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# Chlorogenic Acid Biosynthesis Appears Linked with Suberin Production in Potato Tuber (*Solanum tuberosum*)

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Supporting Information

**ABSTRACT:** Potato (*Solanum tuberosum* L.) is a good source of dietary antioxidants. Chlorogenic acid (CGA) and caffeic acid (CA) are the most abundant phenolic acid antioxidants in potato and are formed by the phenylpropanoid pathway. A number of CGA biosynthetic routes that involve hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase (HQT) and/or hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT) have been proposed, but little is known about their path in potato. CA production requires a caffeoyl shikimate esterase (CSE), and CA serves as a substrate of lignin precursor ferulic acid via the action of caffeic/5-hydroxyferulic acid *O*-methyltransferase (COMT I). CGA is precursor of caffeoyl-CoA and, via caffeoyl-CoA O-methyltransferase (CCoAOMT), of feruloyl-CoA. Feruloyl-CoA is required for lignin and suberin biosynthesis, crucial for tuber development. Here, metabolite and transcript levels of the mentioned and related enzymes, such as cinnamate 4-hydroxylase (C4H), were determined in the flesh and skin of fresh and stored tubers. Metabolite and transcript levels were higher in skin than in flesh, irrespective of storage. CGA and CA production appear to occur via *p*-coumaroyl-CoA, using HQT and CSE, respectively. HCT is likely involved in CGA remobilization toward suberin. The strong correlation between CGA and CA, the correspondence with *C4H*, *HQT*, *CCoAOMT2*, and *CSE*, and the negative correlation of *HCT* and *COMT I* in potato tubers suggest a major flux toward suberin.

**KEYWORDS:** *antioxidant, caffeic acid, flavonoid metabolism, phenolic acids metabolism, Solanum tuberosum, storage*

## INTRODUCTION

Potato (*Solanum tuberosum* L.) is an important food crop worldwide. It is considered a cheap source of high-quality proteins, minerals, and antioxidants, including vitamin C, carotenoids, and phenolic compounds such as chlorogenic acid (CGA) and flavonoids.[1] Phenolic acids have been extensively studied because they are involved in defense mechanisms induced upon biotic and abiotic stresses in plants.[2] Recently, they have been associated with health-promoting effects, mainly related to their antioxidant activity.[3,4] In particular, CGA has been reported to protect against degenerative diseases, cancer, heart disease,[5] hypertension,[6] and viral and bacterial diseases. Since differences in CGA levels of 1−2 orders of magnitude among varieties have been reported,[4] the biosynthesis of CGA in potato appears to be an important subject of study.

CGA and other phenolic acids, as caffeic acid (CA), are derived from the complex phenylpropanoid biosynthesis, part of the secondary metabolism. In the first steps of the "core phenylpropanoid pathway", primary metabolite phenylalanine is converted to *p*-coumaroyl-CoA by the consecutive action of phenylalanine-ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate-CoA ligase (4CL). *p*-Coumaroyl-CoA is the general precursor of the flavonoid metabolism, a precursor of lignin biosynthesis, and the starting point for two routes of CGA production (Figure 1).[7,8] Route I consists of esterification of quinic acid with *p*-coumaroyl-CoA, followed by hydroxylation of *p*-coumaroylquinic acid to form CGA (Figure 1). Route II requires four consecutive steps: esterification of shikimic acid with *p*-coumaroyl-CoA, hydroxylation of *p*-coumaroyl shikimate to form caffeoyl shikimic acid, and then de-esterification to produce caffeoyl-CoA, which in turn is re-esterified with quinic acid to form CGA (Figure 1). It has long been assumed that caffeoyl-CoA can also be synthesized from *p*-coumaric acid (CouA) via CA,[9] which is the second most abundant phenolic in potato tuber. Furthermore, a heteromeric C3H/C4H complex that converts CouA to CA has been identified in *Populus trichocarpa* (route III, Figure 1).[10] A fourth route, producing CGA via caffeoyl-glucose, has been proposed in sweet potato tuber (not shown in Figure 1).[11] Key enzymes appear to be hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase (HQT) and hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT), two very closely related, homologous enzymes. Note that two reactions supposedly require HCT, one reaction may utilize HQT or HCT, and in one reversible reaction HQT and HCT catalyze opposite reactions (Figure 1). Using gene silencing and overexpression, HQT was shown to be the principal enzyme for CGA accumulation in tomato leaves.[8] Tobacco HCT is involved in CGA remobilization toward caffeoyl-CoA, as silenced *HCT* tobacco plants were shown to accumulate large amounts of CGA in the stem.[12]

Recently, caffeoyl shikimate esterase (CSE) was shown to be involved in the biosynthesis of CA and, via 4CL, caffeoyl-CoA in *Arabidopsis thaliana*, bypassing the second HCT reaction.[13] Activity of caffeic/5-hydroxyferulic acid *O*-methyltransferase (COMT I) and caffeoyl-CoA *O*-methyltransferase

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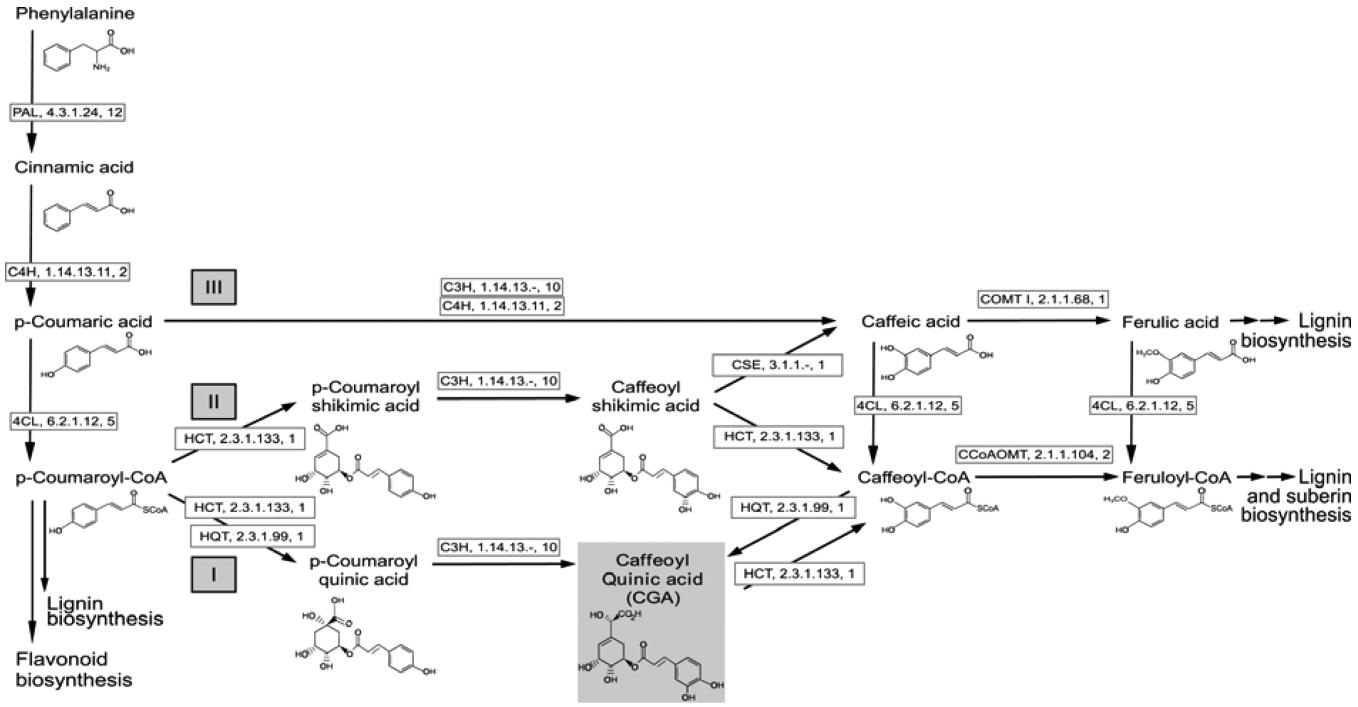


**Figure 1.** Chlorogenic acid biosynthetic pathways in plants. I, II, and III indicate possible biochemical routes toward CGA. The abbreviated enzyme names are boxed and followed by the corresponding EC number and the number of possible isoenzymes identified in the potato genome. Abbreviations: PAL, phenylalanine-ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; HCT, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyltransferase; C3H, *p*-coumarate 3′-hydroxilase; HQT, hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase; CSE, caffeoyl shikimate esterase; COMT I, caffeic/5-hydroxyferulic acid *O*-methyltransferase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase.

(CCoAOMT), respectively, results in ferulic acid (FA), a lignin precursor, and feruloyl-CoA, a precursor of both lignin and suberin (Figure 1).

It has been generally accepted that phenylpropanoid biosynthesis is mostly regulated by transcriptional control.[14] Several studies performed in CGA-accumulating plant families (i.e., Asteraceae, Rubiaceae, and Solanaceae) show that transcript levels reflect metabolites levels.[15−21] Relatively little is known about the regulation of the phenolic acids biosynthesis in potato tuber. André et al.[22] studied the expression of various genes involved in the phenylpropanoid pathway in tubers under normal and drought stress conditions. Constitutive and drought-induced expression profiles of *PAL*, *HCT*, and *C3H* genes were coordinated and correlated with phenolic acids levels, suggesting that also in potato tuber these enzymes are regulated at the transcriptional level; curiously, this was less clear for *C4H* and *HQT*. In a similar study, Payyavula et al.[23] found that phenylpropanoid gene expression appeared coordinately regulated and was well correlated with metabolite pools. In accordance with André et al.,[22] the correlation observed between *HQT* and CGA was less clear than that between the other phenylpropanoid genes and CGA. Enzymes that act downstream of CGA synthesis, such as CCoAOMT, were shown to be involved in suberization of potato tuber skin.[24] However, it is not clear how downstream events catalyzed by COMT I and CCoAOMT affect CGA levels. To the best of our knowledge, there are no studies regarding the role of CSE in CGA synthesis nor secondary metabolism in potato.

The phenolic acids contents of potato tuber are determined by both intrinsic and extrinsic factors. Genotype, developmental stage, and tissue are the major intrinsic factors.[25−36] It should be noted that most studies on the regulation of CGA synthesis have been carried out in whole tubers, although it has been shown that metabolite levels between flesh and skin differ significantly.[25,30,33,34] The major extrinsic factors, cultivation conditions (e.g., location, year of cultivation, and fertilization)[29,37,38] and cold storage, have not yet been studied extensively. The literature suggests that during cold storage, a period in which potatoes may undergo high metabolic changes,[39] their phenolics content either remains constant or increases.[40−42] A similar observation has been reported for antioxidant activity.[43−45] However, there are no studies on the effect of cold storage on transcription levels of genes involved in CGA biosynthesis in potato tubers. The aim of this work was to determine the transcriptional regulation of the synthesis of CGA and related phenolics, such as CA, through a comparative analysis of flesh and skin tissues of fresh and stored tubers of five carefully selected processing potato varieties.

## MATERIALS AND METHODS

**Plant Material.** Twelve white- or yellow-fleshed processing varieties of *Solanum tuberosum* ssp. *tuberosum* were grown in fields located in Balcarce, Buenos Aires, Argentina, during the 2009/2010 campaign of McCain Argentina S.A. All varieties were planted on the same date in random plots and harvested at the end of their respective cycles. For each variety, skin and flesh from 10 tubers that were either freshly harvested ("fresh") or stored for 3 months in darkness at 8 °C and a relative humidity of 80−90% with forced air ventilation ("stored") were pooled to generate a representative sample. The material was immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

**Total Phenolics.** Total phenolics were determined using the Folin−Ciocalteu reagent following the procedure described by Campos et al.[46] with minor modifications. Briefly, a 300 mg sample


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**Table 1. Primer Sequences Used for qRT-PCR**

| gene | primer | sequence (5′−3′) | potato genome contig |
|---|---|---|---|
| *C4H1* | fw | CCAGCTGAAAGCAAAATCTTA | PGSC0003DMS000000597 |
| | rv | GAAAGGAAGAAATCTGAAGTCG | |
| *C4H2* | fw | CTGGATACGATATTCCAGCTGAG | PGSC0003DMS000000597 |
| | rv | ACCGAAAGGAAGAAATCTAAAGTCA | |
| *HCT* | fw | ACGACTTATTCCGGCAGTTG | PGSC0003DMS000000559 |
| | rv | CCATGTGTTGATGAAGTGAAGAC | |
| *HQT* | fw | CACCATGTTTGGAACTTAAGAAAC | PGSC0003DMS000000585 |
| | rv | TGGAGGGATGAGAGACCATC | |
| *CSE* | fw | CCGGATACTTGGACAGGGTT | PGSC0003DMS000001550 |
| | rv | ATAGCCTTGCCCACCATCTT | |
| *COMT I* | fw | CTTTGATTTGCCACATGTTATTGA | PGSC0003DMS000000587 |
| | rv | AAGCAATGTTCATCGCTCCA | |
| *CCoAOMT1* | fw | CTGGCTATTCTCTTCTTGCTACT | PGSC0003DMS000001250 |
| | rv | GTAAAGCAGGTCCCTCTCG | |
| *CCoAOMT2* | fw | TGGCTACTCCCTTCTTGCTACC | PGSC0003DMS000001261 |
| | rv | GCAAAGCAGGGCCTTCTCT | |
| *ACTIN* | fw | CTAGCAGCATGAAGATTAAGGTG | PGSC0003DMS000000503 |
| | rv | GGACAATAGAAGGACCAGATTCG | |
| *L2* | fw | CGAAGGAGCTGTTGTTTGTAAC | PGSC0003DMS000002000 |
| | rv | GGGCACAATCTTTTTGGC | |

was homogenized in a mortar with liquid nitrogen, suspended in 3 mL of 95% ethanol, and subsequently incubated at 4 °C for 24 h. A 0.5 mL aliquot of extract was diluted with 7.5 mL of water, mixed with 0.5 mL of water-diluted Folin−Ciocalteu reagent (1:7), and allowed to react for 3 min. Next, 1 mL of 0.5 M $Na_2CO_3$ was added and allowed to react for 10 min. The absorbance at 725 nm was measured in a visible light spectrophotometer (Hitachi U-1900). Chlorogenic acid (Sigma-Aldrich) was used as standard for the calibration curve, and total phenolics contents were expressed as micrograms of CGA equivalents per gram fresh weight (FW). Blank samples consisted of 95% ethanol.

**Antioxidant Activity.** Antioxidant activity was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay described by Reddivari et al.[28] with minor modifications. A 100 mg sample, homogenized in a mortar with liquid nitrogen, was suspended in 1 mL of methanol and incubated at 4 °C for 24 h. The extract was obtained by centrifugation at 10000*g* for 10 min at 4 °C. A DPPH solution was freshly prepared by dissolving 4 mg of DPPH in 100 mL of methanol to obtain an absorbance of ∼1.1 at 515 nm. Next, 150 μL of the extract was mixed with 2850 μL of DPPH solution and incubated for 24 h at room temperature in the dark. The absorbance at 515 nm was measured using a visible light spectrophotometer (Hitachi U-1900). Blank samples consisted of methanol. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as standard for the calibration curve. The results were expressed as micrograms of Trolox equivalents per gram FW.

**Phenolic Acids Analysis.** One gram of tissue homogenized in a mortar with liquid nitrogen was incubated with extraction buffer (25 mL of 90% methanol with 0.1% HCl) for 60 min at 4 °C in darkness with constant agitation. After centrifugation at 4000*g* for 20 min at 4 °C, the extract was first concentrated using a rotary evaporator, and the remaining solvent was then completely evaporated in a vacuum centrifuge (Savant AES 1010 Automatic Environmental Speed Vac). The residue was dissolved in 1 mL of extraction buffer and filtered through a 0.45 μm PVDF syringe filter. Quantification of phenolic compounds was carried out using an Agilent 1100 system equipped with a quaternary pump, an autosampler, and a diode array detector (DAD). A flow rate of 1 mL $min^{-1}$ was used, and 20 μL samples were injected onto a C-18 Phenomenex Luna column (250 × 4.6 mm i.d.; 5 μm particle size). The mobile phases were (A) acetonitrile and (B) buffer, pH 2.3 (HPLC-grade water acidified with $H_3PO_4$). The solvent gradient was as follows: 0 to 20 min, linear gradient of A 20% to 100% then back to 20% to 25 min and held until 30 min. Identification of phenolic compounds was carried out by comparing retention times and spectra of authentic standards. The external standard method of calibration and peak areas were used for quantitation. Phenolic acids were expressed in μg $g^{-1}$ FW.

**Primer Design.** A BLAST analysis[47] was performed at the Web site of the Potato Genome Sequencing Consortium (http://potato.plantbiology.msu.edu/blast.shtml) in order to identify the phenolic acids' biosynthetic genes, *PAL*, *C4H*, *4CL*, *HCT*, *C3H*, *HQT*, *CSE*, *COMT*, and *CCoAOMT*, from the *Solanum phureja* genome. Housekeeping genes *ACTIN* and *L2*, previously used to normalize transcript levels of genes involved in the phenylpropanoid pathway in potato tubers,[22,23,35,36,48−50] were identified by BLAST at NCBI. Primers were designed with Fast-PCR software[51] and the Primer3 online primer design tool.[52] Amplicon sizes were selected around 150 bp, and temperatures of annealing were set around 60 °C. The *HCT*, *HQT*, *CSE*, *COMT I*, and *CCoAOMT* primers are intron spanning. Primers (sequences given in Table 1) were tested by BLAST at NCBI, and against *Solanum* DNA and cDNA. When possible, primer sequences were designed to anneal in regions conserved among *S. tuberosum*, *S. lycopersicum*, *S. melongena*, *S. pinnatisectum*, and *S. cardiophyllum*.

**RNA Extraction and cDNA Synthesis.** RNA was extracted from tuber samples using the cetyltrimethylammonium bromide (CTAB) method.[53] Samples were ground to fine powders in mortars precooled with liquid nitrogen. Next, 100 mg portions of the powders were transferred to RNase-free tubes filled with 1 mL of prewarmed extraction buffer, including 20 μL of β-mercaptoethanol, and vortex-mixed. The samples were incubated at 65 °C for 20 min and vortex-mixed every 5 min. An equal volume of chloroform/isoamyl alcohol (24:1) was added prior to the final vortex-mix. After centrifugation for 10 min at 15000*g* at 4 °C, the aqueous phase was extracted twice with an equal volume of chloroform/isoamyl alcohol. The supernatant was then mixed with 1/3 volume of 8 M lithium chloride. RNA was precipitated overnight at −20 °C and harvested by centrifugation for 10 min at 15000*g* at 4 °C. The pellet was dissolved in 400 μL of distilled, RNase-free water ($dH_2O$), reprecipitated with 1/10 volume of 3 M sodium acetate (pH 5.2) and 3 volumes of 100% ethanol at −20 °C for 30 min, and harvested by centrifugation for 10 min at 15000*g* at 4 °C. The RNA pellet was washed with 75% ethanol and finally dissolved in 30 μL of $dH_2O$. RNA was quantified by measuring the OD260 and OD280 values using a spectrophotometer (Hitachi U-1900), and quality was assessed by agarose gel electrophoresis. cDNA was synthesized using 2 μg of total RNA, previously treated with RNase-free DNase I (Invitrogen), anchored oligo(dT) 15 VN primers, and MMuLV reverse transcriptase according to the manufacturer's description (Invitrogen).


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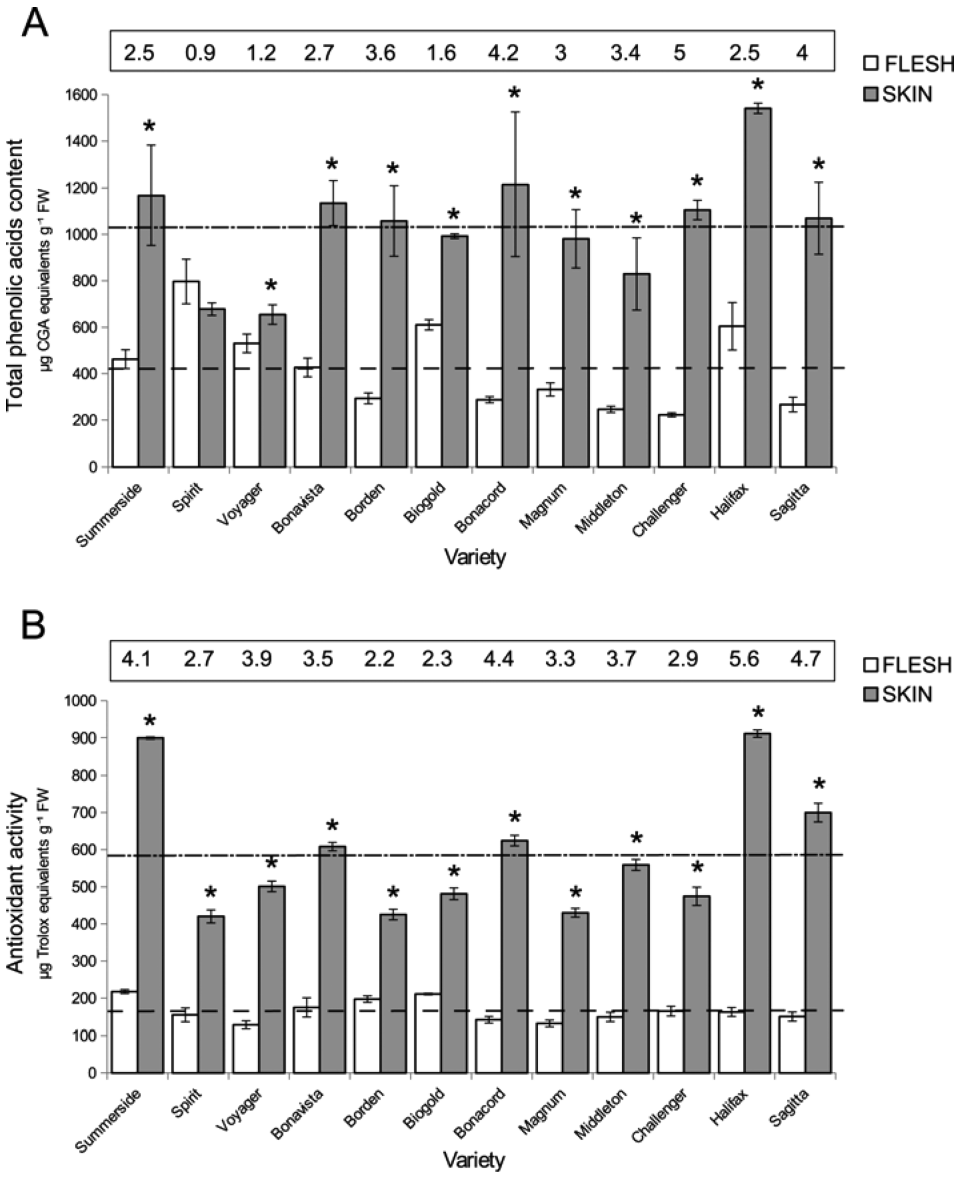


**Figure 2.** Phenolic acids contents and total hydrophilic antioxidant activity in flesh and skin of tubers from 12 processing potato varieties. (A) Total phenolic acids contents determined by the Folin−Ciocalteu reaction, and (B) total antioxidant activity determined by the DPPH assay in flesh (white bars) and skin (gray bars). Values are presented as the mean ± SD of three independent extractions. Dashed and dash-dotted lines indicate the mean values considering the 12 varieties in flesh and skin, respectively. Numbers in the upper boxes represent the skin-to-flesh ratios. Asterisks denote significant differences between flesh and skin by Student's *t* test ($p < 0.05$).

**qRT-PCR Experiments.** Relative transcript levels were determined by qRT-PCR in a 10 μL reaction volume with 20 ng of RNA equivalent cDNA, 300 nM gene-specific primers, and 5 μL of SYBR Green Mix (Roche, Mannheim, Germany). Amplification was done using a StepOne real-time PCR system (Applied Biosystem) according to the manufacturer's description, with a 10 min preincubation step at 95 °C, followed by 40 cycles of 15 s denaturation at 95 °C and 1 min annealing/extension at 60 °C. Relative expression was calculated by the ΔCT method[54] by normalizing the CT levels of target genes to the geometric mean of CT levels of both housekeeping genes, *ACTIN* and *L2*. Specificity of amplification was assessed by dissociation curve analysis and agarose gel electrophoresis. qRT-PCR data represent the normalized means ± SD of two independent experiments with two technical replicates each.

**C4H2 Sequencing.** Genomic DNA (gDNA) was extracted from leaves of *in vitro* grown potato plants using the PureLink genomic plant DNA purification kit (Invitrogen) according to the manufacturer's instructions. The *C4H2* locus was amplified with specific primers C4H2seqfw (5′ GTGCAGCAGTATAGAGGG 3′) and C4H2seqrv (5′ CCTTGCAAATCTTCAAGTAC 3′) from purified gDNA by standard PCR conditions. PCR products were purified from agarose gel using the DNA PuriPrep-GP kit (Inbio, Tandil, Argentina). Purified fragments were sequenced using an internal primer C4H2seqint1rv (5′ AAAGCTCTGAGCCAATCTACTCC 3′) by the sequencing service of Unidad de Genómica, Instituto de Biotecnología, INTA Castelar, Argentina.

**Statistical Analysis.** Pearson product moment correlation coefficients (*r* values) were calculated with Microsoft Excel using the means of metabolite concentrations or relative gene expression values and visualized with HeatMapper Plus.[55] Differences in the contents of metabolites and transcripts between flesh and skin were evaluated with *t* test.[56] For all statistical analyses, normality and homoscedasticity of the data set were tested using the Kolmogorov−Smirnov and Cochran tests, respectively.[56] When necessary, data were transformed to fit parametric assumptions.


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**Table 2. Phenolic Acids Content in Flesh and Skin of Fresh and Stored Tubers of Five Potato Varieties**[a]

| | metabolite | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CGA | | | CA | | | CouA | | | FA | | | total | |
| condition | flesh | skin | *R* | flesh | skin | *R* | flesh | skin | *R* | flesh | skin | *R* | flesh | skin |
| | | | | | | | Summerside | | | | | | | |
| fresh | 9.8 ± 1.1 (95.4) | 142.0 ± 15.4* (95.5) | 14.5 | 0.26 ± 0.04 (2.5) | 6.24 ± 1.27* (4.2) | 24.0 | 0.11 ± 0.03 (1.0) | 0.14 ± 0.04 (0.1) | 1.3 | 0.11 ± 0.02 (1.1) | 0.31 ± 0.09* (0.2) | 2.8 | 10.28 ± 1.19 | 148.69 ± 16.8 |
| stored | 5.6 ± 1.5 (95.1) | 94.7 ± 13.2* (94.7) | 17.0 | 0.15 ± 0.03 (2.6) | 4.88 ± 0.75* (4.9) | 32.5 | 0.05 ± 0.00 (0.9) | 0.18 ± 0.02* (0.2) | 3.5 | 0.09 ± 0.02 (1.5) | 0.20 ± 0.09 (0.2) | 2.2 | 5.89 ± 1.55 | 99.96 ± 14.06 |
| | | | | | | | Voyager | | | | | | | |
| fresh | 9.0 ± 1.5 (96.5) | 97.8 ± 10.1* (95.5) | 10.9 | 0.22 ± 0.09 (2.4) | 4.21 ± 0.11* (4.1) | 19.1 | 0.03 ± 0.00 (0.3) | 0.27 ± 0.10* (0.3) | 9.0 | 0.08 ± 0.02 (0.9) | 0.18 ± 0.03* (0.2) | 2.2 | 9.33 ± 1.61 | 102.46 ± 10.34 |
| stored | 5.3 ± 0.9 (92.9) | 62.0 ± 7.3* (96.0) | 11.6 | 0.22 ± 0.10 (3.8) | 1.73 ± 1.11 (2.7) | 7.8 | 0.11 ± 0.04 (1.9) | 0.07 ± 0.01 (0.1) | 0.7 | 0.08 ± 0.04 (1.4) | 0.81 ± 0.10* (1.3) | 9.7 | 5.71 ± 1.08 | 64.61 ± 8.52 |
| | | | | | | | Challenger | | | | | | | |
| fresh | 4.6 ± 0.1 (93.6) | 91.5 ± 14.7* (94.7) | 20.0 | 0.11 ± 0.03 (2.3) | 4.78 ± 1.10* (4.9) | 43.5 | 0.06 ± 0.01 (1.2) | 0.24 ± 0.03* (0.2) | 4.0 | 0.14 ± 0.03 (2.9) | 0.13 ± 0.05 (0.1) | 0.9 | 4.91 ± 0.17 | 96.65 ± 15.88 |
| stored | 4.4 ± 1.3 (93.4) | 57.2 ± 15.9* (94.4) | 12.9 | 0.07 ± 0.01 (1.5) | 3.00 ± 0.75* (5.0) | 42.9 | 0.05 ± 0.02 (1.1) | 0.04 ± 0.01 (0.1) | 0.8 | 0.19 ± 0.04 (4.1) | 0.36 ± 0.06* (0.6) | 1.8 | 4.71 ± 1.37 | 60.6 ± 16.72 |
| | | | | | | | Halifax | | | | | | | |
| fresh | 14.4 ± 2.1 (97.2) | 175.4 ± 24.9* (92.2) | 12.2 | 0.24 ± 0.00 (1.6) | 14.16 ± 1.51* (7.4) | 59.0 | 0.04 ± 0.01 (0.3) | 0.27 ± 0.07* (0.1) | 6.8 | 0.14 ± 0.02 (0.9) | 0.49 ± 0.14* (0.3) | 3.5 | 14.82 ± 2.13 | 190.32 ± 26.62 |
| stored | 16.5 ± 2.5 (96.8) | 175.7 ± 19.4* (96.1) | 10.6 | 0.15 ± 0.04 (0.9) | 6.8 ± 1.32* (3.7) | 44.3 | 0.28 ± 0.06 (1.6) | 0.10 ± 0.01* (0.1) | 0.4 | 0.11 ± 0.01 (0.6) | 0.20 ± 0.04 (0.1) | 1.9 | 17.04 ± 2.61 | 182.8 ± 20.77 |
| | | | | | | | Sagitta | | | | | | | |
| fresh | 5.9 ± 0.4 (93.0) | 118.4 ± 11.3* (89.6) | 20.1 | 0.18 ± 0.03 (2.8) | 12.86 ± 1.48* (9.7) | 71.4 | 0.08 ± 0.02 (1.2) | 0.56 ± 0.13* (0.4) | 7.4 | 0.19 ± 0.02 (3.0) | 0.27 ± 0.10 (0.2) | 1.4 | 6.35 ± 0.47 | 132.09 ± 13.01 |
| stored | 3.4 ± 0.4 (91.9) | 119.8 ± 34.2* (95.6) | 35.1 | 0.11 ± 0.02 (3.1) | 5.21 ± 1.97* (4.2) | 46.0 | 0.08 ± 0.02 (2.0) | 0.07 ± 0.01 (0.1) | 0.9 | 0.11 ± 0.03 (3.1) | 0.26 ± 0.05* (0.2) | 2.3 | 3.70 ± 0.47 | 125.34 ± 36.23 |

[a]Data represent the mean ± SD from three independent extractions. Metabolite levels were determined by HPLC-DAD and are expressed as $\mu g\ g^{-1}$ FW. Numbers in parentheses indicate the percentage of each metabolite with respect to the total. *R* indicates skin-to-flesh ratio. Abbreviations: CGA, chlorogenic acid; CA, caffeic acid; CouA, *p*-coumaric acid; FA, ferulic acid. Asterisk denotes significant differences between flesh and skin by Student's *t*-test ($p < 0.05$).



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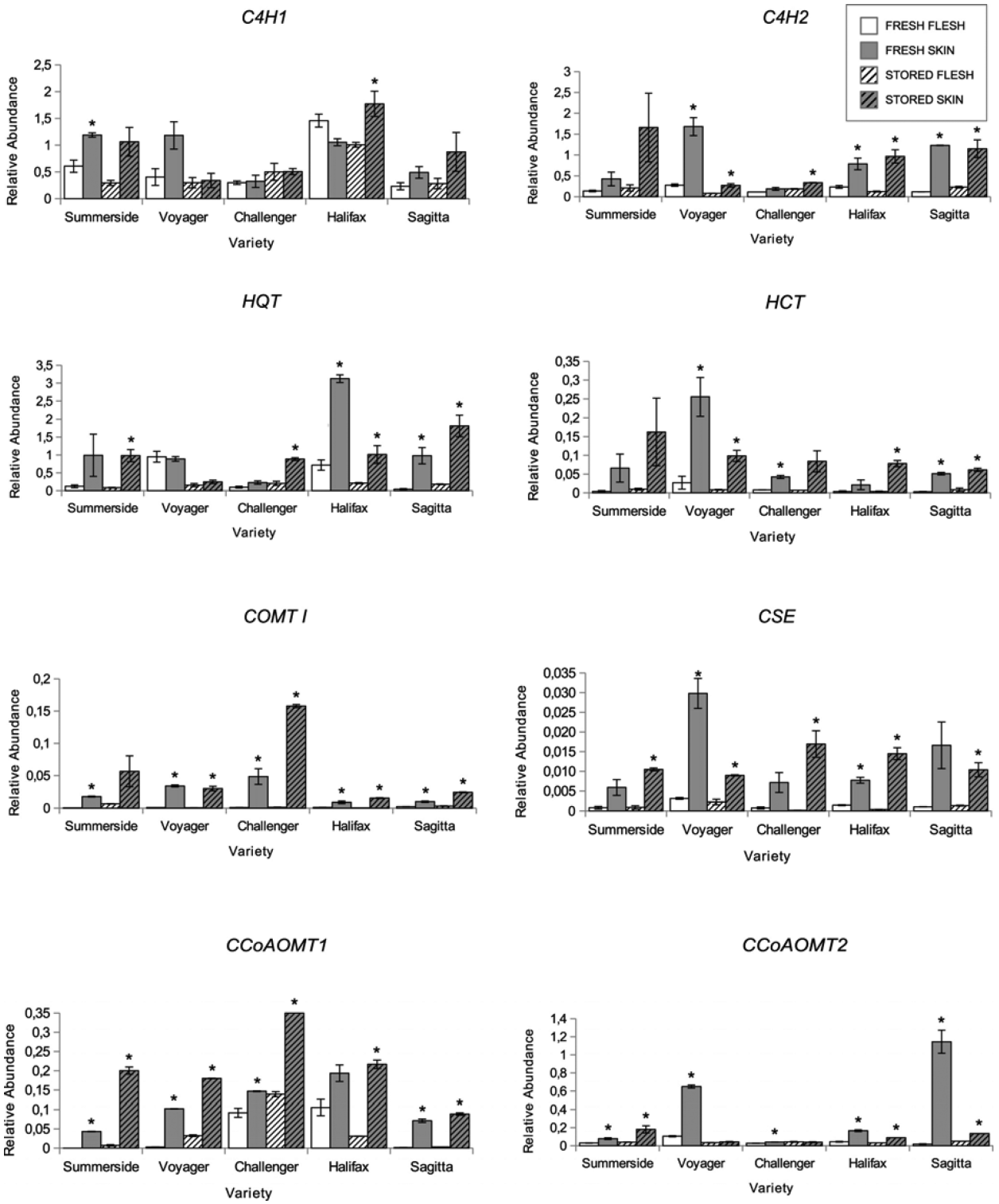


**Figure 3.** Transcript levels of phenolic acids' biosynthetic genes by qRT-PCR in flesh and skin of fresh and stored tubers from five selected processing potato varieties. qRT-PCR data represent the normalized means ± SD of two independent experiments with two technical replicates each. Asterisk denotes significant differences between flesh and skin by Student's *t* test ($p < 0.05$).

## RESULTS

**Selection of Potato Varieties with Different Phenolic Acids Profiles.** In order to study the aspects that determine the levels of CGA, it is necessary to have a group of potato varieties with different levels of CGA and related metabolites. Therefore, and since CGA is the major phenolic acid in potato tuber,[57] varieties with different contents of total phenolic acids were selected, taking into account differences in skin and flesh tissue content. Phenolic acids contents in flesh and skin of fresh tubers of 12 processing potato varieties were determined by means of the Folin−Ciocalteu reaction (Figure 2A). Total phenolic acids in flesh ranged from 220 (Challenger) to 800 (Spirit) $\mu$g CGA equivalents $g^{-1}$ FW (mean concentration 423.2 $\mu$g), whereas in skin the content varied between 650


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(Voyager) and 1550 (Halifax) $\mu$g CGA equivalents $g^{-1}$ FW (mean concentration 1035.6 $\mu$g). Total phenolics were 1.2 (Voyager) to 5 (Challenger) times more abundant in skin than in flesh (Figure 2A). Curiously, Spirit showed slightly higher phenolic acids levels in flesh than in skin.

Five potato varieties were selected for subsequent in-depth analysis on the basis of differences in their skin-to-flesh ratios. Briefly, Voyager shows a low, Summerside and Halifax an intermediate, and Challenger and Sagitta a high skin-to-flesh ratio.

Phenolic acids form a major source of hydrophilic antioxidants. In order to demonstrate the contribution of phenolic acids to antioxidant levels, the hydrophilic antioxidant activity was quantified in both tissues by the DPPH assay (Figure 2B). Antioxidant activity was 2.2 (Borden) to 5.6 (Halifax) times higher in skin than in flesh. Antioxidant activity in flesh ranged from 130 (Voyager) to 220 (Summerside) $\mu$g Trolox equivalents $g^{-1}$ FW (mean concentration 165.4 $\mu$g), while in skin it varied between 420 (Spirit) and 910 (Halifax) $\mu$g Trolox equivalents $g^{-1}$ FW (mean concentration 586.2 $\mu$g; Figure 2B). There is a weak positive correlation between total phenolic content and antioxidant activity in skin but not in flesh (data not shown). These results indicate that there are differences in either the phenolic acids or the non-phenolic acids antioxidant composition.

**Chlorogenic and Caffeic Acids Levels Are Higher in Skin than in Flesh and Largely Independent of Storage.** Because producing fresh potatoes throughout the year is unfeasible, storage is essential[58] but might affect phenolic acids content. Therefore, the levels of CGA, CA, CouA, FA, and cinnamic acid (CinA) were quantified via HPLC-DAD in flesh and skin of both fresh and 3 months stored tubers of the five selected potato varieties (Table 2).

Table 2 shows that CGA is the major phenolic acid in the skin and flesh of both fresh and stored potato tubers (89.6–97.2%). CA, CouA, and FA are minor components (0.1–9.7%), whereas CinA could not be detected by HPLC-DAD. In correspondence to what was found for total phenolics in the initial Folin–Ciocalteu screening, the abundance of most compounds was higher in skin than in flesh. For instance, CGA levels were 10.9 (Voyager) to 20.1 (Sagitta) times more abundant in skin than in flesh. Skin-to-flesh ratios of CA were even higher, reaching up to 71-fold in fresh tubers of the Sagitta variety. In general, CouA and FA exhibited minor differences among tissues, as reflected by their skin-to-flesh ratios. During storage, the levels of CGA and CA either diminished or did not alter significantly. The levels of CouA and FA fluctuated without a clear pattern (Table 2).

**CGA Synthesis Is Regulated at the Transcriptional Level in Potato Tuber and Is the Result of a Dynamic Balance between the Influx via C4H and HQT and the Outward Flux through CSE and HCT, Irrespective of the Effect of Storage.** With the aim to explore the molecular mechanisms that determine the levels of CGA and CA, a qRT-PCR transcript level analysis of key enzymes *C4H*, *HQT*, *HCT*, *CSE*, *COMT I*, and *CCoAOMT* was performed on the skin and flesh of both fresh and stored tubers of the five selected potato varieties. *CCoAOMT* appears to be encoded by two nearly identical (86%) genes, which were both evaluated. *C4H* shows two 98% identical genes, of which *C4H2* initially appeared to be a pseudogene. The genome sequence of *Solanum phureja*[59] shows that *C4H2* has a single nucleotide deletion, resulting in a reading frame shift. SNP analysis of publicly available EST data could not unequivocally show that *C4H2* is a pseudogene in all *S. tuberosum* varieties (data not shown). Part of the *C4H2* locus from the *S. phureja* variety that was sequenced by the Potato Genome Sequencing Consortium as well as two varieties used in our analysis was amplified and sequenced with locus-specific primers. Surprisingly, none of the obtained sequences demonstrates the single nucleotide deletion identified in the genome sequence (see Supporting Information, Figure S1). Hence, gene-specific primers for both *C4H* genes were designed and used in qRT-PCR analysis. *ACTIN* and cytoplasmic ribosomal protein *L2* transcript levels were used for normalization (Figure 3). In general, the results show that transcript levels were higher in skin than in flesh, in both fresh and stored tubers (Figure 3). Levels in stored and fresh tubers were of the same order of magnitude. Transcript levels of *C4H1*, *C4H2*, *HQT*, and *CCoAOMT2* were of the same order of magnitude, although *CCoAOMT2* levels showed larger fluctuations. *HCT* and *CCoAOMT1* levels were about 1–2 orders of magnitude lower (skin and flesh, respectively), whereas the lowest transcript levels were found for *CSE* and *COMT I* (Figure 3). Transcript levels of the genes involved in CGA synthesis were tissue- and condition-specific. Expression patterns of *C4H1*, *C4H2*, and *HQT* were similar in flesh of fresh tubers (Figure 3) and in accordance with the levels of CGA (Table 2) in all studied varieties except for Voyager. Voyager showed the highest expression levels of *C4H2* and *HQT*, but intermediate levels of *C4H1* and CGA (Figure 3 and Table 2). The transcript level of *HQT* reflects CGA levels in skin of fresh potato tubers in four of the five varieties (Figure 3 and Table 2). Nevertheless, Voyager has intermediate transcript levels of *HQT* but lower CGA levels.

A Pearson correlation analysis was performed in order to quantify associations between metabolite and transcript levels. Figure 4 shows the correlation between fresh flesh transcript and metabolite levels as well as fresh skin metabolite levels of four of the five initially selected varieties. The Voyager data were not included since this cultivar exhibits a different behavior which might hinder detecting otherwise obvious trends. The complete set of analysis of all four conditions of the four selected varieties can be found in the Supporting Information, Figure S2.

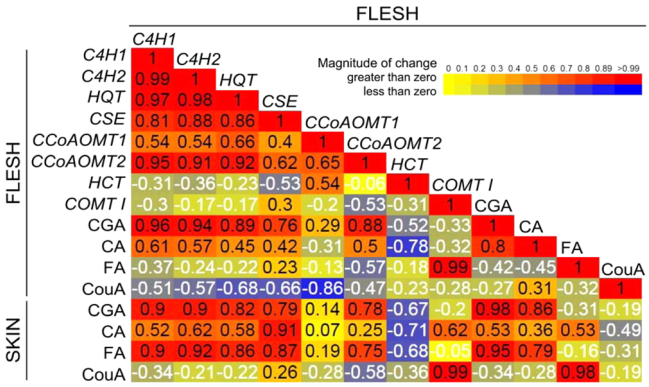


**Figure 4.** Correlation analysis of transcript and metabolite levels of fresh tubers from four selected processing potato varieties. Pearson correlation coefficients were calculated using the means of metabolite concentrations from fresh flesh and/or fresh skin and/or relative gene expression values from fresh flesh and used to generate a heatmap. Abbreviations are listed in Figure 1 and Table 2.



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In fresh flesh, transcript levels of *C4H1*, *C4H2*, *HQT*, *CSE*, and *CCoAOMT2* show mutual positive correlations and exhibit positive correlations with the major metabolites CGA and, to a lesser extent, CA. In contrast, *HCT* and *COMT I* show an almost consistent negative correlation with all other transcripts and most metabolites. An important exception to the latter is the mutual positive correlation between *COMT I* and *CSE* transcripts and FA, the metabolite downstream of the consecutive action of the corresponding enzymes. *CCoAOMT1* shows lower *r* values than *CCoAOMT2* and exhibits a positive correlation with *HCT* (Figure 4).

The metabolite levels in skin are higher than in flesh, in correspondence with transcript levels. Nevertheless, Figure 4 also shows the correlation between fresh flesh transcript and metabolite levels with fresh skin metabolite levels in order to analyze putative metabolite transport. A high correlation between CGA levels in skin and flesh ($r$ = 0.98; Figure 4) suggests metabolite contribution from flesh toward skin. Accordingly, flesh *C4H1*, *CH42*, and *HQT* transcript levels correlate well with skin CGA levels ($r$ = 0.90, 0.90, and 0.82, respectively). Furthermore, skin FA levels show a better correlation with flesh than with skin *C4H* transcript levels (Figures 4 and S2; also see Discussion section).

## DISCUSSION

The present study was directed at the elucidation of the biosynthesis of the dietary antioxidant CGA in the staple crop potato. Metabolite and transcript levels in flesh and skin from fresh and stored tubers from five carefully selected potato varieties were determined. The initial selection of five from 12 varieties was made on the basis of total phenolic acids contents determined by Folin−Ciocalteu assay. Total phenolic acids in flesh were in accordance with reports for white- or yellow-fleshed cultivars by Al-Saikhan et al.[60] and Reddivari et al.[29] Antioxidant activity in flesh was similar to or slightly lower than that in previous reports.[28,61] As with total phenolic acids content, antioxidant activity was higher in skin than in flesh and correlated well with it in skin but not in flesh. Differences in the composition of phenolics and other antioxidants present in methanol extracts may cause the lack of correlation in flesh. It has been shown that CA has higher antioxidant activity than CGA[28] and vitamin C is an abundant, methanol-soluble antioxidant in potato.[62]

The Folin−Ciocalteu assay showed higher values for total phenolic contents than the sum of all the individual phenolic compounds quantified by HPLC-DAD. These results fit with those obtained by other groups[33,63] and can be explained by the fact that compounds like thiol derivatives, vitamins, and amino acids may interact with the Folin−Ciocalteu assay.[64] A strong correlation was found between the results obtained by the two different methods ($r$ = 0.93 for flesh, $r$ = 0.83 for skin), as previously reported by André et al.,[27] even though fold differences between samples appear less clear in the Folin−Ciocalteu assay. This supports the idea that the Folin−Ciocalteu assay remains a rapid and economic alternative for comparative purposes.

**CGA and CA Levels Are Significantly Higher in Skin Tissue.** CGA is the predominant phenolic acid in both skin and flesh. CA contributes significantly, around 1−10%, whereas other phenolic acids contribute little to the total phenolic content. This is in agreement with other reports that show CGA is the most abundant phenolic acid in potatoes, followed by CA,[27,32,33,42,65] with CouA and FA being the least abundant.[33,66] We could not identify CinA in skin nor in flesh. CinA was earlier identified only in tuber skin.[25]

Both CGA and CA levels are 10−70 times higher in skin than in flesh. Similar results were obtained by Im et al.[30] in skin and flesh of five commercial potato varieties. Other previous data indicated that phenolic compounds are found mostly between the cortex and potato skin[67] or in the potato skin and adjoining tissues, with decreasing concentrations toward the center of potato tubers.[68] This is likely related to suberin deposition in skin tissue during tuber growth.[69]

During storage, the levels of CGA and CA diminished or remained unaffected. Conversely, Madiwale et al.[61] reported that 90 days of storage at 4 °C led to a 1.3- to 4-fold increase of CGA and CA content. These differences might be attributed either to cultivar-dependent storage responses or to storage conditions.

**Transcript and Metabolite Levels Are Highly Correlated.** Transcript levels of phenolic acids biosynthetic genes were also, in general, higher in skin than in flesh (see Figure 3). The good correspondence between transcript and metabolite levels suggests that the control of gene expression is essential in the control of CGA biosynthesis and that the enzymes are coordinately regulated, at least in part, at the transcriptional level. This confirms earlier work[14−21] and is fundamental in the use of Pearson correlation analysis for metabolic modeling. Interestingly, *HCT* shows a consistent negative correlation, whereas *COMT I* shows a prevalent negative correlation with most metabolites, FA forming the obvious exception (Figure 4).

Of particular interest is C4H, encoded by two genes. The close proximity of the two genes in the *S. phureja* genome combined with the high sequence identity suggests that these genes are under concerted evolution.[70] Although the genome sequence of *S. phureja* shows *C4H2* has a fatal deletion, resequencing of the locus amplified by locus-specific primers indicates that *C4H2* is a functional gene (see Figure S1). André et al.[22] used indiscriminative primers, which might explain the lack of positive correlation between *C4H* transcript and CGA levels they demonstrated. The consistent positive correlation of the *C4H* transcripts corresponds with a major role for the sink in the metabolism of both CA and CGA. *HQT* transcripts correspond very well with CGA, which indicates that HQT is the principal enzyme for CGA accumulation. This is in correspondence with results found in tomato leaves, where overexpression of *HQT* increased CGA content, whereas *HQT* silencing caused a reduction but had no detectable effects on lignin accumulation.[8] An in-depth study of *HCT*-silenced *Nicotiana benthamiana* plants revealed that lignin biosynthesis depends on HCT activity *in vivo*.[12] In correspondence, we found that CGA shows a negative correlation with *HCT* transcript levels in all studied conditions (Figure S2).

**Toward a Model for CGA and CA Metabolism.** CGA is the major phenolic acid in potato tuber, and its biosynthesis is very well explained by the transcript levels of its structural genes, as shown in Figure 4. We did not analyze transcript levels of structural genes of the flavonoid pathway, but previously CGA was shown to correlate better with flavonoid biosynthetic enzymes than with *HQT* transcript levels.[22,23] Thus, phenolic acid and flavonoid metabolism are linked, under direct transcriptional control, and must be subject to metabolic regulation. Using the Pearson correlation analysis shown in Figure 4, we developed an initial model of CGA and CA biosynthesis that appears to be driven by both the source CouA


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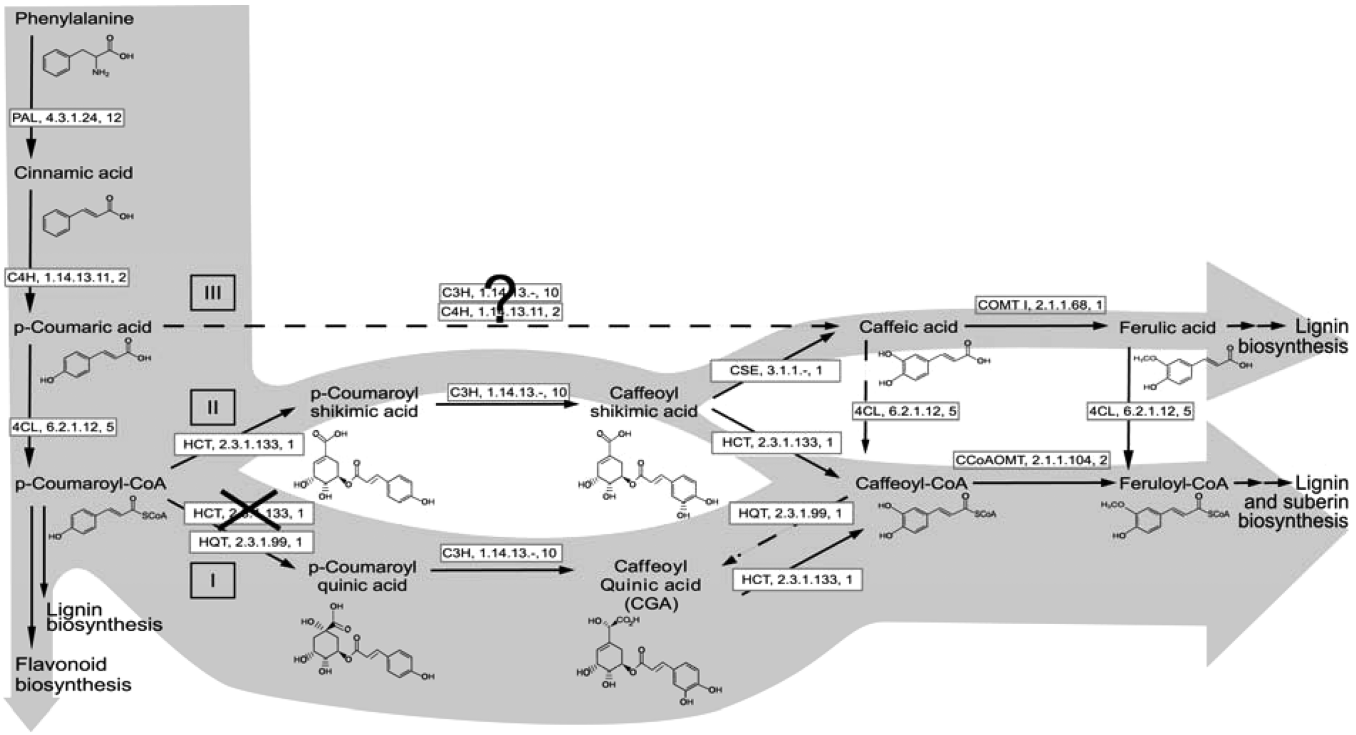


**Figure 5.** Proposed flux model of biochemical pathways leading to CGA and CA production in potato tuber. Dashed lines, question mark, and cross indicate unlikely routes, uncertain enzyme complexes, and unfeasible reaction, respectively. Enzyme names are listed in Figure 1.

and the sink suberin (Figure 5). The model explains the correlation between CA and CGA ($r = 0.8$, see Figure 4) by the high correlation of both metabolites with *C4H* transcript levels and, hence, the sink via CouA. Then, the positive correlation between *CSE* and CA indicates route II is actively involved in CA biosynthesis. Note that HCT and CSE will compete for substrate, which contributes to the negative correlation of *HCT* with CA, which can further be explained by additional or alternative CA formation via route III. Although, theoretically, CGA biosynthesis could occur via CA and caffeoyl-CoA, the negative correlation of *HCT* points toward a role for HCT in CGA remobilization toward lignin and suberin. Thus, route I would be the major route, and HQT is the major enzyme in CGA biosynthesis. Note also that *HCT* transcript levels are consistently low in flesh and show up to ~18-fold induction in skin, where suberin is deposited in the primary cell wall. This suggests that CGA is transported to the skin. The positive correlation of *CCoAOMT*, required for feruloyl-CoA and downstream suberin, and the negative correlation of *COMT I*, required for FA and downstream lignin, also suggest a central role for suberin deposition in potato tuber. It should be mentioned that, given the complexity of the phenylpropanoid, lignin, and suberin biosynthetic pathways, this merely a trend, and other fluxes can and likely will occur.

The model was based on the fresh flesh data set but also explains the fresh skin data set quite well, albeit that the correlation values are not so high. This could be caused by metabolite transport, suggested by the correlations between flesh transcript and skin metabolite levels (Figure 4). Such metabolite transport would have a minor effect on the voluminous flesh tissue but a major effect on skin metabolite levels. On that proposition, the skin data are not reliable resources for model construction. Then, the stored tuber data sets shows less clear patterns. Nevertheless, the stored flesh data correspond with the model. *C4H2*, *HCT*, *CSE*, and *COMT I* correlate well to each other. On the other hand, *C4H1*, *HQT*, and CGA correspond well, whereas *CCoAOMT* appears to have an inconsistent correlation. Within the proposed model, this would mean that, upon storage, there is more emphasis on lignin production, or less emphasis on suberin production. This is not reflected in the levels of CA, CGA, and FA but might be reflected in the levels of caffeoyl-CoA and feruloyl-CoA. It should be mentioned that the model corresponds with poor rather than negative correlations of *HCT*. However, in all likelihood, no model can account for the consistent negative correlation of *HCT*. Then, the model was made on the basis of an incomplete data set since we ignored the Voyager data set in the Pearson correlation analysis, this in order to more easily detect the major trends. However, the Voyager data set does actually also correspond quite well with the model. An increased flux toward lignin and suberin, reflected by the highest levels of *CSE* and *HCT* observed in fresh tubers of Voyager variety, would explain why even though it showed high expression levels of *C4H* and *HQT*, it has intermediate levels of CGA. In other words, CGA levels are the result of a dynamic balance between the influx via C4H and HQT and the outward flux through CSE and HCT.

The model in Figure 5 should be seen as an initial model that requires both substantiation and elaboration. The model is clearly based on the oversimplification that transcript levels reflect metabolite levels and that no post-translational regulation is involved. Hence, for instance, differences in enzyme kinetics between various cultivars cannot be captured by such a simple model but would require a more delicate systems biology approach. The same should be mentioned regarding the proposed metabolite transport and the Voyager data set, which complicate Pearson correlation analysis but can readily be included in more delicate and elaborate analyses such


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as flux balance analysis, thereby contributing to an improved, possibly even semiquantitative model.

## ■ ASSOCIATED CONTENT

### Supporting Information

Figure S1, showing multiple sequence alignment of the *C4H2* subsequences surrounding the nucleotide deletion reported in the genome sequence of *S. tuberosum* group *phureja* (2_PGSC_Sp); Figure S2, showing correlation analysis of transcript and metabolite levels in tubers from four selected processing potato varieties. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jf505777p.

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS USED

qRT-PCR, quantitative real-time polymerase chain reaction; HPLC-DAD, high-performance liquid chromatography with diode-array detection; CGA, chlorogenic acid; CA, caffeic acid; CoA, coenzyme A; CouA, *p*-coumaric acid; FA, ferulic acid; CinA, cinnamic acid; PAL, phenylalanine-ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; HCT, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyltransferase; C3H, *p*-coumarate 3′-hydroxylase; HQT, hydroxycinnamoyl-CoA quinate hydroxycinnamoyltransferase; CSE, caffeoyl shikimate esterase; COMT I, caffeic/5-hydroxyferulic acid *O*-methyltransferase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase

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