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Research article

# Molecular cloning and identification of a flavanone 3-hydroxylase gene from *Lycium chinense*, and its overexpression enhances drought stress in tobacco

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ABSTRACT

Flavonoids, as plant secondary metabolites, are widespread throughout the plant kingdom and involved in many physiological and biochemical processes. Drought resistance is attributed to flavonoids with respect to protective functions in the cell wall and membranes. The flavanone 3-hydroxylase (*F3H*) gene which encodes flavanone 3-hydroxylase, is essential in flavonoids biosynthetic pathway. *Lycium chinense* (*L. chinense*) is a deciduous woody perennial halophyte that grows under a large variety of environmental conditions and survives under extreme drought stress. A novel cDNA sequence coding a *F3H* gene in *Lycium chinense* (*LcF3H*, GenBank: KJ636468.1) was isolated. The open reading frame of *LcF3H* comprised 1101 bp encoding a polypeptide of 366 amino acids with a molecular weight of about 42 kDa and an isoelectric point of 5.32. The deduced LcF3H protein showed high identities with other plant F3Hs, and the conserved motifs were found in LcF3H at similar positions like other F3Hs. The recombinant protein converted naringen into dihydrokaempferol in vitro. Since studies have shown that amongst flavonoids, flavan-3-ols (catechin and epicatechin) have direct free radical scavenging activity to maintain the normal physiological function of cells in vivo, these data support the possible relationship between the oxidative damage and the regulation of *LcF3H* gene expression in *L. chinense* under drought stress. In order to better understand the biotechnological potential of *LcF3H*, gene overexpression was conducted in tobacco. The content of flavan-3-ols and the tolerance to drought stress were increased in LcF3H overexpressing tobacco. Analysis of transgenic tobacco lines also showed that antioxidant enzyme activities were increased meanwhile the malondialdehyde (MDA) content and the content of $H_2O_2$ were reduced comparing to nontransformed tobacco plants. Furthermore, the photosynthesis rate was less decreased in the transgenetic plants. These results suggest that *LcF3H* plays a role in enhancing drought tolerance in *L. chinense*, and its overexpression increases tolerance to drought stress by improving the antioxidant system in tobacco.



## 1. Introduction

Drought is one of the most serious environmental stresses that account for crop yield reduction (Abeles et al., 1988; Zhu, 2002). It has been reported that a series of physiological and biochemical response including stomatal closure, inhibition of cell growth, repression of photosynthesis will be stimulated when plants are subjected to drought stress (Pizzi et al., 1986; Shinozaki and Yamaguchi-Shinozaki, 2007; Tattini et al., 2004). Drought also leads to the generation of reactive oxygen species (ROS) in plants (Munne-Bosch and Penuelas, 2003). Oxidative damage, caused by the interaction of ROS and proteins, lipids and deoxyribonucleic acid further impairs the normal functions of cells (Foyer and Fletcher, 2001). A number of earlier studies have shown that drought stress also affected the functionality of both photosystem II (PSII) and photosystem I (PSI), and this may be due to the stress-induced impairment in pigment biosynthetic pathway or in

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pigment degradation (Ashraf and Harris, 2013; Farooq et al., 2012). During the long history of evolution, plants have evolved varieties of molecular and biochemical mechanisms such as non-enzymatic ROS scavenging to adapt to oxidative damage caused by ROS. Flavonoids that are employed by plants to avoid oxidative damage belong to non-enzymatic ROS scavenging mechanism.

Flavonoids are a large family of polyphenolic secondary metabolites and widely distributed in higher plants. They are involved in numerous physiological and biochemical processes that are regulated by a complex signaling network triggered by internal metabolic cues and external signals such as plant hormones, pathogen interaction, pollination, UV and drought stress (Izaguirre et al., 2007; Moore et al., 2005; Napal et al., 2009; Xu et al., 2012). The strong free radical scavenging ability towards highly reactive oxygen species (ROS) is a common shared property of these different flavonoids (Schroeter et al., 2006). Despite their diversity of functions and structures, all the flavonoids are synthesized through the phenylpropanoid (PAL) pathway and most enzymes involved in this process have been characterized in several plant species, including maize, *Antirrhinum*, tobacco, *Petunia* and *Arabidopsis* (Dixon and Steele, 1999; Forkmann and Martens, 2001; Holton and Cornish, 1995; Winkel-Shirley, 2001).

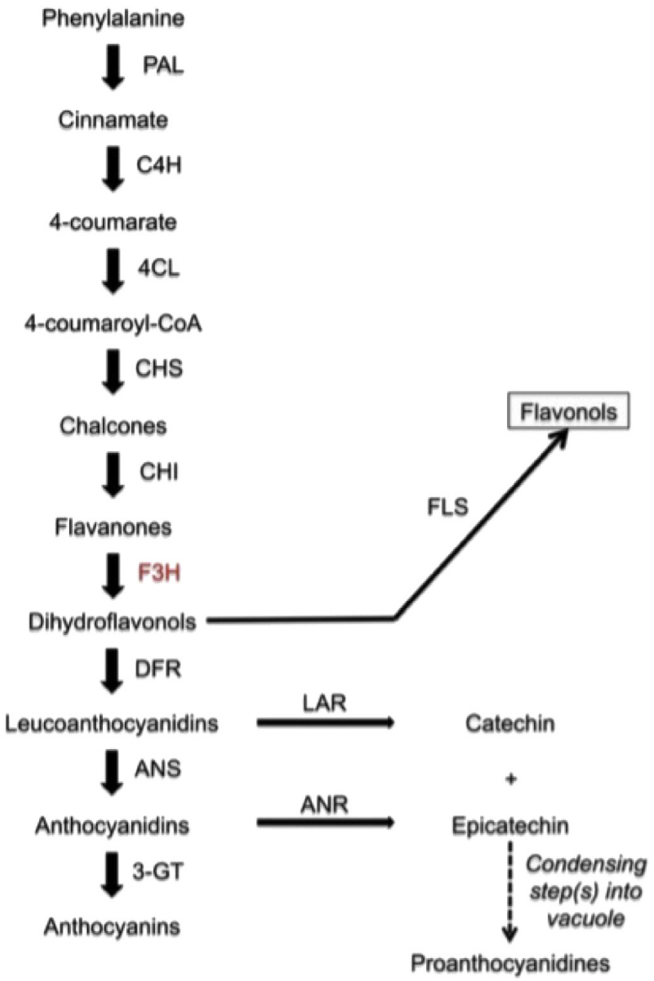


**Fig. 1.** Biosynthetic pathway of flavonoids starting with the general phenylpropanoid pathway. PAL, Pheammonialyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductas; 3-GT, Flavonoid-3-glucosyltransferase.

Flavanone 3-hydroxylase (F3H, EC 1.4.11.9) catalyzes the synthesis of early and unbranched segment of the flavonoid (Fig. 1). F3H belongs to a family of 2-oxoglutarate-dependent dioxygenases (2-ODDs) (A. G. Prescott & P. John, 1996), it catalyzes a critical step in the biosynthesis of flavonoids by hydroxylating (2S)-naringenin in the 3 position to form the respective (2R,3R)-dihydroflavonols. F3H is a key enzyme in the flavonoid biosynthetic pathway and has been cloned from many species (Liu et al., 2013; Lukačin and Britsch, 2004; Nishihara et al., 2005; Sparvoli et al., 1994; H. R. Zhang et al., 2014). Studies have shown that *F3H* not only regulates the types and quantities of flavonoids and the colors and flavors of flowers and fruits but also plays important roles in resistance to abiotic stress (F. Jiang et al., 2013; Mahajan and Yadav, 2014; H. R. Zhang et al., 2014). It was reported that the expression level of *F3H* was increased when potato was subjected to drought stress during growth (Watkinson et al., 2006). When *Reaumuria soongorica*, a desert plant, was exposed to UV-B radiation and drought stress, the expression of *F3H* was significantly increased (Liu et al., 2013). *Arabidopsis* grown in nutrient-deficient soil also resulted in increased *F3H* expression (Misson et al., 2005; Morcuende et al., 2007; Scheible et al., 2004). These results suggested that the increase in *F3H* gene expression and the content of flavonoids could play a positive role in stress resistance.

*Lycium chinense*, a deciduous woody perennial halophyte that grows in a large variety of environmental conditions and accumulates extraordinary high content of flavonoids in its leaves can survive under extreme drought stress. Having this characteristic, various physiological and ecological characteristics and molecular regulation mechanisms involved in tolerance to abiotic stress might have been evolved in *L. chinense*. Due to the large amount of flavonoids in the leaves of *L. chinense*, and the potential correlation between increased expression of *F3H* gene and tolerance to drought stress, it is hypothesized that F3H may involved in increasing drought resistance in *L. chinense*. However, until now, little has been done to investigate the relationship between the expression levels of *F3H* and tolerance to drought stress in *L. chinense*.

In this study, we presented the previously undescribed coding sequence of *F3H* from *L. chinense*. A detailed statement of this gene encoding F3H in *L. chinense* by bioinformatics analysis and its function identification by purified enzyme in the prokaryotic expression system. Analysis of *LcF3H* expression pattern demonstrated its pivotal role in the biosynthesis of flavonoids in *L. chinense* under drought stress and provides evidence for understanding the positive link between drought stress and the expression of *F3H* gene. Overexpression of *LcF3H* in tobacco leading to increased tolerance to drought stress.

## 2. Materials and methods

### 2.1. Plant materials and treatment

The *L. chinense* seedlings used for the investigation were cultivated at 18–25 °C in a controlled-growth chamber (12 h light/12 h dark), the photosynthetic photon flux density of 500 μmol (photon)/$m^2$/s and the relative humidity of 70–75% in the greenhouse.

The fresh root, stem, primordial leaf, young leaf, mature leaf and flower were collected separately from 15 weeks old plants with 35–40 leaves, which were not treated with any stress, frozen immediately in liquid nitrogen and stored at −80 °C prior to total RNA extraction. Total RNA was extracted TRIzol™ reagent according to manufacturer instructions (Tiangen, China). Genomic DNA was extracted from leaves of *L. chinense* following protocol (L. Jiang and Cai, 2000). The quality and concentration of the RNA and genomic DNA were all then quantified by spectrophotometry and