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1 **Down-regulation of cinnamoyl-CoA reductase in tomato (*Lycopersicon***
2 ***esculentum* Mill.) induces dramatic changes in soluble phenolic pools**

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4 Benoît van der Rest, Saïda Danoun, Alain-Michel Boudet, Soizic F Rochange

5
6 UMR 5546 CNRS-Université Paul Sabatier "Surfaces Cellulaires et Signalisation chez les
7 Végétaux", Pôle de Biotechnologie Végétale, 24 chemin de Borderouge, BP 42617, F-31326
8 Castanet, France

9
10 Author to whom correspondence should be sent:

11 Soizic Rochange

12 UMR UPS/CNRS 5546, Pôle de Biotechnologie Végétale, 24 chemin de Borderouge,
13 BP42617, F-31326 Castanet, France

14 Tel: +33 562193531

15 Fax: +33 562193502

16 E-mail: rochange@scsv.ups-tlse.fr

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21
22 Key words : antioxidant, cinnamoyl-CoA reductase, flavonoid, hydroxycinnamate, lignin,
23 *Lycopersicon esculentum*, phenolic, tomato.

24
25 **Abbreviations**

26 CCR: cinnamoyl-CoA reductase; ESI: ElectroSpray Ionization; ABTS: 2,2'-azinobis(3-
27 ethylbenzothiazoline-6-sulfonate); Trolox: 6-hydroxy-2,5,7,8-tetramethylchroman-2-
28 carboxylic acid.

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ABSTRACT

Health-beneficial properties of many plant secondary metabolites have driven much interest into the control of their biosynthesis in crop species. Phenolic compounds, including flavonoids, hydroxycinnamates and tannins, make up an important group of such phytonutrients. They are formed via the phenylpropanoid pathway and share common precursors with lignin, an insoluble cell wall-associated polymer. In this study, we aimed at reducing lignin biosynthesis to enhance availability of these precursors and thereby stimulate the production of soluble, potentially health-promoting, phenolic compounds in tomato (*Lycopersicon esculentum* Mill.).

We first identified and characterized two tomato genes encoding cinnamoyl-CoA reductase (CCR), a key enzyme in the formation of lignin monomers. Transgenic plants exhibiting a reduced lignin content were subsequently obtained through an RNAi strategy targeting one of these genes. As anticipated, the total level of soluble phenolics was higher in stems and leaves of the transformants as compared to control plants. This was correlated with an increased antioxidant capacity of the corresponding plant extracts. Analysis of the soluble phenolic fraction by HPLC-MS revealed that vegetative organs of CCR down-regulated plants contained higher amounts of chlorogenic acid and rutin, and accumulated new metabolites undetectable in the wild type, such as N-caffeoyl putrescine and kaempferol rutinoside. In fruits, CCR down-regulation triggered the moderate accumulation of two new compounds in the flesh, but the total phenolic content was not affected. Although the prospects of exploiting such a strategy for crop improvement are limited, our results provide further insight into the control of the phenylpropanoid pathway in Solanaceae.

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INTRODUCTION

There is growing awareness that a diet rich in fruit and vegetables contributes to the prevention of chronic diseases. Evidence coming from epidemiology, feeding studies and *in vitro* analyses suggests that particular classes of phytonutrients exert protective effects against cancer or cardiovascular disease, for example (Liu, 2004). Among putative beneficial phytonutrients, ascorbic acid (vitamin C), tocopherol (vitamin E), carotenoids and phenolics (including flavonoids, hydroxycinnamates, lignans *etc*) are found at high levels in a wide variety of plants. This makes it difficult to assign a protective role to a particular compound, and the issue is further complicated by the extensive modifications undergone by these molecules prior to and during human absorption (Rice-Evans *et al.*, 2000; Stahl *et al.*, 2002). Likewise, the molecular mechanisms underlying these health-promoting effects are still largely unknown. For example, the beneficial effects of many phytonutrients have long been attributed to their antioxidant properties, but evidence is gathering that some antioxidant compounds such as flavonoids may in fact exert their effect by interfering with signalling pathways (Williams *et al.*, 2004). Nevertheless the interest for such compounds and their occurrence in the diet is growing.

In the past decade, there have been several successful attempts to increase the content of phytonutrients in crop species, as providing plant products enriched in certain compounds is considered more efficient than the use of food supplements (Cooper, 2004). Biotechnology is often considered the method of choice for the improvement of such characters, although traditional breeding can offer an interesting alternative by recruiting favourable genes/alleles from wild species closely related to crop plants (Willits *et al.*, 2005). Recent achievements in the design of crops with added nutritional value include enhanced vitamin content in various crops through metabolic engineering (reviewed by Herbers, 2003), production of tomatoes with high flavonol content following overexpression of two key transcription factors (Bovy *et al.*, 2002), and the production of phytoestrogens in non-legume species by expression of a heterologous isoflavone synthase gene (Yu *et al.*, 2000). Recently, Davuluri *et al.* (2005)

81 reported enhancement of both carotenoid and flavonoid content in tomato via silencing of the
82 transcription factor DET1.

83 These approaches usually involve modifying the expression of key enzymes catalyzing the
84 synthesis of target compounds (e.g. chalcone isomerase for the production of flavonols; Muir
85 *et al.*, 2001), and/or of transcription factors that control the expression of these enzymes. The
86 latter approach is often more efficient as whole biosynthetic pathways can be controlled by a
87 small number of transcription factors (Borevitz *et al.*, 2000; Bovy *et al.*, 2002). In contrast,
88 little is known about the possibility to enhance availability of precursors involved in the
89 synthesis of the requested molecule(s) by rerouting metabolic flow.

90 Lignin represents a major carbon sink in vascular plants, and is built from three main
91 monomers termed monolignols. The biosynthesis of monolignols involves two specific steps
92 branching off the general phenylpropanoid pathway. Therefore, decreasing lignin synthesis
93 should make more precursors available for the synthesis of other phenolic compounds. Down-
94 regulating cinnamoyl-CoA reductase (CCR, EC 1.2.1.44), the first committed enzyme of the
95 monolignol biosynthetic pathway (Lacombe *et al.*, 1997), appears to be one of the most
96 efficient ways to decrease lignin biosynthesis (Piquemal *et al.*, 1998; Anterola and Lewis,
97 2002). Limiting carbon flow down the monolignol pathway should enhance availability of
98 coumaroyl-CoA esters, which are some of the substrates of chalcone synthase (CHS), the first
99 catalytic step towards flavonoid synthesis.

100 Tomato is one of the preferred targets for metabolic engineering because it is widely
101 consumed in many Western countries, which makes it one of the principal sources of
102 phytonutrients (Canene-Adams *et al.*, 2005), and it is amenable to biotechnological
103 modifications. In tomato fruits, the main carotenoid is lycopene. Phenolics are represented by
104 a wide variety of compounds, including chlorogenic acid isomers (Niggeweg *et al.*, 2004).
105 Flavonoids, mainly represented by naringenin chalcone and rutin, are found in the peel in
106 relatively small amounts (Bovy *et al.*, 2002).

107 In this paper, we report our experiments aiming to reorientate the carbon flow from lignin
108 formation to the synthesis of soluble phenolic compounds in transgenic tomato plants. We

109 show that down-regulating CCR through an RNAi strategy leads to important quantitative and
110 qualitative changes in the soluble phenolic content of extracts from fruits and vegetative
111 organs. In stems and leaves, the observed modifications are strongly correlated with an
112 increase of antioxidant capacity. Factors that limit the applicability of this approach for crop
113 improvement are discussed.

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MATERIALS AND METHODS

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Plant material and culture conditions

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All experiments were performed on *Lycopersicon esculentum* Mill. cv. Microtom, a miniature
122 tomato cultivar commonly used as a model system (Meissner *et al.*, 1997). Plants were grown
123 in soil in a culture room (16h/8h light/dark regime, 25°C day/22°C night, with 80% humidity)
124 unless stated otherwise.

125

126

Cloning of *LeCCR1* and *LeCCR2*

127

Total RNA was extracted from stems, flowers and leaves using Extract'All reagent (Eurobio,
128 France) according to the manufacturer's instructions, and cDNAs were obtained by reverse
129 transcription using an oligo-dT primer. The coding sequence for *LeCCR2* was amplified by
130 PCR on the basis of the EST sequences found in the TIGR database (<http://www.tigr.org>). The
131 3' end of *LeCCR1* was amplified using an oligo-dT primer and a primer derived from the
132 partial coding sequence deduced from ESTs. Following sequencing of the 3' end, the whole
133 coding sequence of *LeCCR1* could be amplified. Full-length amplification products for both
134 *LeCCR1* and *LeCCR2* were sequenced, and the sequences were deposited into GenBank under
135 accession numbers DQ019125 and DQ019126, respectively.

136

137 **RNAi construct**

138 An expression cassette comprising the CaMV 35S promoter and CaMV polyadenylation
139 signal was introduced into pGreen0029 binary vector (Hellens *et al.*, 2000). A 220-bp intron
140 (Vancanneyt *et al.*, 1990) was then inserted between the promoter and the polyadenylation
141 signal. The resulting plasmid, pSR02, allows the generation of RNAi constructs designed to
142 produce self-complementary RNA which will be targeted for degradation.

143 A 420 bp fragment corresponding to the 5' region of *LeCCR1* cDNA was amplified by PCR
144 using upstream primer ^{5'}GTCGTCTGTGTTACCGGTG^{3'} and downstream primer
145 ^{5'}ATCAGGATCGCTCCAGCA^{3'}. It was then introduced upstream and downstream of the
146 intron in pSR02 in the antisense and sense orientations, respectively. The resulting vector,
147 pVDR01, was introduced by the freeze-thaw method (Holsters *et al.*, 1978) into
148 *Agrobacterium tumefaciens* strain LBA4404 containing pSoup (Hellens *et al.*, 2000).

149
150 **Plant transformation**

151 Tomato transformation through *Agrobacterium tumefaciens* with vectors pSR02 and pVDR01
152 was performed as described in Ling *et al.* (1998). Rooted primary (T0) transformants were
153 transferred to soil, grown in the greenhouse and allowed to self-pollinate. Non-fertile
154 transgenic plants were maintained by vegetative propagation: stem cuttings were dipped in
155 Rootone F[®] (Bayer) hormone rooting powder and transferred to soil.

156
157 **Recombinant proteins**

158 Full-length coding sequences for *LeCCR1* and *LeCCR2* were amplified from mixed leaf and
159 stem cDNAs. The PCR products were cloned into pGEX 4T-1 vector (Amersham), to obtain
160 GST-CCR fusion proteins. These constructs were then introduced into *Escherichia coli* strain
161 BL21 (Invitrogen). Bacteria were grown in liquid LB medium supplemented with 50 mg L⁻¹
162 ampicillin for a few hours at 37°C, until OD reached 0.5. Production of recombinant protein
163 was then induced by 0.2 mM IPTG, at 23°C for 3 h. Bacterial cells were harvested by
164 centrifugation, resuspended in PBS buffer and lysed by sonication. The supernatant (crude

165 extract) containing soluble recombinant protein was collected after centrifugation at 10000 g.
166 Recombinant proteins were purified on a glutathione resin (Amersham) according to the
167 manufacturer's instructions.
168 Activity of purified recombinant proteins was measured spectrophotometrically according to
169 Wengenmayer *et al.* (1976) using 2 µg of purified recombinant protein for each test. The
170 reaction was carried out in 100 mM sodium citrate buffer pH 6.2 with 70 µM
171 hydroxycinnamoyl-CoA and 40 µM NADPH. The activity was deduced from the decrease of
172 absorbance corresponding to the transformation of hydroxycinnamoyl-CoA (for feruloyl-CoA:
173 $\epsilon_{366} = 13.0 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) into its corresponding hydroxycinnamaldehyde (for coniferaldehyde:
174 $\epsilon_{366} = 10.0 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) and to the oxidation of NADPH ($\epsilon_{366} = 3.0 \text{ mM}^{-1} \cdot \text{cm}^{-1}$). The four
175 substrates: feruloyl-CoA, sinapoyl-CoA, caffeoyl-CoA and coumaroyl-CoA, were synthesized
176 according to the method of Stöckigt and Zenk (1975).

177

178 **RNA gel blot analysis**

179 Total RNA was extracted from plant samples using Extract'All reagent (Eurobio, France). Ten
180 µg RNA of each sample was run on a formaldehyde denaturing gel and blotted onto a nylon
181 membrane (Hybond N+, Schleicher and Schuell). Blots were hybridized according to Church
182 and Gilbert (1984) with a $\alpha^{32}\text{P}$ -labelled DNA probe corresponding to the 200 bp 3'-end of
183 *LeCCR1* coding sequence.

184

185 **Histochemistry**

186 Stem transverse sections obtained with a vibratome (100 µm thick for phloroglucinol, 200 µm
187 thick for autofluorescence) and fruit longitudinal sections were cleared by overnight
188 incubation in 95% ethanol and were either mounted directly on a glass slide for fluorescence
189 microscopy or stained for 30 minutes with phloroglucinol-HCl reagent (Prolabo, France).
190 Sections were observed using an inverted microscope (Leitz DMIRBE, Leica, Heidelberg,
191 Germany) as described by Chabannes *et al.* (2001).

192

193 **Determination of lignin content**

194 Whole stems were harvested from three-month-old wild-type and transgenic plants, frozen in
195 liquid nitrogen and freeze-dried. Cell wall residue (CWR) was prepared as described
196 previously (Chabannes *et al.*, 2001), and Klason lignin content was determined using a micro-
197 Klason technique (Whiting *et al.*, 1981). Two plants from each line were pooled for CWR
198 extraction, and measurements were performed in triplicate for each CWR sample.

199

200 **HPLC analysis of phenolic compounds**

201 When the first fruits reached maturity, leaves, stems and fruits at different ripening stages
202 were collected, ground in liquid nitrogen and freeze-dried. Tissue samples were taken from at
203 least two individual plants from each line and extracted separately. Soluble phenolics were
204 extracted from 50 µg of lyophilized powder by stirring the sample three times in 500 µL of
205 methanol/water (2:1, v/v), so that the final extract volume was 1.5 mL. Prior to injection, a 50
206 µL aliquot of each extract was rotary evaporated under vacuum and subsequently resuspended
207 in the same volume of water/methanol (4:1; v/v). For each run, 20 µL of sample was injected
208 into the HPLC column. HPLC separation was performed in duplicate for each extract.

209 HPLC analysis was carried out on a Spectra Physics system with an SP8800 ternary pump.
210 UV spectra from 240 to 380 nm were monitored with a UV-3000 (Spectra-system) detector.
211 Analyses were performed using a 250 x 4.6 mm RP-C₁₈ Nova-Pak column (Waters). Mobile
212 phase A consisted of water/methanol/5 M HCl (80:20:0.1; v/v/v) and solvent B was
213 acetonitrile. The following linear gradient was run at a flow rate of 0.6 mL.min⁻¹ for 60 min:
214 95A:5B from 0-10 min, decreasing linearly to 50A:50B by 35 min, held for a further 5 min
215 and back to 95A:5B by 45 min. The last 15 min (45-60 min) at 95A:5B ensured re-
216 equilibration of the column. Compound quantification was based on the area under peak,
217 determined at 320 nm.

218

219 **LC-MS and LC-MS/MS characterization**

220 HPLC separation was carried out with an Agilent 1100 system with detection at 320 nm. The
221 C₁₈ Nova-Pak (Waters) column and the mobile phase were identical to those used for HPLC-
222 UV analysis with the following exceptions: 0.05% aqueous formic acid was substituted for
223 0.1% HCl 5M in the mobile phase A and the volume of sample injected was reduced to 5 µL.
224 Capillary voltage was set at 4200 V and source temperature at 400°C. Atmospheric pressure
225 ionization mass spectrometry analysis was performed on a Q-Trap mass spectrometer operated
226 using the electrospray ionization interface in the negative mode (ESI⁻), and the data were
227 analysed using Analyst 1.4 software (Applied Biosystems). For LC-MS-MS, selected ions
228 were fragmented using a collision energy of -25 V, except compound 6 which was fragmented
229 at -15 V. Mass spectrometric data were acquired in the full scan mode over the *m/z* 150-800
230 range. Sensitivity of the mass spectrometer was optimized using a chlorogenic acid standard
231 (Acros) in the 150-400 range and a quercetin-rutinoside standard (Extrasynthese, France) in
232 the 400-800 range.

233

234 **Total phenolic content and antioxidant capacity**

235 Methanol/water extracts (2:1; v/v) obtained as described previously were evaporated under
236 vacuum, the dry residue was resuspended in the same volume of water and centrifuged for 2
237 min at 12000g. Total phenolic content was estimated by the Folin-Ciocalteu method adapted
238 from Dicko *et al.* (2002). Assays were performed in duplicate for each extract by mixing 20 µ
239 L supernatant, 20 µL water and 100 µL 50 % Folin-Ciocalteu's reagent. After 5 min
240 incubation at room temperature, 100 µL of 20% (w/v) Na₂CO₃ and 560 µL water were added.
241 The mixture was incubated for a further 30 min at room temperature and absorption at 720 nm
242 was determined using a Cary100-UV spectrophotometer (Varian, Australia). A calibration
243 curve was obtained with freshly prepared solutions of gallic acid. Results were calculated as µ
244 g gallic acid equivalents per mL extract.

245 Antioxidant capacity was determined in the same extracts by a TEAC assay, based on the
246 capacity of samples to scavenge 2,2'-azinobis(3-ethylbenzothiozoline-6-sulfonate) (ABTS^{•+})
247 radical cations. Measurements were performed in triplicate for each extract as described by

248 Braca *et al.* (2003). Antioxidant capacity was determined relative to the reactivity of Trolox
249 (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Aldrich) as a standard, and
250 expressed as nmol Trolox equivalent per μL extract.

251 For both measurements (total phenolic content and antioxidant capacity), each extract
252 represented an individual plant from one of the lines, and the whole experiment was repeated
253 with similar results.

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RESULTS

Cloning of two tomato CCR genes and characterization of recombinant proteins

The aim of this study was to reduce lignin biosynthesis in tomato plants through down-regulation of CCR activity, and to evaluate subsequent impacts on phenylpropanoid metabolism. The first gene encoding CCR was cloned from *Eucalyptus gunnii* (Lacombe *et al.*, 1997). Since then, a number of CCR genes have been identified in dicot as well as monocot species (reviewed by Boudet *et al.*, 2004). Despite the existence of a large set of sequences annotated as "CCR-like proteins" (Raes *et al.*, 2003), *bona fide* CCR enzymes seem to be encoded by a small number of closely related genes: so far, no more than two genes have been demonstrated experimentally to encode CCR in one species. The *E. gunnii* CCR sequence was used to search the TIGR EST database (<http://tigrblast.tigr.org>) for tomato homologs. This search yielded two contigs with a very high homology to the target sequence (80 % identity at the amino acid level), plus a few other contigs that were less similar (45 to 48% identity). The two sequences with highest homology to *E. gunnii* CCR were named *LeCCR1* and *LeCCR2*. While *LeCCR2* likely corresponded to a full-length CCR sequence, *LeCCR1* lacked the 3' end, which we cloned by a 3'RACE approach. Complete reconstituted sequences of *LeCCR1* and *LeCCR2* were used to design oligonucleotide primers at the 3' and 5' ends. Full-length cDNAs corresponding to both genes were then amplified by RT-PCR. Their exact sequences were determined and deposited in Genbank under accession numbers DQ019125 and DQ019126. Both coding sequences are 999 bp long, and encode proteins with a predicted molecular mass of 36.8 kDa. They share 83% and 87% identity at the nucleotide and amino-acid levels, respectively. By comparison with CCR genes from other plant species, we can conclude that *LeCCR1* and *LeCCR2* are closer to each other than to any other CCR gene (data not shown).

Previous studies have shown that one of the two CCR genes is usually predominant in lignin biosynthesis, and that down-regulating that gene can efficiently trigger a decrease in lignin content (Lauvergeat *et al.*, 2001; Jones *et al.*, 2001; Goujon *et al.*, 2003). Therefore, we

283 wanted to investigate which of the newly cloned tomato CCR genes would be more
284 appropriate as a silencing target. Towards this end, recombinant GST-fusion proteins were
285 produced in *Escherichia coli* and purified to >90% homogeneity, as estimated by SDS-PAGE
286 (data not shown). Specific activities of purified, soluble fusion proteins were determined for
287 four potential CCR substrates: feruloyl-CoA, sinapoyl-CoA, caffeoyl-CoA and coumaroyl-
288 CoA (Table 1). These results clearly show that LeCCR1 is able to use the four substrates albeit
289 with a strong preference for feruloyl-CoA, while LeCCR2 only uses feruloyl-CoA, with a
290 lower efficiency than LeCCR1. Data obtained on recombinant proteins are likely to reflect the
291 properties of the corresponding native proteins, as was shown for *E. gunnii* CCR enzymes
292 (Lacombe *et al.*, 1997).

293 Expression patterns for *LeCCR1* and *LeCCR2* were examined by RT-PCR. While
294 *LeCCR2* was found to be expressed only in roots and stems, *LeCCR1* was expressed in stems,
295 petioles, leaves and fruits at the expanding, green and red stages (data not shown). These
296 observations combined with the higher CCR activity exhibited by LeCCR1 recombinant
297 protein led us to select this gene as a target for RNAi silencing.

298

299 **Phenotype of CCR down-regulated tomato plants**

300

301 *Generation of transgenic lines*

302 In order to obtain RNAi-mediated silencing of *LeCCR1*, a construct designed to produce
303 dsRNA under the control of the Cauliflower Mosaic Virus (CaMV) 35S promoter was
304 introduced into tomato plants through *Agrobacterium tumefaciens*. Sixteen independent
305 transformants were regenerated. Three of them were selected for further analysis on the basis
306 of a strongly reduced *LeCCR1* expression (Fig. 1) and the ability to produce fruits. However in
307 these selected lines seed formation was strongly affected and the very small seeds produced
308 were unable to germinate. Therefore, these lines had to be maintained by vegetative
309 propagation. In these plants steady-state levels of *LeCCR2* transcript were also reduced, albeit
310 to a lesser extent than *LeCCR1* (data not shown). A transgenic line carrying the empty vector

311 pSR02 was also generated as a control and taken to the T2 generation. Transformants were
312 subsequently compared with both wild-type and control plants.

313

314 *Plant morphology and development*

315 All transgenic plants with reduced *LeCCR1* expression exhibited a dramatic reduction of size
316 as compared to the wild type or control plants (Fig. 2A). Furthermore, the leaves of CCR
317 down-regulated lines were darker and smaller (they contained one to five leaflets vs. up to
318 nine in the wild type). The three selected lines, L2, L5 and L15 could only produce a limited
319 number of small fruits (Fig. 2B).

320

321 *Microscopic examination and histochemistry*

322 Sections were taken from stems and fruits and observed either for lignin autofluorescence
323 under UV light or after phloroglucinol staining. Under UV light, xylem vessels in stems and
324 petioles of CCR down-regulated plants appeared partially collapsed with the cell walls
325 apparently buckling under mechanical stress, as compared to control vessels which exhibited a
326 round-open shape (Fig. 3 A and B). The differences between CCR down-regulated and wild-
327 type or control lines were even more obvious after staining with phloroglucinol: xylem tissue
328 was hardly stained in the CCR-deficient plants while it exhibited a strong pink-red coloration
329 in wild-type and control stems (Fig. 3 C and D). Similar observations were made on vascular
330 bundles from fruits (Fig. 3 E and F). Another distinctive feature of CCR down-regulated plants
331 was the brown-orange coloration of the xylem ring in the basal part of the stem, visible
332 without any staining (not shown).

333

334 *Lignin content*

335 Klason lignin content was determined in the cell wall residue extracted from whole stems.
336 This experiment allows the quantification of sulfuric acid-insoluble lignin material in plant
337 samples (Whiting *et al.*, 1981). Table 2 shows that Klason lignin content is markedly
338 decreased (by 20 to 37 %) in CCR-deficient plants as compared to wild-type and control lines.

339

340 **Analysis of soluble phenolic compounds**

341 In order to evaluate the potential impact of CCR down-regulation on different pathways of
342 phenolic metabolism, methanol-soluble phenolic compounds were quantified by a global assay
343 then separated by HPLC. Vegetative organs (stems and leaves) as well as ripe fruits were
344 studied separately.

345

346 *Determination of total phenolic content*

347 Following reduction of CCR expression and of lignin synthesis, we expected the
348 phenylpropanoid metabolic flow to be reoriented towards the production of soluble phenolics.
349 To investigate whether this was the case, we estimated the total soluble phenolic content of
350 extracts from different organs using Folin-Ciocalteu's method (Dicko *et al.*, 2002). Results
351 reported in Fig. 4 clearly show the accumulation of soluble phenolics at higher levels in stems
352 and leaves of CCR down-regulated lines as compared to control plants. Differences are
353 significant according to Student's t-test ($\alpha=0.05$), and correspond to 3.1- and 1.4-fold increases
354 in total phenolic content in stems and leaves, respectively. In contrast, no significant
355 difference could be observed in total phenolic contents of fruit extracts ($P=0.09$).

356

357 *Global comparison of phenolic profiles*

358 To further characterize modifications of phenolic metabolism in CCR down-regulated plants,
359 methanol-soluble fractions were subjected to HPLC separation. As important differences were
360 detected, we focused on the most abundant compounds. Major peaks detected in extracts from
361 all organs were numbered consecutively according to their order of elution on the HPLC
362 column. Chromatograms from wild-type plants (Fig. 5) show that some of the major phenolic
363 compounds are present in stems, leaves and fruits, while others are specific to a particular
364 organ (e.g. compound **3**). Differences between wild-type and CCR down-regulated plants can
365 be observed by comparing HPLC profiles, and were confirmed by quantification of individual
366 compounds (Table 3). In stems, some of the major compounds were hyper-accumulated in the

367 transformants (compounds **1**, **4** and **9**), and four new compounds appeared that were
368 undetectable in the wild type (**2**, **5**, **6** and **11**; Fig. 5, Table 3). Leaf extracts also exhibited
369 differences with the wild-type, albeit to a lesser extent. The same compounds specifically
370 observed in CCR down-regulated stems were also present (peaks **2**, **5**, **6** and **11**), together with
371 at least three new compounds (**7**, **8** and **12**; Fig. 5, Table 3). In contrast, HPLC profiles from
372 fruits did not show significant differences between lines. Amounts of specific phenolic
373 compounds in the fruit varied to a much larger extent than in vegetative organs: important
374 variations were observed between several fruit samples from the same line, whereas extracts
375 from vegetative parts were essentially similar. Technical replicates (i.e. repeated extraction
376 and analysis of the same sample) gave consistent results, indicating that variation was not due
377 to the analytical procedure. This observation suggests that phenolic compound accumulation
378 in the fruit is more subject to biological variation, and could be strongly influenced by slight
379 differences in developmental stage. For this reason, it was difficult to detect significant
380 differences between transgenic and control fruits. Yet, compounds **5** and **6** were consistently
381 detected in the fruits from CCR-deficient lines and absent from wild-type and control fruits
382 (Fig.5, Table 3). Separate analysis of the phenolics extracted from the flesh and peel of these
383 fruits revealed that this trait was more conspicuous in the flesh, while several other fruit
384 phenolics were more abundant in the peel (Fig. 6).

385

386 *Compound identification*

387 We attempted identification of phenolic compounds differentially accumulated between
388 control and transformed lines, using information derived from UV/Vis absorption spectra,
389 HPLC retention times and LC-MS or LC-MS/MS data. Table 3 summarizes the data available
390 for major compounds: abundance in CCR-deficient lines relative to the wild type, retention
391 time on HPLC, UV/Vis absorption maxima, molecular mass, MS/MS fragmentation ions, and
392 compound name if determined. Identity of compounds was confirmed by spiking with the
393 corresponding standard when available.

394

395 Peaks **1**, **3** and **4** exhibited UV spectral characteristics similar to chlorogenic acid isomers,
396 and displayed, after LC-MS analysis, an m/z of 353 $[M-H]^{-1}$ and a major fragmentation ion at
397 191 $[M-H]^{-1}$ likely corresponding to the quinic acid moiety. Peak **4** was confirmed to
398 correspond to chlorogenic acid (5-caffeoylquinic acid) by spiking with a standard. Compounds
399 **1** and **3** therefore likely correspond to chlorogenic acid isomers. On the basis of elution times
400 and by analogy with previous work (Chabannes *et al.*, 2001), we propose that compound **1** is
401 3-chlorogenic acid (neochlorogenic acid) and compound **3** is 4-chlorogenic acid
402 (cryptochlorogenic acid).

403 Compound **2**, though abundant in the CCR down-regulated lines, could not be identified by
404 LC-MS since the modification of the elution protocol (the use of formic acid instead of HCl)
405 resulted in the disappearance of this compound from the LC chromatogram.

406 Compound **5**, with an m/z of 355 $[M-H]^{-1}$ and fragmentation ions of 193 $[M-H]^{-1}$ and 179 $[M-$
407 $H]^{-1}$ was tentatively identified as a hexoside of ferulic acid. Compound **6** exhibited an m/z of
408 251 $[M+H]^{+1}$. Its UV spectral properties are very similar to those reported for
409 hydroxycinnamic acid amide conjugates (Whitaker and Stommel, 2003). On the basis of this
410 observation and the molecular mass of this compound, we propose that it is N-caffeoyl
411 putrescine. UV spectra of compounds **7**, **8** and **12** were very similar to that of chlorogenic
412 acid, suggesting that they may contain a hydroxycinnamoyl moiety, however their identity
413 could not be determined precisely by LC-MS.

414 Peak **9** exhibited an m/z of 609 $[M-H]^{-1}$ and was identified as quercetin-*O*-rutinoside (rutin) by
415 co-elution with a standard.

416 Compounds **10** and **13**, with an m/z of 515 $[M-H]^{-1}$, fragmentation ions at 353 $[M-H]^{-1}$ and 191
417 $[M-H]^{-1}$, and UV spectra similar to chlorogenic acid, were tentatively identified as two isomers
418 of dicaffeoylquinic acid.

419 Peak **11**, with an m/z of 593 $[M-H]^{-1}$, was identified as kaempferol-*O*-rutinoside by
420 comparison with a standard.

421 One of the compounds that accumulated at similar levels between WT and CCR- lines was
422 also identified: peak **14** exhibited an m/z of 271 $[M-H]^{-1}$ and was identified as naringenin
423 chalcone by spiking with a standard.

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426 **Antioxidant properties of plant extracts**

427 Since the general aim of this work was to improve antioxidant content in tomato, and despite
428 the fact that few changes were observed in the phenolic patterns of the edible part of the
429 tomato plant: the fruit, we measured antioxidant properties of methanol extracts from different
430 organs using a TEAC assay. This method measures the capacity of a solution to scavenge
431 $ABTS^{+}$ radicals, and is suitable for the determination of antioxidant properties of phenolics
432 such as hydroxycinnamates and flavonoids (Re *et al.*, 1999). As shown in Fig. 7A, total
433 antioxidant capacity is increased respectively 3.4- and 1.6-fold in stem and leaf extracts from
434 CCR down-regulated plants as compared to control lines. As these results are very similar to
435 those reported for total phenolic content (Fig. 4), we examined a possible correlation between
436 these two traits. Fig. 7B shows a plot of TEAC values *vs.* total phenolic content in stem
437 extracts from various plants, and reveals a remarkable correlation between these two
438 measurements ($R^2=0.999$). A similar observation can be made on leaf extracts ($R^2=0.986$). In
439 fruit samples, antioxidant capacity did not differ significantly between control and transformed
440 lines, as was also observed for total phenolic content.

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444 **DISCUSSION**

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446 Two tomato CCR genes, *LeCCR1* and *LeCCR2*, were cloned on the basis of a high sequence
447 homology with *E. gunnii* CCR. The identity of these genes was subsequently confirmed by
448 analysis of the catalytic properties of corresponding recombinant proteins produced in *E. coli*.

449 The two enzymes exhibited contrasting activities on the four cinnamoyl-CoA substrates tested:
450 while LeCCR1 could use all four substrates, LeCCR2 could only reduce feruloyl-CoA.
451 Furthermore, LeCCR1 was more active than LeCCR2 on feruloyl-CoA, and was expressed in
452 a wider range of organs. This situation is similar to that reported in *Arabidopsis thaliana* by
453 Lauvergeat *et al.* (2001): one of the *Arabidopsis* CCR enzymes, encoded by At1g15950,
454 exhibits higher activity on different cinnamoyl-CoA thioesters than the other (At1g80820).
455 Expression studies as well as other reports based on the analysis of mutants or transgenic
456 plants revealed that the former is most likely involved in constitutive lignification, while the
457 latter seems to be preferentially expressed when the plant is challenged with a bacterial
458 pathogen. Based on the results of expression and recombinant protein studies, we chose
459 *LeCCR1* as a target for our silencing strategy. Of the 16 transformants obtained following
460 tomato transformation with a *LeCCR1* RNAi construct, many were unable to set fruit. Since
461 this organ was of particular interest for our study, we decided to select three transgenic lines
462 that exhibited a strong reduction of *LeCCR1* expression but were still able to produce fruits.
463 Lignin content determined in vegetative organs by the micro-Klason method (Whiting *et al.*,
464 1981) was 20 to 37% lower in these transgenic lines as compared to control plants. Lignin
465 content could not be quantified in fruits, but absence of staining of vascular traces by
466 phloroglucinol revealed that lignin synthesis was also affected in this organ. This reduction in
467 lignin content was an expected outcome of transformation with the *LeCCR1* RNAi construct,
468 since extinction of *CCR* genes in other species has already been reported to bring about
469 important decreases in lignin content. In our case, the differences were not as important as
470 those reported for tobacco plants (about 50% decrease in Klason lignin content; Piquemal *et*
471 *al.*, 1998). This may be due to the selection of lines with an intermediate level of CCR down-
472 regulation, since the lines exhibiting the most severe phenotype were discarded because they
473 did not set fruit. Alternatively, this discrepancy may be related to the fact that Piquemal *et al.*
474 (1998) analysed isolated xylem tissue, which was not possible for very small tomato plants.
475 When whole stems from *Arabidopsis* CCR antisense lines were studied (Goujon *et al.*, 2003),
476 the reduction in Klason lignin content was similar to our results. In agreement with their

477 decreased lignin content, transgenic lines L2, L5 and L15 exhibited a morphological
478 phenotype typical of CCR down-regulated plants: stunting, collapsed vessels, absence of
479 staining by phloroglucinol of vascular tissue, coloration of xylem in unstained basal stems.
480 Collapsing of vessels can be explained by the lower resistance to negative pressure of cell
481 walls containing less lignin. This in turn may affect plant growth and development by
482 decreasing water and solute transport efficiency (Piquemal *et al.*, 1998). Effects of CCR
483 down-regulation on fertility observed in tomato are the most severe described so far, although
484 reduced seed viability and decreased fertility at high temperatures have already been reported
485 in *Arabidopsis* (Goujon *et al.*, 2003; Jones *et al.*, 2001). At the moment we do not know if the
486 altered fertility and seed production is linked to the reduction in lignin content or to other
487 induced metabolic changes that occur in these plants.

488 Since we succeeded in decreasing lignin synthesis in transgenic tomato plants, we next
489 examined the consequences of this modification on soluble phenolic content. Total phenolic
490 content was markedly increased in stems and leaves of transformants as compared with
491 control plants, indicating that the pool of precursors left unused by the monolignol
492 biosynthetic pathway had been reallocated to the synthesis of other phenolic compounds.
493 However this was not the case in fruits, most likely because this organ is poorly lignified. This
494 observation suggests that soluble phenolic compounds accumulated in vegetative organs as a
495 result of CCR down-regulation are not transported towards the fruit.

496 In order to further characterize the modifications that occurred in phenolic metabolism,
497 methanol extracts were subjected to separation by HPLC. In stems and leaves, the observed
498 accumulation of soluble phenolic compounds corresponded to both quantitative and qualitative
499 changes: some compounds already present in wild-type plants accumulated to higher levels in
500 the transgenics (e.g. chlorogenic acid and rutin), and new compounds undetectable in the wild
501 type appeared as major contributors to the phenolic profile in the transformants.

502 Most of the differentially accumulated compounds that could be tentatively identified on the
503 basis of UV-Vis spectral properties and LC-MS data are hydroxycinnamate derivatives, at the
504 exception of two flavonoids, rutin and kaempferol rutinoside. Interestingly, the majority of

505 these compounds are potent radical scavengers, as has been documented for several
506 hydroxycinnamate derivatives (Sawa *et al.*, 1999; Niggeweg *et al.*, 2004). This is also
507 illustrated by the remarkable correlation observed between the increases in total phenolic
508 content and antioxidant capacity measured in extracts from transgenic plants. Indeed,
509 stimulating the synthesis of some of these compounds is considered a target of interest to
510 improve fruit nutritional quality (Niggeweg *et al.*, 2004). In our case however, the strong
511 morphological phenotype of transformants as well as the absence of soluble phenolic
512 accumulation in fruits make CCR down-regulation an inappropriate approach.

513 From a more fundamental point of view, the identity of the differentially accumulated
514 compounds provides new insight into the control of phenylpropanoid metabolism in tomato.
515 First, all hydroxycinnamate derivatives identified in this study comprise a caffeic acid or
516 ferulic acid moiety (Table 3). While the accumulation of a ferulic acid derivative could be
517 expected, feruloyl-CoA being the preferred substrate of both tomato CCR isoforms (table 1),
518 the predominance of caffeate derivatives was more intriguing. Caffeate and caffeoyl-CoA are
519 synthesized upstream of CCR, and are methylated into ferulate and feruloyl-CoA,
520 respectively, by a caffeate O-methyltransferase (COMT) and a caffeoyl-CoA O-
521 methyltransferase (CCOMT). The abundance of caffeate derivatives in CCR down-regulated
522 plants could therefore be attributed to a regulation of the metabolic flow (either by feedback
523 inhibition or transcriptional control) at this O-methylation step. In contrast, the absence of
524 identifiable coumarate derivatives suggests that the flow entering the 3'-hydroxylation step
525 (from coumarate to caffeate) is poorly affected by CCR down-regulation. This may explain the
526 limited accumulation of end products deriving from coumaroyl-CoA, such as flavonoids.
527 Alternatively, other limiting factors may restrain flavonoid synthesis: availability of malonyl-
528 CoA, chalcone isomerase activity (Verhoeven *et al.*, 2002) or activity of other flavonoid
529 biosynthetic enzymes. Indeed, up-regulation of the corresponding genes via overexpression of
530 key transcription factors leads to the accumulation of flavonoids regardless of precursor
531 availability (Schijlen *et al.*, 2004).

532 Second, several of the differentially accumulated compounds have been described as
533 predominant phenolic metabolites in Solanaceae. For example, chlorogenic acid (compounds
534 **1**, **3** and **4**) is the most abundant phenolic compound in tobacco leaves and stems, and may
535 correspond to a storage form that can be used when necessary to build lignin or other
536 downstream phenolics. Its increased abundance in CCR down-regulated transformants may
537 involve hydroxycinnamoyl-CoA: shikimate/quinic acid hydroxycinnamoyl transferase (HCT;
538 Hoffmann *et al.*, 2003), since this enzyme can synthesize chlorogenate back from caffeoyl-
539 CoA, a substrate of CCR. Caffeoyl putrescine (compound **6**), on the other hand, is not
540 constitutively present in tomato extracts but it has been shown to accumulate to important
541 amounts in Solanaceae in response to jasmonate treatment and fungal infection (Keinänen *et*
542 *al.*, 2001; Keller *et al.*, 1996). In our case, its accumulation may either correspond to rerouting
543 of the metabolic flow following blocking of the monolignol pathway, or to a stress response
544 due to the severe developmental alterations observed in the transformants.

545 In conclusion, we report for the first time down-regulation of CCR expression in a fruit
546 species. This modification leads to the accumulation of antioxidant soluble phenolic
547 compounds in vegetative organs, but not in the fruit, and our results highlight the tight
548 relationship between soluble phenolic abundance and antioxidant properties of plant extracts.
549 Precursors made available by CCR down-regulation are only partly used for the synthesis of
550 flavonoids, which shows the existence of complex regulatory networks within
551 phenylpropanoid metabolism. Finally, our work confirms that as previously demonstrated for
552 other metabolic pathways, rerouting metabolic flows can lead to unexpected effects at the
553 biochemical and/or developmental levels. As illustrated by our results this can include the
554 synthesis of novel compounds not detected in normal plants. This remarkable plasticity of
555 plant secondary metabolism could potentially be exploited in future metabolic engineering
556 studies for the production of high-value pharmaceutical compounds.

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Substrate	LeCCR1		LeCCR2	
	V_{\max} (nkat/mg protein)	k_{cat} (sec^{-1})	V_{\max} (nkat/mg protein)	k_{cat} (sec^{-1})
Feruloyl-CoA	221	14.2	25	1.6
Caffeoyl-CoA	5.2	0.33	n.d.	n.d.
Coumaroyl-CoA	6.7	0.43	n.d.	n.d.
Sinapoyl-CoA	15	0.96	n.d.	n.d.

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Table 1. Specific activities of recombinant LeCCR1 and LeCCR2 enzymes

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Activity of purified recombinant LeCCR1 and LeCCR2 proteins was measured against a range of cinnamoyl-CoA thioester substrates. Catalytic activities (V_{\max}) and turnover numbers (k_{cat}) were determined in the conditions described in Wengenmayer *et al.* (1976). n.d.: non detectable.

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	WT	CT	L2	L5	L15
Lignin content (% CWR)	14.3 ± 0.74	14.2 ± 0.92	9.0 ± 0.53	11.4 ± 0.98	11.3 ± 0.53

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Table 2. Determination of lignin content

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Cell wall residue (CWR) was prepared from whole freeze-dried wild-type (WT), control (CT) and transgenic plants (L2, L5 and L15). Acid-insoluble lignin content in CWR was estimated following the micro-Klason method (Whiting *et al.*, 1981). Values correspond to the mean of three replicates, ± standard error of the mean.

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Compound number	Elution time (min)	[M-H] ⁻¹ ion, MS ² daughter ions	UV absorbance peaks (nm)	Compound identity	Relative accumulation CCR/WT		
					Stems	Leaves	Fruits
1	8.7	353 (191)	(298) 327	3-caffeoyl quinic acid	1.4 -3.3	0.47-1.0	n. d.
2	9.3		294, 320	unknown	<i>ccr</i> only (>10 x)	<i>ccr</i> only (> 6 x)	0.8 - 3.0
3	12.7	353 (191)	(298) 327	4-caffeoyl quinic acid	n. d.	n. d.	0.2 - 2.0
4	13.7	353 (191)	(298) 327	5-caffeoyl quinic acid	3.4 - 14	0.9 – 1.9	0.2 – 1.5
5	14.1	355 (193, 179)	(300) 330	ferulic acid hexoside	<i>ccr</i> only (> 10 x)	<i>ccr</i> only (> 6 x)	<i>ccr</i> only (d.l.)
6	15.6	249	(294) 320	N-caffeoyl putrescine	<i>ccr</i> only (> 8 x)	<i>ccr</i> only (> 6 x)	<i>ccr</i> only (d.l.)
7	23.6		(300) 327	unknown	n. d.	<i>ccr</i> only (> 2 x)	n. d
8	24.7		(300) 327	unknown	n. d.	<i>ccr</i> only (> 3 x)	n. d
9	27.8	609	257, 356	quercetin- <i>O</i> -rutinoside	2.2 – 4.2	1.1 – 1.7	0.2– 0.9
10	28.7	515 (353, 191)	(298) 327	dicafeoyl quinic acid	n. d.	n. d.	0.3 – 0.9
11	29.7	593	265, 346	kaempferol- <i>O</i> -rutinoside	<i>ccr</i> only (> 2 x)	<i>ccr</i> only (> 1.5 x)	0.3 - 1.0
12	31.3		(292)320	unknown	n. d.	<i>ccr</i> only (> 2 x)	n. d
13	32.7	515 (353, 191)	(298) 327	dicafeoyl quinic acid	n. d.	n. d.	0.2 – 1.4
14	35.8	271	226, 370	naringenin chalcone	n. d.	n. d.	0.3 - 5.0

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Table 3. Identification and occurrence of major soluble phenolic compounds in control and CCR down-regulated plants

Elution times correspond to the separation by HPLC. Molecular mass was determined by MS as m/z following ESI in the negative mode. Whenever possible, compounds were fragmented in MS-MS experiments and the m/z values for the main daughter ions are given between brackets. UV absorbance peaks correspond to the two main wavelengths at which the compounds absorb. Numbers between brackets indicate the second major peak of absorbance. Relative accumulation values correspond to the ratio of the content of each compound in a transgenic plant vs the mean value obtained for the wild type. At least two plants were analysed for each line, and HPLC separation and quantification were performed in duplicate for each extract. The two values indicate the lowest and highest ratios obtained among the three transgenic lines. n.d.: non detectable. d.l. : detection limit. CCR⁻ only: compounds

719 detected only in CCR down-regulated transformants. Between brackets : minimum fold
720 accumulation over the detection limit observed in the transgenics, on the basis of HPLC
721 quantification.

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FIGURE LEGENDS

Figure 1. RNA blot analysis of *LeCCR1* steady-state transcript levels in control and transgenic plants

Total RNA was extracted from stems of 3-month-old wild-type (WT), control (CT) and transgenic plants (L2, L5 and L15). Following separation on a denaturing gel, RNA was hybridized to a *LeCCR1* probe (A) and UV fluorescence of 18S rRNA was visualized (B) to estimate loading of the different samples.

Figure 2. Phenotype of CCR down-regulated transgenic plants

Transgenic tomato plants from different lines showing severe down-regulation of *LeCCR1* expression were compared to wild-type (WT) and control (CT) plants of the same age.

A: whole plants. B: ripe fruits.

Figure 3. Microscopic examination of sections from control and CCR down-regulated plants

Sections were taken from stems (A to D) and fruits (E and F) of control and transgenic plants (transformant L2). Sections were observed under UV light (A and B) or following phloroglucinol staining (C to F). Similar results were obtained with transgenic plants L5 and L15. Arrows indicate phloroglucinol-stained vascular traces in the fruit. A to D: bar=100 mm; E and F: bar=2 mm.

Figure 4. Total phenolic content in extracts from different organs

Total phenolic content in the methanol-soluble fraction extracted from stems, leaves and fruits of control and CCR down-regulated plants was determined using Folin-Ciocalteu's method. Assays were performed on extracts obtained from individual plants representing each line. Values represent the mean \pm s.e.m. for each group (WT, CT: wild-type and empty vector

749 transformant; CCR⁻: transgenic plants L2, L5 and L15). The whole experiment was duplicated
750 with similar results.

751
752 **Figure 5. HPLC separation of methanol-soluble phenolic compounds**

753 Chromatograms correspond to extracts from stems, leaves and whole fruits. For each organ a
754 representative chromatogram from CCR down-regulated (CCR⁻) and control (CT) plants is
755 shown.

756
757 **Figure 6. Separate HPLC analysis of fruit flesh and peel**

758 Tomato fruit peel was manually separated from the flesh and the soluble phenolics were
759 methanol-extracted from each sample. Chromatograms are representative of flesh (A) and peel
760 (B) samples from wild-type (WT) and transgenic (CCR⁻) fruits.

761
762
763 **Figure 7. Effect of CCR down-regulation on antioxidant capacity**

764 Methanol extracts obtained from stems, leaves and fruits of control and transgenic plants were
765 analysed using the TEAC assay (Trolox Equivalent Antioxidant Capacity). WT, CT: wild-type
766 and empty vector transformant; CCR⁻: transgenic plants L2, L5 and L15.

767 A: Antioxidant capacity of extracts from different organs. Assays were performed on extracts
768 obtained from individual plants representing each line. Values correspond to the mean \pm
769 s.e.m. for each group. The whole experiment was duplicated with similar results.

770 B: Plot of total phenolic content as determined by Folin-Ciocalteu's method vs. antioxidant
771 capacity. Results correspond to wild-type and control plants, and three CCR down-regulated
772 transgenic plants.

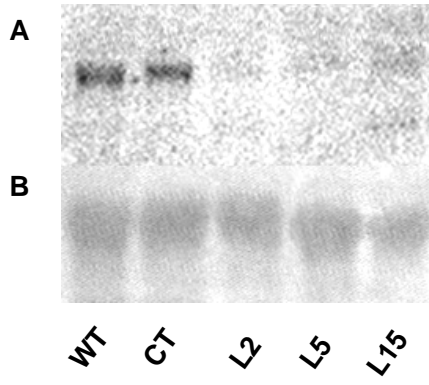


Figure 1

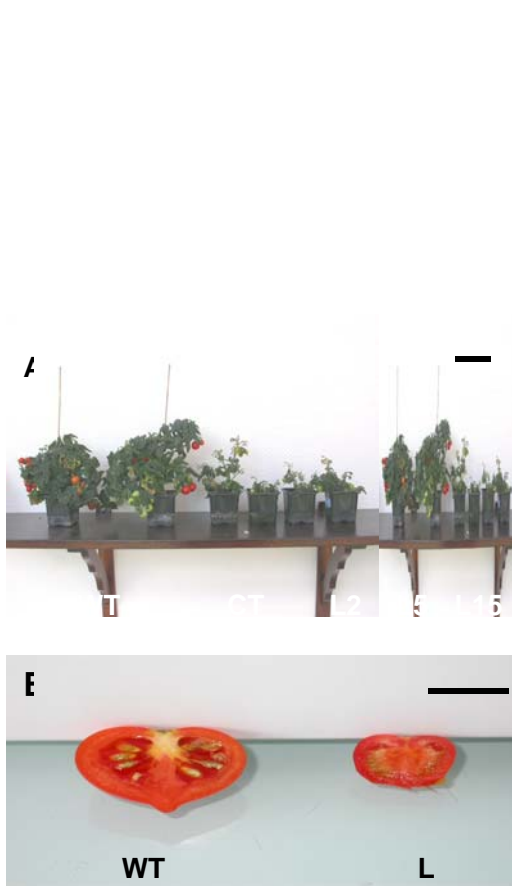


Figure 2

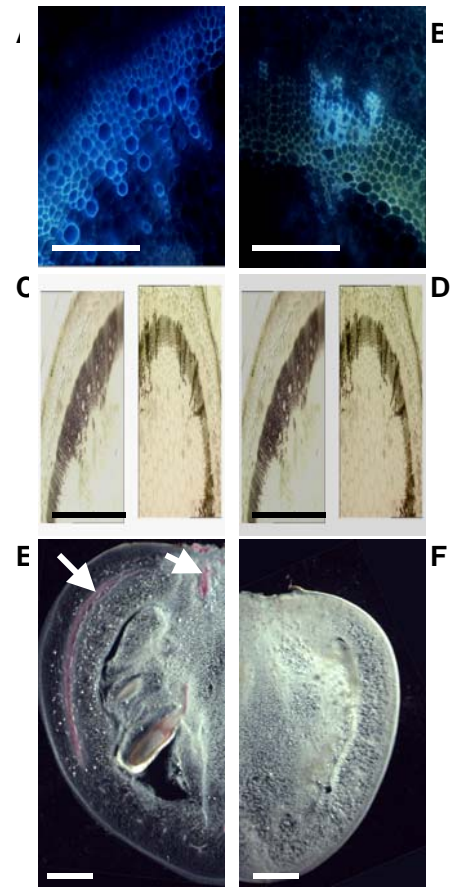


Figure 3

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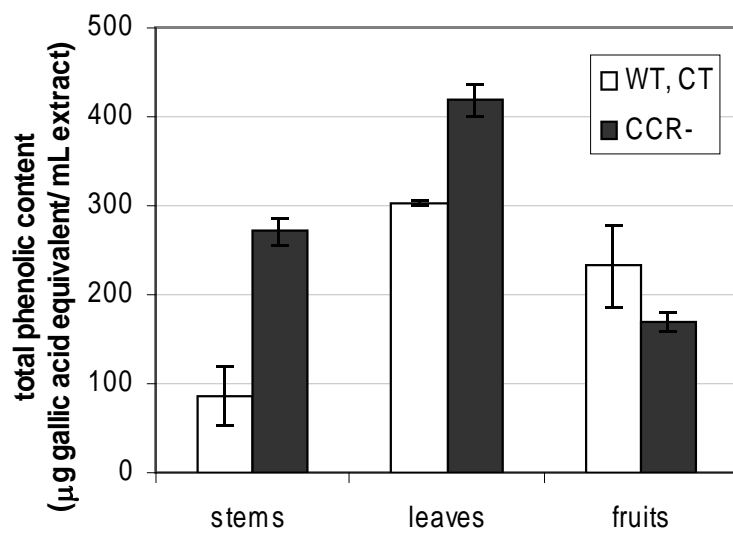


Figure 4

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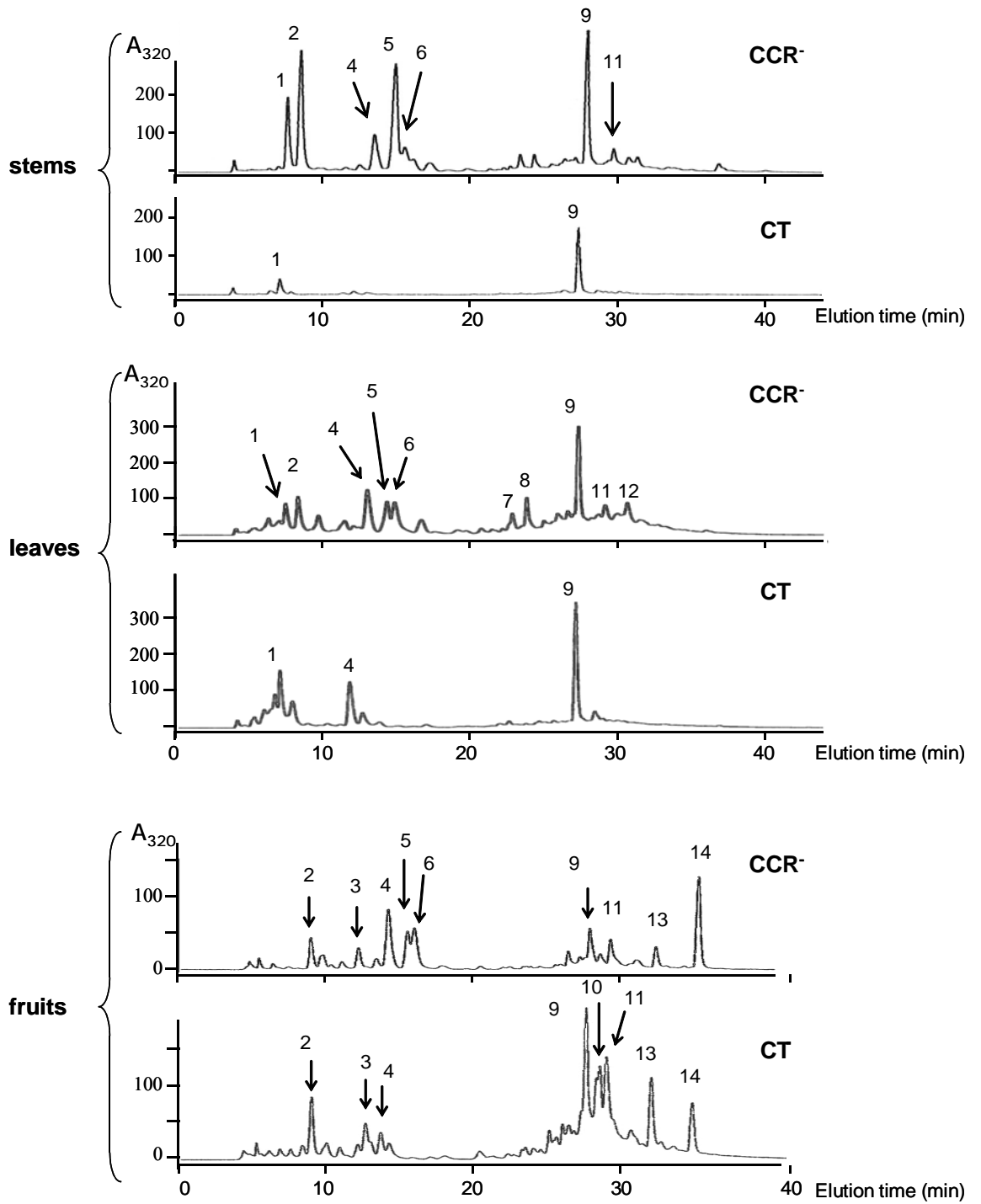


Figure 5

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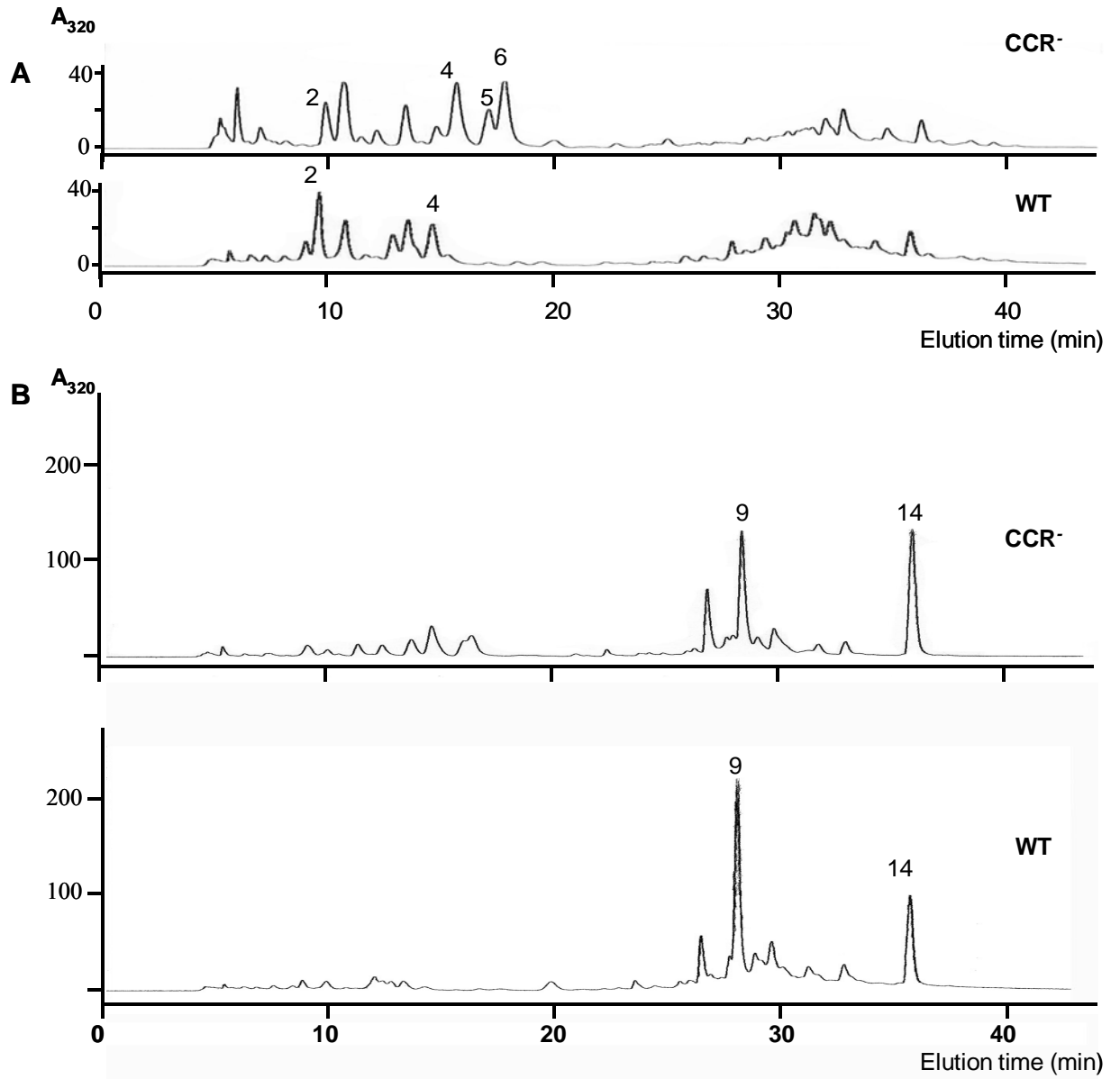


Figure 6

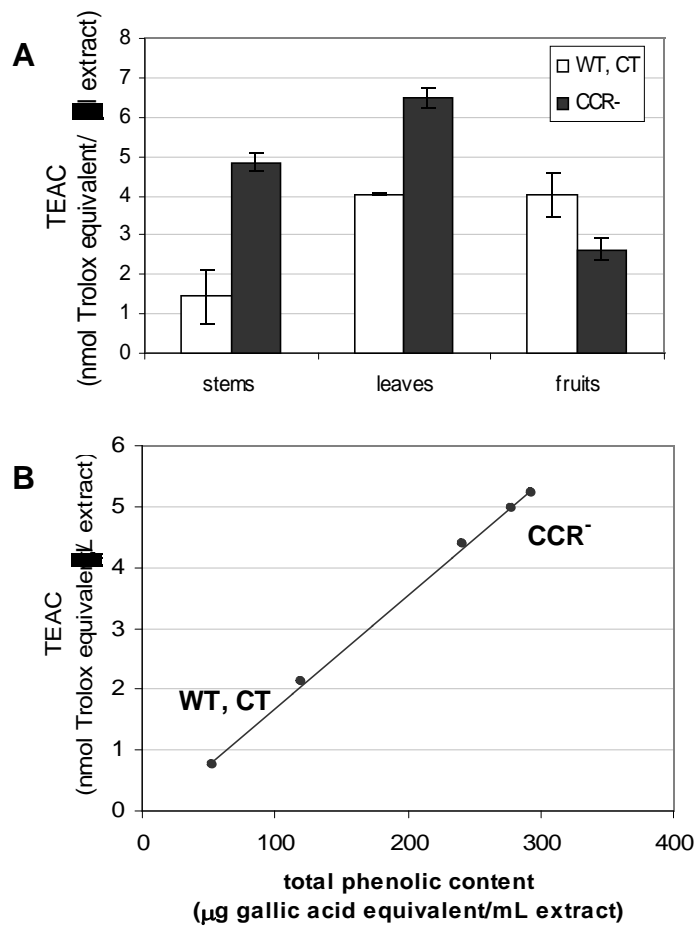


Figure 7