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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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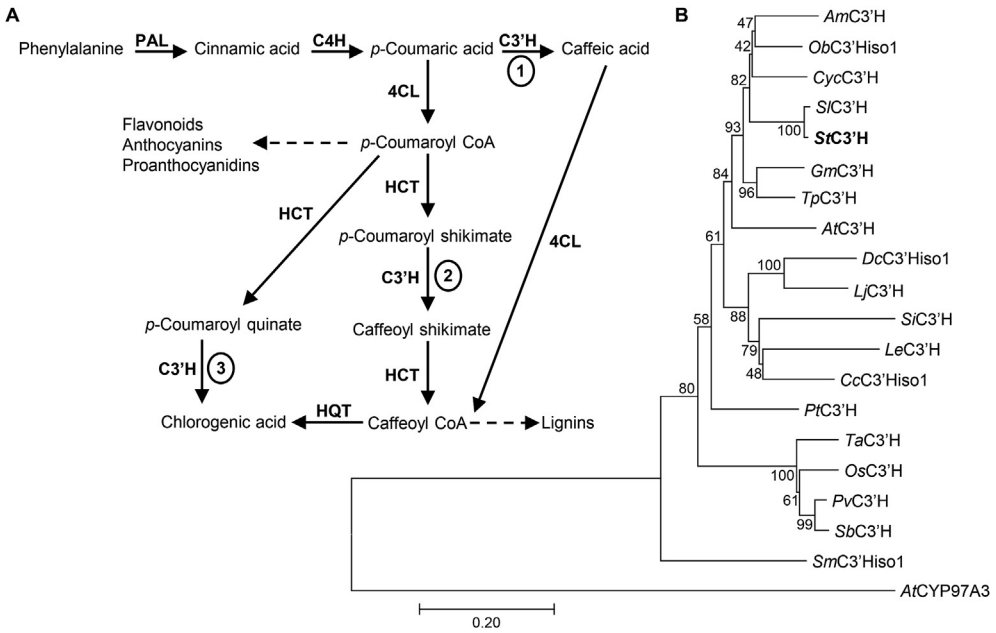

Fig. 1. A. Three proposed routes for chlorogenic acid (CGA) formation in potato. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; C3′H, *p*-coumaroyl quinate/shikimate 3′-hydroxylase; 4CL, *p*-coumaroyl:CoA ligase; HCT, hydroxycinnamoyl CoA shikimate/quinate:hydroxycinnamoyl transferase; 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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coumaroyl quinate from *p*-coumaroyl CoA and quinic acid) [5], and pYeDP60-*StC3′H*- or pYeDP60-expressing yeast microsomes. The initial reaction (1 mL) included 0.35 mg *p*-coumaric acid, 0.2 mg coenzyme A, 11.5 μL of 100 mM ATP, and 5.5 μg of purified, His-tagged *At*4CL1 proteins in 100 mM sodium phosphate buffer, pH 7.5. After incubating at room temperature for 1 h, an additional 0.2 mg coenzyme A and 11.5 μL of 100 mM ATP were added to the reaction mixture, followed by continued incubation at room temperature overnight. On the next day, the above reaction was divided into 100 μL aliquots, to which 0 (used as control) or 5 μL of purified *Pv*HCTa1 proteins (at 2 mg mL^{-1}), 500 μM quinic acid, and 500 μM DTT were added. This reaction was incubated at room temperature for 2 h. To the above reaction approximately 100 μg of pYeDP60-*St*C3′H- or pYeDP60-containing microsomal proteins were added along with 600 μM NADPH. The reaction was incubated at 30 °C for 3 h before adding 10 μL of 17.5 M acetic acid. After centrifugation at 13,000 × *g* for 10 min, 100 μL of methanol was added to the collected supernatant prior to HPLC analysis.

2.4. Potato transformation

A 2.5-kb B33 patatin promoter [16] was amplified from potato cv. Atlantic and cloned into the pCAMBIA2300 vector. The *StC3′H* coding sequence was then cloned downstream from the patatin promoter in the AS orientation in pCAMBIA2300. The recombinant pCAMBIA2300-*patatin promoter*-AS *StC3′H* plasmid and the empty pCAMBIA2300 vector were transformed into the electrocompetent *Agrobacterium tumefaciens* LBA4404 cells (Invitrogen).

Agrobacterium-mediated potato transformation was carried out using microtuber discs and internode explants following an established method [17]. Nine transgenic potato lines were produced, including 2 empty vector controls (EV) and 7 AS *StC3′H* lines. Incorporation of the AS *StC3′H* transgene was confirmed by PCR amplification using primers that are specific for the pCAMBIA2300 vector and *StC3′H* in the AS orientation; the PCR products were verified by DNA sequencing.

2.5. Molecular characterization of transgenic potato plants

Potato seedlings were grown in a temperature-controlled greenhouse in a randomized complete block design. Potato tubers were harvested at 110 d after planting in soil, which is approximately when CGA concentrations stabilize in the tuber tissue [18]. Total tuber yield at harvest was measured for four biological replicates of the transgenic and control plants. After the tubers were cleaned with water and 75% ethanol, the peel/cortex tissue (approximately 4 mm from the surface) was removed using a potato peeler. A core borer was then used to collect a plug of the medullary/core tissue (diameter = 1 cm, length = 2 cm). Tissues from three tubers were pooled for each plant, which constitute one biological replicate; three biological replicates were collected for metabolite and gene expression analyses. The tuber tissues were ground in liquid nitrogen and stored at −80 °C until further analysis.

Total RNA was extracted from the ground tuber tissue using Trizol reagent (Invitrogen) and treated with DNase I (Fermentas, Glen Burnie, MD). Reverse transcription (RT) reactions were carried out using 650 ng of total RNA with the Superscript IV cDNA synthesis kit (Invitrogen). The oligo$(dT)_{12\text{-}18}$ primer was used in the RT reaction to amplify both sense and AS *StC3′H* transcripts. To reverse transcribe specifically the AS *StC3′H* transcript, a gene specific primer (5′-ATGGCTATTCCCATTTCTTTACCTG-3′) was used in the RT reaction. The sense and/or AS *StC3′H* transcripts were amplified using 1.5 μL of cDNA template and AmpliTaq DNA polymerase (Thermo Scientific, Waltham, MA). The cycling parameters were 1 cycle of 5 min at 94 °C, 28 cycles of 30 s at 94 °C, 30 s at 63 °C, and 90 s at 72 °C, followed by 1 cycle of 10 min at 72 °C. The PCR products were separated on a 1% agarose gel.

2.6. HPLC and MS/MS analyses

*St*C3′H enzyme assay products and metabolite extracts from potato tubers were separated on a reverse-phase HPLC using a gradient between A (0.1% formic acid in water) and B (acetonitrile) at a flow rate of 1 mL min^{-1}. For analysis of assay products, the gradient was 0–15 min, 88-65% A and 15–15.5 min, 65–88% A. The retention time and absorption spectra of reaction products were compared to the authentic *p*-caffeoyl shikimate (provided by Prof. John Ralph) and CGA (Sigma Aldrich) standards. For analysis of potato metabolite extracts, the HPLC gradient was 0–5 min, 95% A; 5–15 min, 95-80% A; 15–34.5 min, 80-45% A; 34.5–35 min, 45–95% A. Individual peaks were collected, concentrated in a vacuum centrifuge, and subjected to tandem Mass Spectrometry (MS) analysis as described in Ref. [19].

2.7. Disease assay

Disease assays with *Pectobacterium carotovorum* subsp. *carotovorum* were performed following a previously established method [20]. Briefly, each tuber was inoculated with the bacterial suspension at three points from the basal to apical end to a depth of 1 cm. The tubers were placed on paper towels wetted with sterile water and incubated in dark at room temperature for 4 d. At the end of the incubation period, tubers were cut longitudinally, photographed, and lesions were measured with a ruler at their widest diameter to the nearest 0.5 mm. All lesions for each tuber were averaged. There were four biological replicates for the transgenic and control potato lines.

2.8. Statistical analysis

The tuber yield, metabolite, and disease assay data were analyzed by one-way ANOVA followed by Tukey's HSD test using JMP vers. 11 (SAS Institute, 2014).

3. Results

3.1. StC3′H is closely related to dicot C3′Hs

*St*C3′H, a C3′H homolog, was identified in potato through homology-based searches and its full-length sequence was obtained using RACE-PCR. To understand the evolutionary relationship of *St*C3′H with other C3′Hs, phylogenic analysis was conducted using *St*C3′H and (putatively) identified plant C3′Hs (Fig. 1B). The lycophyte *S. moellendorffii* C3′H (*Sm*C3′H) is ancestral to both monocot and dicot C3′Hs. *St*C3′H falls in the dicot C3′H clade, and associates most closely with the tomato C3′H (*Sl*C3′H) that is 99% identical to *St*C3′H (Fig. 1B).

3.2. StC3′H demonstrated p-caffeoyl shikimate and CGA forming activities in in vitro enzyme assays

To determine the catalytic activity of *St*C3′H, the recombinant *St*C3′H protein was expressed in yeast strain WAT11, which contains an *A. thaliana* cytochrome P450 reductase for testing the activities of cytochrome P450 enzymes. The *St*C3′H-expressing yeast microsomes were incubated with substrates of three possible routes, including *p*-coumaric acid, *p*-coumaroyl shikimate, and *p*-coumaroyl quinate (derived from sequential activities of *At*4CL1 and *Pv*HCTa1 using *p*-coumaric acid as substrate). Although *St*C3′H did

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not exhibit any activity towards *p*-coumaric acid (Fig. 2A), it converted *p*-coumaroyl shikimate near completely to *p*-caffeoyl shikimate (Fig. 2B). CGA was formed as the final product when *p*-coumaric acid was incubated with *At*4CL1, *Pv*HCTa1, and *St*C3′H, indicating that *St*C3′H is active towards *p*-coumaroyl quinate and can convert it to the hydroxylated derivative CGA (Fig. 2C). These *in vitro* assay results collectively suggested that routes 2 and 3 involving *p*-coumaroyl shikimate and *p*-coumaroyl quinate can lead to the formation of CGA in potato (Fig. 1A).

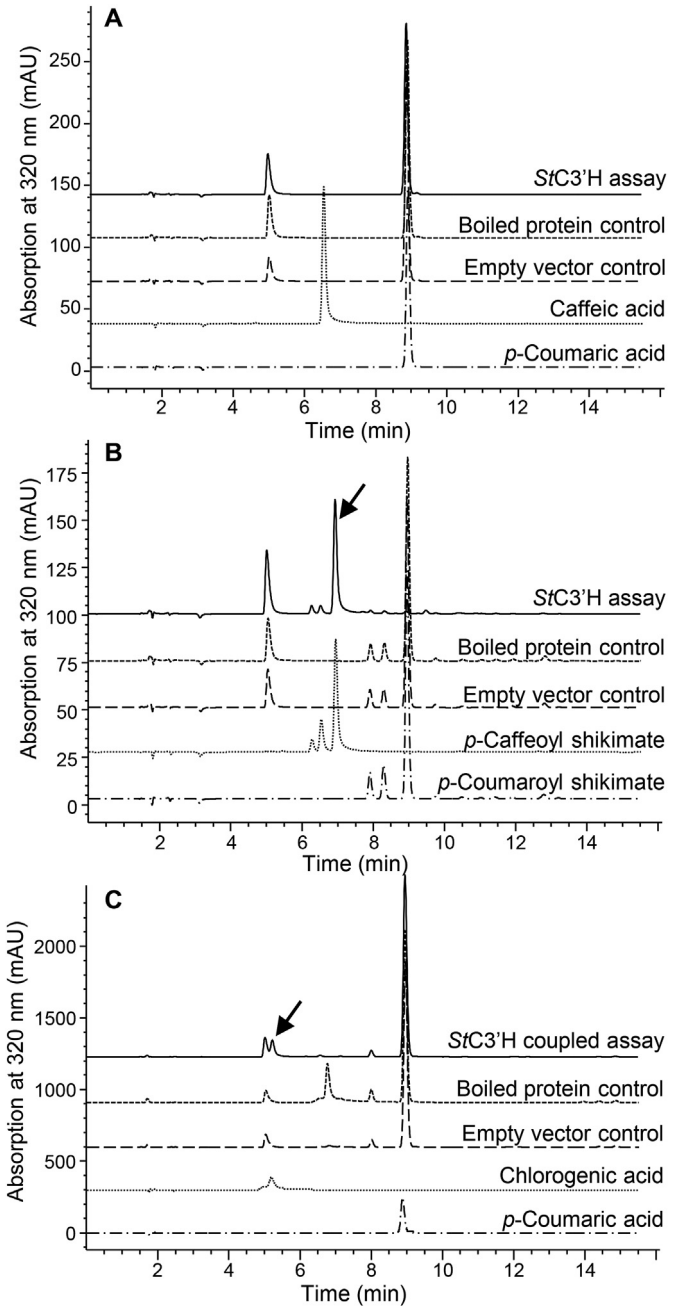

Fig. 2. Enzyme activity assays of *St*C3′H using *p*-coumaric acid (A), *p*-coumaroyl shikimate (B), and *p*-coumaroyl quinate (C) as substrates. HPLC elution profiles of the authentic standards and reaction mixtures are shown. Reaction products are highlighted with arrows. The *p*-coumaroyl quinate substrate in (C) was derived from coupled reactions catalyzed by the *Arabidopsis thaliana* *p*-coumaroyl:CoA ligase (*At*4CL1) and the *Panicum virgatum* hydroxycinnamoyl transferase (*Pv*HCTa1).

3.3. AS StC3′H transcripts accumulated in transgenic potato tubers

To understand the *in vivo* function of *StC3′H*, an AS *StC3′H* construct was transformed in potato tubers to block its translation into proteins. To prevent pleiotropic effects from downregulating *StC3′H* expression in aboveground tissues, a tuber-specific patatin promoter was used to direct AS *StC3′H* expression. Incorporation of AS *StC3′H* in potato plants was confirmed by PCR amplification of the AS *StC3′H* transgene, using primers located in the patatin promoter and the *StC3′H* gene in AS orientation, in the seven AS *StC3′H* transgenic, but not wild type (WT) or EV control lines (Fig. 3A).

To determine whether AS *StC3′H* transcripts were expressed in transgenic potato lines, AS strand-specific RT-PCR was performed (Fig. 3B). AS *StC3′H* transcripts accumulated in all seven lines, with strong AS *StC3′H* expression in the core tissues of AS5, AS6, AS14, and AS16 tubers (Fig. 3B). In a separate set of reactions intended to detect both sense and AS *StC3′H* transcripts, a similar trend of AS *StC3′H* transcript accumulation/enrichment was also observed in the AS *StC3′H* transgenic potato lines (Fig. 3C).

3.4. AS StC3′H potato plants showed moderate changes in tuber yield and soluble phenolics in the core tuber tissue, but similar responses to a bacterial pathogen, compared to the controls

Although no obvious changes were observed in the growth of aboveground tissues (data not shown), there was an around 26%

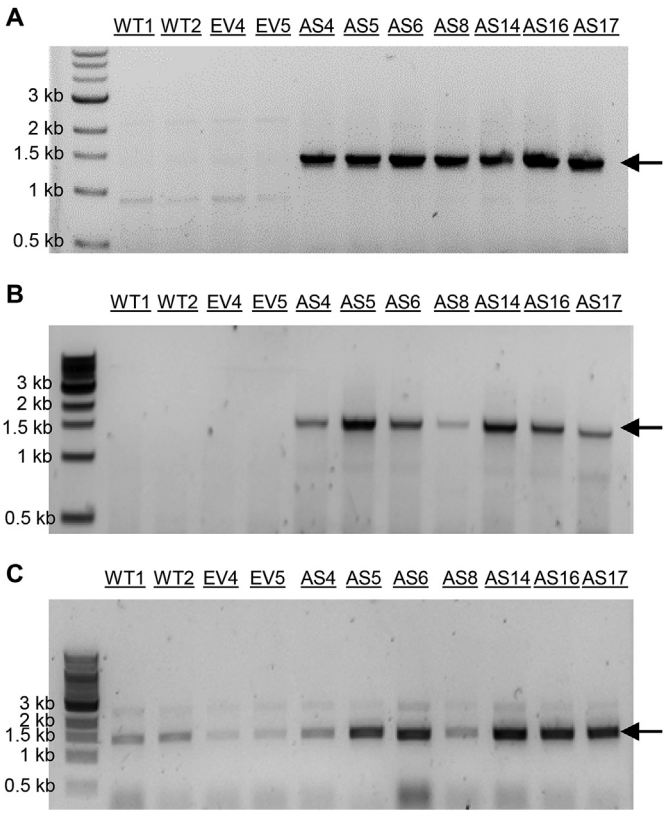

Fig. 3. Molecular characterization of the antisense (AS) *StC3′H* transgenic and control potato plants. A. Amplification of the AS *StC3′H* transgene using primers located on the patatin promoter and *StC3′H* in the AS orientation. B. Amplification of AS *StC3′H* transcripts. A primer that specifically amplifies the AS *StC3′H* transcript was used in the reverse transcription (RT) reaction. C. Detection of sense and AS *StC3′H* transcripts. The oligo(dT)$_{12-18}$ primer was used in the RT reaction. Each experiment was performed with three biological replicates and a representative image of each experiment is shown. The expected *StC3′H* gene/transcript amplification product is indicated with an arrow.

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reduction in total tuber yield for AS5 (238.7 g/plant) and a trend of decreased tuber yield for AS4, AS6, AS16, and AS17 lines (Fig. 4A). Overall, the AS *StC3′H* lines on average (269.3 g/plant) had a 17% decrease in total tuber yield compared to the EV control plants (324.4 g/plant) (Fig. 4A).

To determine the impact of AS *StC3′H* expression on CGA accumulation and phenolic metabolism in potato tubers, metabolite analysis was performed on soluble phenolics extracted from the core tissue of potato tubers. When the peak areas were compared among the control (WT and EV) and AS *StC3′H* lines, only peaks 1 and 2 showed significant differences (in particular, increases) from the controls in 2 and 4 of the 7 AS lines, respectively (Fig. 4B and Fig. S1). Peak 1 was determined to be CGA based on the UV absorption spectra and MS fragmentation patterns, which are similar to the authentic standard as well as those shown in previous reports (Fig. S1) [21]. Peak 2 was determined to be a mix of *trans*-5-*O*-feruloyl quinic acid and an unknown feruloyl conjugate, with a larger contribution from *trans*-5-*O*-feruloyl quinic acid, based on comparison to previous work in potato (Fig. S1) [22].

To evaluate the disease resistance property of the transgenic plants, tubers from the control and AS *StC3′H* lines were subjected to disease assays with *P. carotovorum* subsp. carotovorum, one of the main disease-causing agents of soft rot in potato tubers (Fig. 4C). Despite changes in potato tuber yield and soluble phenolics, AS *StC3′H* lines showed similar lesion sizes to the control lines when inoculated with *P. carotovorum* (Fig. 4C).

4. Discussion

Stunted growth was reported for Arabidopsis and poplar plants with reduced *C3′H* gene expression in all tissues [23,24]. To avoid such pleiotropic effect on plant development, AS *StC3′H* was expressed specifically in potato tubers and we did not observe any obvious reduction in the growth of aboveground tissues in the transgenic potato plants. However, total tuber yield was significantly decreased in AS5 and to a less extent in four other AS *StC3′H* lines (Fig. 4A). The reduction in tuber yield could be a result of metabolic perturbations in this tissue. It is also possible that, in maintaining normal aboveground tissue growth, carbons were reallocated between the source (aboveground) and sink (tuber) tissues. As such, less photosynthate was available to drive tuber growth, which led to decreased tuber yield in AS *StC3′H* lines.

Interestingly, while most soluble phenolics accumulated at comparable levels between the AS *StC3′H* and control plants, CGA and feruloyl conjugates were moderately increased in the AS *StC3′H* plants (Fig. S1). A search of potato transcriptome sequences (Potato Genomics Resources; http://solanaceae.plantbiology.msu.edu/) identified four open reading frames that are 62–74% identical to *St*C3′H at the protein sequence level (Fig. S2). In the future, these *St*C3′H-like proteins can be biochemically and genetically (e.g. via overexpression and downregulation of gene expression) characterized to determine whether they can functionally compensate for the missing C3′H activity in AS *StC3′H* plants. Another possible explanation for the increased CGA and feruloyl conjugates could be that the initial reduced CGA production from AS *StC3′H* expression limits carbon flux towards lignin (wall-bound phenolics; uses caffeoyl CoA as a precursor) biosynthesis (Fig. 1A). As a result, the backed up carbons positively feedback regulate CGA biosynthesis. The intricate interplay of soluble and wall-bound potato phenolics should be further investigated in future research.

CGA and ferulic acid conjugates have shown to be associated with potato resistance to *P. carotovorum* [2]. However, the slight increases in these metabolites in AS *StC3′H* tubers may not be sufficient for suppression of *P. carotovorum* growth as observed in the disease assays (Fig. 4C).

In summary, this work elucidated the biochemical activity of *St*C3′H towards *p*-coumaroyl shikimate/quinate. AS downregulation of *St*C3′H protein expression in potato tubers provided interesting new insights into the complex control of CGA and phenolic metabolism in potato and posed new questions for future

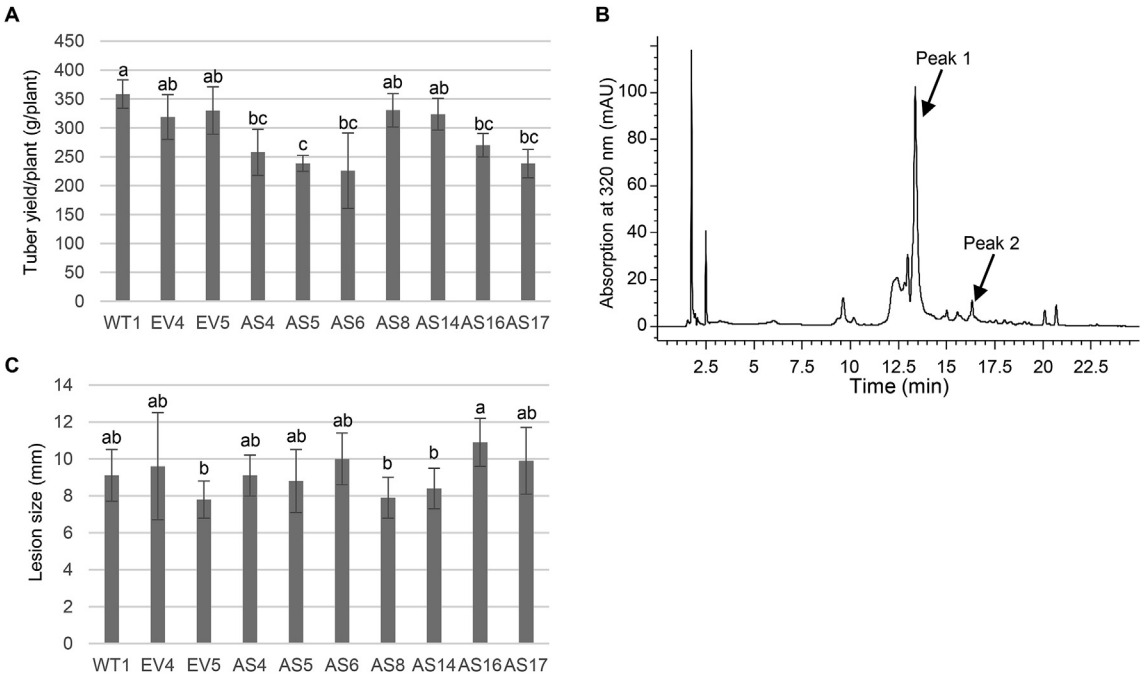

Fig. 4. A. Total tuber yield of the transgenic and control potato lines. Data shown are the average of four biological replicates and the standard deviation. B. A representative HPLC elution profile of phenolic metabolites extracted from the core tissue of potato tubers. C. Disease assays with *Pectobacterium carotovorum* subsp. *carotovorum*. The average lesion sizes of four biological replicates and standard deviations are shown.

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studies. Knowledge obtained from such investigations will assist in the rational design of breeding and metabolic engineering strategies for modulation of CGA content in potato tubers.

Conflicts of interest

The authors have no conflict of interest.

Author contribution

BJK, HL and LT designed the experiment; BJK, JJL and SY performed the experiment; BJK, JJL, SY, HL and LT analyzed the data; BJK and LT wrote the draft; All authors read and approved the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.2018.01.075.

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