Plant Physiology and Biochemistry 115 (2017) 107–118

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Isolation and characterization of a novel chalcone synthase gene family from mulberry

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ARTICLE INFO

Article history:
Received 4 January 2017
Received in revised form
18 March 2017
Accepted 22 March 2017
Available online 23 March 2017

Keywords:
Mulberry
Flavonoids
Chalcone synthase
Stilbene synthase
Co-expression
Transgenic

ABSTRACT

Chalcone synthase (CHS) is the pivotal enzyme that catalyzes the first committed step of the phenylpropanoid pathway leading to flavonoids. Here, five *CHS* genes were determined in mulberry (*Morus atropurpurea* Roxb.). Interestingly, phylogenetic analysis tended to group three MaCHSs in the stilbene synthase (STS) family and initially annotated these as MaSTSs. A co-expression system that harbored a 4-coumarate:CoA ligase gene and one of the candidate genes was established to determine the functions of this novel gene family. The fermentation result demonstrated that *MaSTS* in fact encoded a CHS enzyme, and was consequently retermed *MaCHS*. Tissue-specific expression analysis indicated that *MaCHS1*/*MaCHS2* was highly abundant in fruit, and *MaCHS4* had significant expression in root bark, stem bark and old leaves, while *MaCHS3* and *MaCHS5* were more expressed in old leaves. Subcellular localization experiments showed that MaCHS was localized to the cytoplasm. Transcription levels suggested *MaCHS* genes were involved in a series of defense responses. Over-expression of *MaCHS* in transgenic tobacco modified the metabolite profile, and resulted in elevated tolerance to a series of environmental stresses. This study comprehensively evaluated the function of *MaCHS* genes and laid the foundation for future research on MaCHS in mulberry.

1. Introduction

Flavonoids are a large family of secondary metabolism products derived from phenylalanine metabolic pathways. They are the most common natural compounds in the plant kingdom and act as phytoalexins that protect against fungal infections (Synder and Nicholson, 1990), as ultraviolet protectants (Gläßgen et al., 1998; Vidovic et al., 2015) and as regulators of auxin transport (Kucerova et al., 2015; Peer et al., 2001). In addition, many flavonoids play important roles as pigments in flowers and fruit (Hahlbrock and Scheel, 2003). The presence of flavonoids makes fruit attractive, the fall beautiful, and the world colorful. Recent study revealed that flavonoids also have various bioactivities beneficial to the human body, such as antioxidant (Chang et al., 2011; Wang et al., 2014), anti-inflammatory (Bischoff, 2008) and antitumor action (Shih et al., 2007) and suppression of cancer cell proliferation (Zhang et al., 2005). Thus, flavonoids have been increasingly popular dietary supplements in recent years, and some have been developed into health care products (Liu et al., 2010).

Chalcone synthase (CHS) is a pivotal enzyme that catalyzes the first committed step of the phenylpropanoid pathway leading to branches that produce flavonoids in plants (Fig. 1). CHS utilizes a tetraketide intermediate by condensation with one 4-coumaroyl-CoA and three malonyl-CoAs to form naringenin chalcone (Pang et al., 2005; Yu et al., 2005). This is the first step for carbon in phenylpropanoid metabolic pathways to biosynthesize flavonoids. Naringenin chalcone is usually spontaneously converted to naringenin *in vitro* or catalyzed by chalcone isomerase (CHI) *in vivo*, then further modified by other downstream enzymes to produce a wide variety of colors (Hanumappa et al., 2007).

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http://dx.doi.org/10.1016/j.plaphy.2017.03.014
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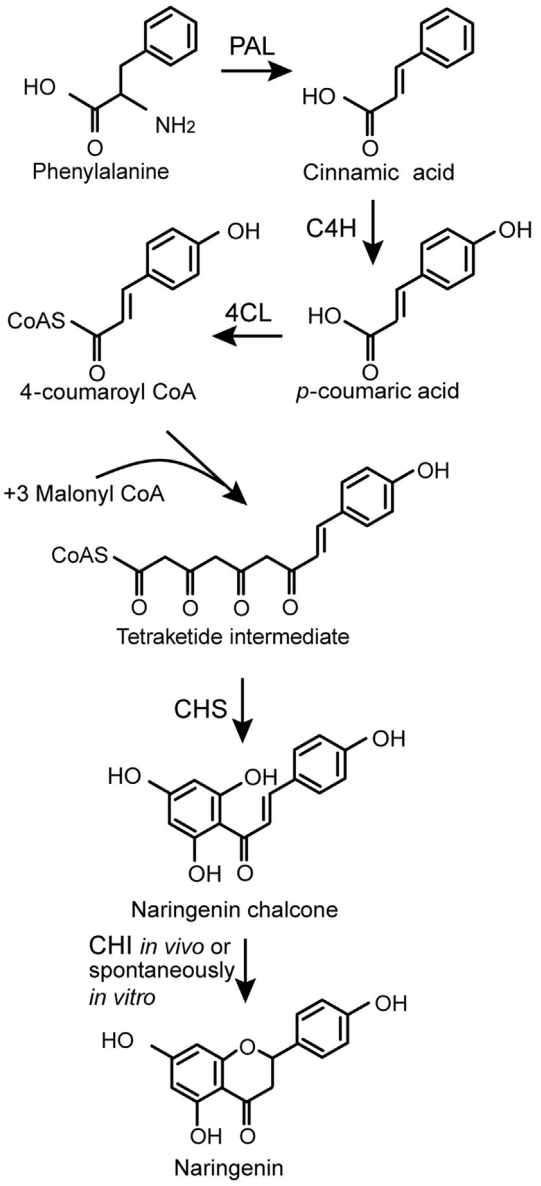

Fig. 1. Schematic overview of the flavonoid biosynthetic pathway. The enzymes of the flavonoid biosynthetic pathway consist of phenylalanine ammonia-lyase (PAL), cinnamic 4-hydroxylase (C4H), 4-coumarate:CoA ligase (4CL), Chalcone synthase (CHS) and chalcone isomerase (CHI). Chalcones are usually spontaneously converted to flavanones *in vitro*.

Mulberry (*Morus atropurpurea* Roxb.) is widely distributed around the world, and is well known as natural food for silkworms (*Bombyx mori* L.). Recent studies revealed that mulberry fruit has abundant flavonoids and phenolic compounds in comparison to other fruits and vegetables (Lin and Tang, 2007; Park et al., 2011; Wang et al., 2014). The flavonoids of mulberry extracts exhibited significant protectant low-density lipoproteins and scavenging effects on free radicals to limit oxidative damage (Chang et al., 2011; Katsube et al., 2006). Moreover, mulberry fruit is famous for its delicious taste and in human nutrition, and is especially rich in bioactive compounds, including anthocyanins, stilbenes and 1-deoxynojirimycin, which reportedly have significant health-promoting effects on the body (Chang et al., 2011; Dugo et al., 2001; Liu et al., 2015; Park et al., 2011). Two *MaCHS* genes, *MaCHS1* and *MaCHS2*, have been well characterized in mulberry (Li et al., 2014; Qi et al., 2014). However, surprisingly, their transcription levels were unexpectedly low in roots and stems, considering that roots and stems have abundant flavonoids (Chang et al., 2011; Wang et al., 2014). Consequently, we presumed the existence of other previously unreported *MaCHS* genes.

Due to the high degree of similarity at amino acid level of stilbene synthase (STS) and CHS, numerous STSs and CHSs have been confusingly annotated as each other in different public databases (Yu et al., 2005). Previously, we annotated a family of three *MaSTS* genes, *MaSTS1–3* (ALS20361, ALS20362 and ALS20363) using bioinformatics. In the present study, we mainly addressed the function of these three genes using a novel metabolic engineering tool. The resulting data demonstrated that *MaSTS1–3*, in fact, encoded a CHS enzyme. Therefore, we renamed *MaSTS1–3* as *MaCHS3–5*, respectively. Moreover, we also evaluated the response to multiple abiotic stresses of transgenic tobacco harboring *MaCHS*.

2. Materials and methods

2.1. Data retrieval and cloning cDNAs

To search for *CHS* genes in mulberry, amino acid sequences of *Medicago sativa* CHS2 (P30074), *Ginkgo biloba* CHS (AAT68477) and *Vaccinium ashei* CHS (BAO58434) were used as queries for BLAST searches against the *Morus* Genome Database (http://morus.swu.edu.cn/morusdb/). Five candidate genes, *MaCHS1*, *MaCHS2* and *MaSTS1–3* (ALS20361, ALS20362 and ALS20363) were selected for further study, and cDNA was cloned from cv. Guiyou No. 62 and a new cv. Jialing No. 40 (both *Morus atropurpurea* Roxb.) (see RT-PCR primers in Supplementary Table 1 and cDNA see in Supplementary file 1). Because *MaCHS1* and *MaCHS2* were highly similar, the same qRT-PCR primers were used for both, and simplify the *MaCHS1*/*MaCHS2* as *MaCHS1* (see qRT-PCR primers in Supplementary Table 2).

2.2. Sequence alignment and phylogenetic analysis

Multiple sequence alignment was performed using MEGA 5.0 software, and a phylogenetic tree constructed using MUSCLE 3.6 software based on the NJ method with 1000 resampling replicates. The other CHS amino acid sequences for the phylogenetic reconstruction were retrieved from GenBank (http://www.ncbi.nlm.nih.gov) (see accession numbers in Supplementary file 2). The subcellular localization was predicted using website tools on http://www.genscript.com/wolf-psort.html.

2.3. RNA extraction, cDNA synthesis and quantitative real-time PCR

To investigate the *MaCHS* expression in fruit, the different developmental stages (S1–S7) of fruit materials were collected from Jialing No. 40. The other tissues were collected from Guiyou No. 62 in the Southwest University mulberry garden. RNA was isolated separately using a RNA Extraction Kit (TaKaRa, Dalian, China), and the total RNA of mulberry fruit was extracted using the RNA Extraction Kit TransZol Plant Kit (TransGen Biotech, Beijing, China). All RNA was digested by DNase I (TaKaRa), and purified RNA was used to synthesize cDNA by using moloney murine leukemia virus reverse transcriptase (M-MLV-RT) (Promega, Madison, WI, USA). All cDNA was diluted six-fold for the following quantitative real-time PCR (qRT-PCR). Primers for qRT-PCR were designed using the online tool of GeneScript Company (Nanjing, China) (http://www.genscript.com.cn/index.html) (Supplementary Table 2). Reactions were tested according to the manufacturer's protocol using SYBR® Premix Ex Taq™ II (TaKaRa) and 2 μL of diluted cDNA used as a template in 20-μL reactions. The qRT-PCRs were conducted in a StepOne qRT-PCR System (Applied Biosystems, Foster City, CA, USA)