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Overexpression of and RNA interference with hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase affect the chlorogenic acid metabolic pathway and enhance salt tolerance in *Taraxacum antungense* Kitag

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ABSTRACT

Dandelions (*Taraxacum* spp.) are wild plants with a long history of use as a functional food and as medicine. Chlorogenic acid (CGA) and caffeic acid (CA) are organic acid components of *Taraxacum antungense* Kitag. that exhibit antioxidant, anti-inflammatory, and other pharmacological effects. This study aimed to determine the regulatory mechanism of CGA biosynthesis and the role of the CGA biosynthetic gene (*HQT*), encoding hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase, in *T. antungense*. Quantitative real-time polymerase chain reaction revealed significant changes in the tissue distribution of *HQT* expression in *T. antungense* transgenic lines overexpressing *HQT*. CGA but not CA concentrations differed significantly between the wild-type *T. antungense* and transgenic lines overexpressing *HQT*. Further, the CGA concentration was significantly higher in the leaves than in the roots. The upregulation of *TaHQT* elevated the CGA levels by up to 82.49% in the leaves, whereas RNA interference (RNAi) resulted in 51.48% reduction. Comparison of physiological parameters (malondialdehyde, proline, and total chlorophyll concentrations) under salt stress conditions revealed that the overexpression lines were more salt resistant than the wild-type and RNAi lines. These findings indicate that *TaHQT* positively regulates the CGA biosynthesis and enhances salt tolerance in the overexpression lines.

1. Introduction

Dandelions (*Taraxacum* spp.) are wild plants that has long been used as a traditional medicine for the treatment of several diseases, such as hepatitis B, since these plants have antimicrobial and antioxidant properties (Martinez et al., 2015). The Pharmacopoeia of the People's Republic of China (China Pharmacopeia Commission, 2015) indicates that caffeic acid (CA) is the main pharmacologically active constituent in *Taraxacum*. Further, chlorogenic (or 5-*O*-caffeoylquinic) acid (CGA), an ester of caffeic acid and quinic acid (Gauthier et al., 2016; Kühnl et al., 1987; Sato et al., 2011), is also one of the main active constituents of *Taraxacum* spp., and it exhibits strong antioxidant properties when CGA-containing food is consumed (Silveira et al., 2017; Liang and Were, 2018). Both CGA and CA show antioxidant properties *in vivo* and are considered beneficial for the functioning of the stomach, liver, and gallbladder (Nam et al., 2017; Shi et al., 2016; Zhu et al., 2011), reduction of the risk of heart disease and type 2 diabetes, and slowing down the release of glucose into the blood stream (Liu et al., 2013). In addition, they may have chemopreventive and hypotensive properties. Recently, *Taraxacum antungense* Kitag. which is mainly cultivated in northeastern China, has been shown to have higher CGA and CA concentrations than those in other *Taraxacum* species, such as *T. mongolicum* Hand.-Mazz., *T. coreanum* Nakai, and *T. ohwianum* Kitam. (Ning et al., 2014). Apomictic characteristics of *T. antungense* make it suitable for extensive cultivation in northeastern China (Ning et al., 2014; Wu et al., 2015).

Abbreviations: 6-BA, 6-benzylaminopurine; CA, caffeic acid; CGA, chlorogenic acid; HCT, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyltransferase; HQT, hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase; HPLC, high-performance liquid chromatography; HPT, hygromycin phosphotransferase; Kan, kanamycin; MDA, malondialdehyde; MS, Murashige and Skoog; NAA, naphthalene acetic acid; qPCR, quantitative real-time polymerase chain reaction; RNAi, RNA interference

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CGA synthesis is regulated by various enzymes in the phenylalanine pathways, such as phenylalanine ammonia-lyase, cinnamate 4-hydroxylase, 4-hydroxycinnamoyl-CoA ligase, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT), and hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase (HQT;Howles and Dixon, 1996; Peng et al., 2010). Three CGA biosynthetic pathways have been proposed, among which the HQT-mediated pathway may be the principal one, as has been reported for artichoke (Comino et al., 2009; Menin et al., 2010) and *Lonicera japonica* (Zhang et al., 2017). The overexpression of HQT in tomato was found to increase the accumulation of CGA (Niggeweg et al., 2004). *Agrobacterium tumefaciens*-mediated transformation in plants such as tomato, potato, tobacco, and *L. japonica* was used to induce the overexpression of *HQT*, leading to the increase in CGA levels compared with those in untransformed control groups, whereas RNA interference (RNAi) suppression of *HQT* resulted in a more than 90% reduction in CGA (Payyavula et al., 2015; Zhang et al., 2017). Lepelley et al. (2007) have found that higher expression of *HQT*, but not of the *HCT*, cinnamate 3-hydroxylase 1 (*C3H1*), and caffeoyl-CoA *O*-methyltransferase 1 (*CCoAOMT1*) genes, was more closely correlated with the CGA accumulation in different tissues at different developmental stages in coffee. The *HQT* expression and CGA levels increased in tomato plants transformed with *Arabidopsis thaliana MYB12* (Luo et al., 2008). Furthermore, the AtMYB11 transcription factor was found to increase the CGA levels and could also increase the expression of *HQT*, as revealed by quantitative real-time polymerase chain reaction (qPCR) analysis(Li et al., 2015).

Plants have evolved a variety of adaptive mechanisms to respond to salt stress (Lee et al., 2013; Saeedipour, 2013), including antioxidant enzyme activities and non-enzymatic antioxidant defense systems. CGA is an antioxidant constituent that under salinity stress can be increased in *L. japonica* (Rezazadeh et al., 2012; Yan et al., 2016). However, whether increased CGA concentrations can be beneficial to improve the salt tolerant of plants has not been reported. Proline is one of the most important osmolytes for osmotic adjustment, allowing plants to cope with osmotic stresses such as salt stress, and it also plays a regulatory role during plant growth (Mattioli et al., 2008). Increased levels of endogenous proline accumulation in plants are correlated with enhanced salt tolerance (Boscaiu et al., 2012; Sripinyowanich et al., 2013). Lipid peroxidation is a marker of oxidative damage under high salinity, and malondialdehyde (MDA) can indirectly indicate the degree of damage to the membrane system and the resistance of the plant. In addition, salinity can decrease the chlorophyll concentration. Thus, MDA, proline, and chlorophyll concentrations can be important indicators of plant resistance to salt stress (Karagözler et al., 2008; Yan et al., 2016).

We have previously found that *T. antungense* had high concentrations of CA and CGA (Ning et al., 2014), but their metabolic pathways have not yet been investigated in this species. We have previously determined a partial *HQT* gene sequence (GenBank accession number: KX909887.1). In this study, we determined the full-length sequence of the *T. antungense HQT* gene. The *Agrobacterium* GV3101 strain with two eukaryotic expression vectors (*HQT*-overexpressing and RNAi vectors) was transformed into *T. antungense* leaves, and the relationship between the HQT expression and CGA concentration was investigated in the transgenic lines generated. The MDA, proline, and total chlorophyll concentrations were investigated in the overexpression *T. antungense* lines subjected to salt stress to determine their stress tolerance level.

2. Materials and methods

2.1. Plant material and HQT gene isolation

A voucher specimen of *T. antungense* has been deposited in the herbarium at the Exsitu Conservation Garden Evaluation Centre of Wild Vegetable Germplasm (EWVG) in Northeast China under the Ministry of Agriculture since 2011. Individual plants were grown in a greenhouse at the EWVG (41°49′N, 123°33′E, altitude 74 m). Total RNA was extracted from approximately 100 mg of fresh tissue using the TRIzol reagent (Takara, Biomedical Technology (Beijing) Co., Ltd.), following the manufacturer's instructions. The final RNA concentrations were determined spectrophotometrically, and RNA integrity was assessed by electrophoresis on a 1.0% (w/v) agarose gel. cDNA was synthesized using a PrimeScript™ RT-PCR kit (Takara), according to the manufacturer's instructions.

Table 1
The list of primers used in this study.

Primer	Nucleotide Sequence
Degenerate F1	5'-ATG RSW AGT GRW VAA ADG-3'
Degenerate R1	5'-YYA AAA STC RTA CAA RHAC-3'
Degenerate F2	5'- ATG ACT ARY GAW SRA ARK -3'
Degenerate R2	5'- CYA AAA BTC RTA CAA RHAC-3'
Degenerate F3	5'-ATG GGW AGT GAW SRA ARK -3'
Degenerate R3	5'-TYA RAA YTC RTA CAA AMAC-3'
PDF1	5'-GAT GGW YTM TCS TTG MTCC-3'
PDR1	5'-RTT CAG RTT VGG RCT CGC-3'
HQT1F1	5'- ATG GGT AGT GAT CAA AAG -3'
HQT1R1	5'- TTA AAA TTC ATA CAA AAAC -3'
TaHQTF	5'-CG GTC GAC ATG GGT AGT GAT CAA AAG-3', SalI site underlined
TaHQTR	5'-CG GGT ACC TTA AAA TTC ATA CAA AAAC-3', KpnI site underlined
35S-promoter	5'-CTATCCTTCGCAAGACCCTTC-3'
M13 F(-47)	5'-CGCCAGGGTTTTCCCAGTCACGAC -3'
M13R(-48)	5'-AGCGGATAACAATTTCACACAGGA-3'
Ta1F	5'-CGGGATCCCGTGAACCACCTACC-3', BamHI site underlined
Ta1R	5'-GGACTAGTGGATTCCGCCATT-3', SpeI site underlined
Ta2F	5'-GGGGTACCGGATTCCGCCATT-3', KpnI site underlined
Ta2R	5'-CCGAGCTCCGTGAACCACCTACC-3', SacI site underlined
qTaHQTF	5'-TGGCAGGGAGAATGGATA-3'
qTaHQTR	5'-AGTCAACAGTCGGAGTAAGA-3'
ACTIN R	5'-AGCAGCTTCCATTCCGATCA-3'
ACTIN F	5'-GGTTACATGTTCACCACCAC-3'

We conducted homology searches using available sequences from other species of the Asteraceae family (*Cichorium intybus* L., accession number: ANN12610.1; *Cynara cardunculus* L., accession numbers: ACJ23164.1 and ADL62854.1) and *Nicotiana tabacum* L. (accession number: AJ582651.1) and designed three pairs of degenerate primers, from the 5′- and 3′-ends of the open reading frames, using the Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA). We also designed one pair of degenerate primers (PDF1 and PDR1) through two motifs (DGLSS and SPNLN) with highly homologous core sequences. The primer sequences are listed in Table 1. PrimeSTAR® GXL DNA polymerase (Takara) was used for PCR, and PCR errors were identified by sequencing multiple clones derived from independent PCR reactions. Next, we amplified the *HQT* sequence with the primers HQT1F1 and HQT1R1 using cDNA as the template. A total of 25 ng of cDNA and 5 pmol of each primer were used for PCR, which was carried out in a thermocycler programmed as follows: 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min and a final extension at 72 °C for 10 min. The PCR product was purified, ligated to the pMD19-T vector (Takara), which was used to transform *Escherichia coli* DH5α, and sequenced. Double enzyme sites were added by amplifying the full-length *TaHQT1* gene by PCR with the TaHQTF and TaHQTR primers (Table 1). For sequence analysis, the ortholog was searched for using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/). A multiple sequence alignment was performed using ClustalX (version 1.81), and the MEGA program (version 7.0) was used to construct a molecular phylogenetic tree using a neighbor-joining method.

2.2. Plasmid construction for overexpression and suppression of the HQT gene

For *TaHQT* overexpression, pMD19T-TaHQT and the plant expression vector pRI101-an (Takara) were digested with the same enzymes