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Cloning and functional characterization of a *p-coumaroyl quinate/shikimate 3′-hydroxylase* from potato (*Solanum tuberosum*)

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ABSTRACT

Chlorogenic acid (CGA) plays an important role in protecting plants against pathogens and promoting human health. Although CGA accumulates to high levels in potato tubers, the key enzyme *p*-coumaroyl quinate/shikimate 3′-hydroxylase (C3′H) for CGA biosynthesis has not been isolated and functionally characterized in potato. In this work, we cloned *StC3′H* from potato and showed that it catalyzed the formation of caffeoylshikimate and CGA (caffeoylquinate) from *p*-coumaroyl shikimate and *p*-coumaroyl quinate, respectively, but was inactive towards *p*-coumaric acid in *in vitro* enzyme assays. When the expression of *StC3′H* proteins was blocked through antisense (AS) inhibition under the control of a tuber-specific patatin promoter, moderate changes in tuber yield as well as phenolic metabolites in the core tuber tissue were observed for several AS lines. On the other hand, the AS and control potato lines exhibited similar responses to a bacterial pathogen *Pectobacterium carotovorum*. These results suggest that *St*C3′H is implicated in phenolic metabolism in potato. They also suggest that CGA accumulation in the core tissue of potato tubers is an intricately controlled process and that additional C3′H activity may also be involved in CGA biosynthesis in potato.

1. Introduction

Potato (*Solanum tuberosum*) is widely consumed in the world. Chlorogenic acid (CGA), a conjugate of caffeic acid and quinic acid, constitutes the most abundant phenolic compound found in potato tubers [1]. A high-level accumulation of CGA in plants has been linked to their increased resistance to a variety of microbial pathogens [2]. Additionally, CGA has also been associated with multiple bioactivities in humans, such as lowering the risks of cancer and type 2 diabetes [3]. To exploit the pathogen defense and human health-promoting benefits of CGA in potato, a better understanding of its biosynthetic mechanism is needed in this plant.

Three possible routes have been proposed for CGA biosynthesis in plants, all of which involve the action of *p*-coumaroyl quinate/shikimate 3′-hydroxylase (C3′H) while using *p*-coumaric acid (route 1), *p*-coumaroyl shikimate (route 2) or *p*-coumaroyl quinate (route 3) as a substrate (Fig. 1A) [4]. To date, several C3′H enzymes from different plant species have been biochemically characterized [5–8]. These C3′Hs exhibited substantial activities towards *p*-coumaroyl shikimate and *p*-coumaroyl quinate, but very little or no activity towards *p*-coumaric acid, suggesting that routes 2 and 3 likely lead to the formation of CGA (Fig. 1A). When the expression of *hydroxycinnamoyl CoA quinate:hydroxycinnamoyl transferase* (*HQT*) was downregulated via RNAi, up to a 90% reduction in CGA was observed in tubers of the transgenic potato plants [9]. This result suggests that route 2 involving *p*-coumaroyl shikimate may play a major role in CGA biosynthesis in potato tubers (Fig. 1A) [9]. However, C3′H genes have not been cloned and biochemically characterized in potato.

Abbreviations: AS, antisense; CGA, chlorogenic acid; C3′H, *p*-coumaroyl quinate/shikimate 3′-hydroxylase; EV, empty vector; HPLC, high performance liquid chromatography; HQT, hydroxycinnamoyl CoA:quinate hydroxycinnamoyl transferase; MS, Mass Spectrometry; MUSCLE, Multiple Sequence Comparison by Log-Expectation; RACE, rapid amplification of cDNA ends; RT, reverse transcription; WT, wild type.

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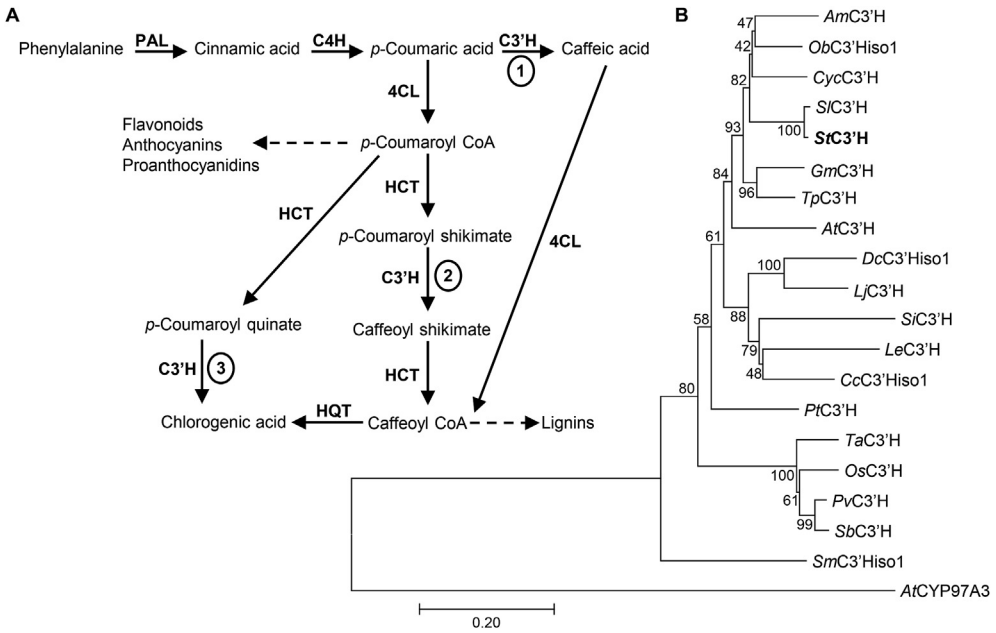

Fig. 1. A. Three proposed routes for chlorogenic acid (CGA) formation in potato. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; C3′H, *p*-coumaroyl quinate/shikimate 3′-hydroxylase; 4CL, *p*-coumaroyl:CoA ligase; HCT, hydroxycinnamoyl CoA shikimate/quinate:hydroxycinnamoyl transferase; HQT, hydroxycinnamoyl CoA quinate:hydroxycinnamoyl transferase. Dotted arrows denote multiple enzymatic reactions. B. A neighbor-joining phylogenetic tree of selected plant C3′H proteins. *St*C3′H is highlighted in bold. *At*CYP97A3 is used as an outgroup for the analysis.

To investigate CGA biosynthesis and assess the role of C3′H on phenolic metabolism in potato, we cloned a *StC3′H* gene and determined its activity towards *p*-coumaric acid, *p*-coumaroyl shikimate, and *p*-coumaroyl quinate in *in vitro* enzyme assays. In addition, an antisense (AS) *StC3′H* was expressed in potato tubers under the control of a tuber specific patatin promoter. Tuber yield, soluble phenolic accumulation, and response to a bacterial pathogen were determined in the AS *StC3′H* transgenic as well as wild type and vector-transformed control potato tubers.

2. Materials and methods

2.1. Cloning of StC3′H

A partial *StC3′H* cDNA sequence was retrieved from the SOL Genomics Network (https://solgenomics.net/). Total RNA was extracted from leaves of potato cv. Atlantic using TRIzol reagent (Invitrogen, Carlsbad, CA) and used for the synthesis of first strand cDNA. The full-length *StC3′H* was obtained by 5′ rapid amplification of cDNA ends (RACE) PCR using the SMARTer RACE kit (Clontech, Mountain View, CA). Primer sequences used for RACE PCR, yeast expression, cloning of the patatin promoter, and generation of AS *StC3′H* for expression in potato, as well as amplification of AS *StC3′H* transgene and *StC3′H*/AS *StC3′H* transcripts are listed in Table S1.

2.2. Phylogenetic analysis

Selected plant C3′H protein sequences were aligned using Multiple Sequence Comparison by Log-Expectation (MUSCLE) [10]. A neighbor-joining tree was constructed using the sequence alignment with MEGA7 [11]. The GenBank accession numbers are: *Ammi majus* (AAT06912), *Arabidopsis thaliana* (NP850337), *Coffea canephora* isoform 1 (DQ269126), *Cynara cardunculus* (KU711508), *Daucus carota* isoform 1 (KU757478), *Glycine* max (AAB94587), *Lithospermum erythrorhizon* (BAC44836), *Lonicera japonica* (KC765076), *Ocimum basilicum* (AAL99200), *Oryza sativa* (AAU44038), *Panicum virgatum* (AB723823), *Pinus taeda* (AAL47685), *Selaginella moellendorffii* isoform 1 (XM_002982586), *Sesamum indicum* (AAL47545), *S. lycopersicum* (XP_004230046), *S. tuberosum* (XM_006347618), *Sorghum bicolor* (AAC39316), *Trifolium pratense* (GQ497816), and *Triticum aestivum* (CAE47489). The accession number for *St*C3′H is a predicted mRNA sequence in GenBank, which is identical to the cDNA sequence obtained in this work. *At*CYP97A3 (NP_564384) was used as an outgroup for the phylogenetic analysis.

2.3. Recombinant protein expression in yeast and in vitro enzyme assays

The open reading frame of *StC3′H* was cloned into the *Bam*HI and *Eco*RI restriction sites of the yeast expression vector pYeDP60 [12]. pYeDP60-*StC3′H* and the empty pYeDP60 vector were transformed into yeast (*Saccharomyces cerevisiae*) WAT11 cells [12]. Yeast microsomes were prepared as described [13]. Protein concentrations of the microsomes were determined using the Bradford assay [14].

Reactions using *p*-coumaric acid (Sigma Aldrich, St. Louis, MO) and *p*-coumaroyl shikimate (provided by Prof. John Ralph, University of Wisconsin, Madison) were performed as follows. The 100 μL reaction included 400 μM *p*-coumaric acid or *p*-coumaroyl shikimate, 600 μM NADPH, and approximately 100 μg of microsomal proteins in 100 mM sodium phosphate buffer, pH 7.5. The reaction was incubated at 30 °C for 3 h and stopped by addition of 10 μL of 17.5 M acetic acid. The reaction mixture was centrifuged at 13,000 × *g* for 10 min. The supernatant was collected and mixed with an equal volume of methanol for high performance liquid chromatography (HPLC) analysis.

Since *p*-coumaroyl quinate was not commercially available, coupled enzyme assays were carried out using the *A. thaliana* *p*-coumaroyl:CoA ligase (*At*4CL1; for formation of *p*-coumaroyl CoA from *p*-coumaric acid and coenzyme A) [15], the *P. virgatum* hydroxycinnamoyl transferase (*Pv*HCTa1; for formation of *p*-

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