Marcelo and Toni, 2008) with minor modification. Leaf samples were ground to fine power with liquid nitrogen. 0.5 g samples were extracted with 1.5 mL mixture of methanol/hydrochlori acid/water (25:5:70, v/v/v) in shakers (32 °C, 150 r/min) for 4 h. The supernatant was collected after centrifuged at 10,000× *g* for 20 min. The absorbance of supernatant was then determined at 525 nm and 657 nm. The anthocyanin content was calculated as: An = (OD525 − 0.25 × OD657)/FW.

2.5. Analysis of flavonoid components by HPLC

Flavonoids were determined by HPLC with a modification of the method described by Revilla and Ryan (Revilla and Ryan, 2000). Plant tissues ground to fine powder in liquid nitrogen were extracted in methanol by ultrasonication. After centrifugation, the resulting supernatants were pools. 2M HCL were added to the supernatants and kept at 40 °C for 40 min. After reaction, 100% methanol was added to prevent aglycone sedimentation. After centrifugation, the resulting supernatants were pools, evaporated to dryness, and re-suspended in 2 ml of methanol for HPLC analysis. HPLC was performed on a Welch UltimateAQ-C18 column (4.6 × 250 nm) with a gradient of solvent A [water containing 1% (v/v) HAC] and solvent B (100% methanol) for 36 min at a flow rate of 6 ml/min. The solvent gradient was programmed as follows: at 0–13 min, 53.5% A; at 13–23 min, 37.5% A; at 23–36 min, 53.5% A. Quantification of the flavonoid compounds was based on peak areas and calculated as equivalents of representative standard