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Title

Investigation of the isomerization of *trans*- and *cis*-cinnamic acid in Arabidopsis using stable-isotope-labeled cinnamic acid isomers

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36 **Abstract**
37 Cinnamic acid (CA) is a widely distributed metabolite in plant species and is a precursor of many
38 important plant molecules, including lignin, flavonoids, and coumarins. CA exists as both *trans* and
39 *cis* isomers; the *trans* isomer is more stable and common in nature. Previous reports have revealed
40 that the *cis* isomer of CA (*cis*-CA) has auxin-like activity when exogenously applied. However, it has
41 also been reported that *cis*-CA does not function as an auxin but affects its transport. Although these
42 reports suggest a crucial role for *cis*-CA as an endogenous signaling molecule, its exact function and
43 mechanism of isomerization from *trans*-CA remain unclear. Here, we report the chemical synthesis
44 of stable-isotope-labeled *trans*- and *cis*-CA. Using these labeled compounds as internal standards, we
45 developed a quantification method of CA using LC–MS/MS. Moreover, we monitored the
46 endogenous conversion from *trans*- to *cis*-CA using the labeled compounds, demonstrating the
47 UV-dependent and UV-independent CA isomerization in Arabidopsis. Additionally, we identified
48 *cis*-CA in diverse plant species, including liverwort.
49
50
51
52 **Keywords**:
53 Cinnamic acid, internal standard, isomerization, UV, LED
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74 **Introduction**

75 Cinnamic acid (CA) is a widely distributed metabolite in the plant kingdom and a precursor of
76 many important plant molecules, including lignin, flavonoids, and coumarins. CA has a sidechain
77 structure, which contains a carbon–carbon double bond. Therefore, CA exists as both *trans* and *cis*
78 isomers, of which the *trans* isomer is more thermodynamically stable and common in nature. It has
79 been reported that UV irradiation of *trans*-CA induces its isomerization to *cis*-CA. Interestingly,
80 *cis*-CA, but not *trans*-CA, exhibits auxin-like activity when exogenously applied to Arabidopsis. At
81 low concentrations, such as less than 1 μM, *cis*-CA promotes lateral root development in Arabidopsis
82 and *Nicotiana benthamiana*, which leads to increased leaf weights [1,2]. However, at higher
83 concentrations, such as 10 μM, *cis*-CA severely inhibits plant growth. The structure of *cis*-CA is
84 partially similar to that of a major auxin, indole-3-acetic acid (IAA). However, *cis*-CA does not
85 induce auxin receptor complex formation between the degron domain of AUX/IAA7 and TIR1 or
86 AFB5 [2]. However, *cis*-CA inhibits polar auxin transport, similar to the well-known transport
87 inhibitor, 1-naphthylphthalamic acid (NPA) [2].

88 *In planta*, CA is biosynthesized from phenylalanine by phenylalanine ammonia lyase, which
89 produces *trans*-CA. However, it is thought that a portion of the *trans* isomer is converted into the *cis*
90 isomer in a manner dependent on UV radiation, which is present in fluorescent light and sunlight.
91 Indeed, in *in vitro* reactions, UV irradiation of *trans*-CA stimulates its isomerization to *cis*-CA [3,4].
92 However, *cis*-CA exists even when Arabidopsis is grown under LED light, which does not contain
93 the UV portion of the spectrum [5]. Therefore, the isomerization mechanism of CA *in planta* and its
94 triggers remain unclear. The biological activity of *cis*-CA as explained above suggests *cis*-CA may
95 function as an endogenous plant growth regulator. To understand the endogenous role of *cis*-CA and
96 its mechanism of isomerization, it would be effective to develop a quantitative analytical method for
97 *trans*- and *cis*-CA. In this paper, we report the chemical synthesis of stable-isotope-labeled *trans*-
98 and *cis*-CA. Using these labeled compounds as internal standards, we developed a quantitative
99 method for determining endogenous levels of *cis*- and *trans*-CA by using liquid
100 chromatography–quadruple/time-of-flight tandem mass spectrometry (LC–Q-TOF-MS). Moreover,
101 we examined the endogenous conversion of exogenously applied labeled *trans*-CA into its *cis* isomer
102 under fluorescent (+UV) and LED (−UV) light.

103

104

105 **RESULTS**

106 **Chemical synthesis of d_5-labeled *trans*- and *cis*-CA**

107 To establish a quantitative method for determining endogenous levels of *trans*- and *cis*-CA, we
108 synthesized stable-isotope-labeled *trans*- and *cis*-CA as the internal standard. The synthetic scheme

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109 is shown in Fig.1. Starting from commercially available d_5-benzaldehyde, we first extended the
110 sidechain by the Horner–Wadsworth–Emmons reaction to yield the ethyl ester d_5-*trans*-CA [6]. We
111 then removed the ethyl group by a simple hydrolysis reaction, which yielded d_5-*trans*-CA. The
112 MeOH solution of d_5-*trans*-CA was irradiated with 254-nm UV light to obtain the *cis* isomer of
113 d_5-CA. The resulting d_5-*cis*-CA was purified by HPLC. The purity of each isomer was determined to
114 be >99% by NMR for both the *trans* and *cis* isomers (Fig. S1).
115
116 **Evaluation of the stabilities of *trans*- and *cis*-CA**
117 Generally, the *trans* form of a double-bond structure is more stable than its corresponding *cis*
118 form. In some cases, a *cis*-configuration bond can be isomerized to the *trans* form by UV irradiation.
119 Therefore, we considered the possibility that *cis*-CA is converted into *trans*-CA *in vitro*. To quantify
120 the endogenous levels of small molecule compounds such as these, LC–MS/MS analysis is
121 appropriate. To do this, it was necessary to prepare the samples, which usually includes several steps
122 of purification using solid-phase extraction. Therefore, we analyzed the stability of *trans*- and *cis*-CA
123 throughout the sample preparation process. As a result, we found that even *cis*-CA was stable during
124 the sample preparation process (Fig. 2).
125
126 **Development of a quantitative method for determining endogenous levels of *trans*-/*cis*-CA**
127 After confirming the stability of CA during purification, we established a quantification
128 method for both *trans*- and *cis*-CA. In a previous report, GC was used to determine the contents of
129 CA after methyl esterification [5]. However, it is possible that the methyl-esterified form of CA
130 exists in plant tissue. Thus, LC–MS/MS analysis without derivatization was thought to be better for
131 accurately measuring levels of CA. After the extraction of Arabidopsis whole plant tissue, the
132 deuterium-labeled CA internal standards were added to the sample, and the extract was filtrated and
133 evaporated. The samples were purified by HLB and WAX column chromatography to collect the
134 acidic compounds, including CA. The resulting acidic fractions were analyzed by LC–MS/MS in
135 negative-ion mode. The retention time of the d_5-*trans*-CA or d_5-*cis*-CA was slightly earlier than the
136 CA peaks identified in the plant extracts because of the presence of the isotope (Fig. 3). However, the
137 MS/MS spectrum confirmed the chemical identity of *trans*- and *cis*-CA (Fig. 3). Using this
138 established method, we successfully detected the *cis*-CA peak in the plant extract sample (Fig. 3).
139
140 **Quantification of *trans*- and *cis*-CA in Arabidopsis grown under different light conditions**
141 As mentioned above, we successfully detected endogenous *trans*- and *cis*-CA by LC–MS/MS

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142 analysis from Arabidopsis extracts. To determine whether *cis*-CA is produced only under
143 UV-containing light, we grew Arabidopsis on MS agar medium under fluorescent light (+UV) or
144 LED light (−UV) (Fig. S2). The shoot and root parts of two-week-old plants were used to quantify
145 the endogenous levels of *trans*- and *cis*-CA (Fig. 4). We detected *cis*-CA in Arabidopsis grown under
146 both fluorescent and LED light; there was no significant difference in *cis*-CA levels between the
147 plant extracts grown under fluorescent or LED light conditions. However, by comparing the ratios of
148 *cis*-CA in total CA, we noticed that *cis*-CA content was slightly higher when seedlings were grown
149 under fluorescent light, and that levels of CA were much higher in roots than in shoots (Fig. 4).
150 These results demonstrate that isomerization from *trans*-CA to *cis*-CA can occur in a
151 UV-independent manner.
152

153 **Bioconversion of *trans*-CA into *cis*-CA under different light conditions**

154 The abovementioned results suggest that the *trans*-to-*cis* isomerization of CA can occur in a
155 UV-independent manner. To determine whether such a conversion can occur *in planta*, we performed
156 experiments in which we fed d_5-*trans*-CA to Arabidopsis Col-0 WT under either fluorescent or LED
157 light conditions. In the negative control sample, d_5-*trans*-CA incubated in water, we did not see any
158 conversion into *cis*-CA under either light condition (Fig. 5). When d_5-*trans*-CA was incubated with
159 an Arabidopsis plant, we detected d_5-*cis*-CA in both the culture medium and tissue extracts. When
160 the plants were grown under fluorescent light, *cis*-CA was detected at much higher levels than when
161 plants were grown under LED light (Fig. 5). These results strongly suggest that *trans*-to-*cis*
162 isomerization of CA can occur, even under LED light conditions, but this isomerization may be
163 accelerated by UV-light irradiation. Alternatively, it is possible there are two isomerization pathways:
164 UV-dependent and UV-independent.
165

166 **Distribution of *cis*- and *trans*-CA in some plant species**

167 We then analyzed the levels of *trans*- and *cis*-CA in various plant species. We used rice (*Oryza*
168 *sativa*, Nipponbare and Shiokari), tobacco (*Nicotiana benthamiana*), tomato (*Solanum lycopersicum*),
169 and liverwort (*Marchantia polymorpha*) as plant materials. We chose both monocot and dicot, as
170 well as a basal land plant species to see whether the CA isomerization system is conserved
171 universally in plant kingdom. After growing for1-2 weeks, shoot and root parts were separated, and
172 *trans*- and *cis*-CA levels were quantified using the abovementioned method. As a result, we
173 successfully detected *cis*-CA in all the analyzed plants, including the basal land plant, *M.*
174 *polymorpha* (Table 1). Interestingly, the *trans*/*cis* ratio differed among plant species. In particular, the

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175 ratio of *cis*-CA in total CA was higher in *S. lycopersicum* roots and *M. polymorpha*. Moreover, even
176 within the same plant species, such as rice, there were significant differences in *trans*-/*cis*-CA ratios
177 between the two cultivars tested.
178

179 **DISCUSSION**

180 In this report, we chemically synthesized deuterium-labeled *trans*- and *cis*-CA. Using these
181 labeled compounds as internal standards, we developed a quantitative analytic method for CA using
182 LC–MS/MS. In a previous report, CA levels were analyzed by GC–MS after methyl esterification,
183 which cannot be distinguished from the naturally existing CA methyl ester [5]. In contrast, our
184 method can accurately quantify the amount of endogenous *trans*- and *cis*-CA. Using this newly
185 developed method, we simultaneously quantified the endogenous levels of *trans*-CA and *cis*-CA. The
186 results demonstrate that levels of *cis*-CA are not increased in Arabidopsis plants grown under
187 fluorescent light compared with plants grown under LED light. However, the feeding experiment of
188 d_5-*trans*-CA indicated that more *trans*-to-*cis* isomerization of CA occurred under fluorescent light
189 than LED light conditions. These results suggest that CA isomerization can occur in a
190 UV-independent manner, which may be promoted by UV irradiation. There may also be both
191 UV-dependent and -independent mechanisms of CA isomerization *in planta*. Although further
192 experiments are needed to understand the CA isomerization mechanism *in planta* and the exact
193 endogenous function of *cis*-CA, our newly developed method for CA quantification could be
194 effective for addressing these questions. Moreover, stable-isotope-labeled CAs could be an effective
195 tool to dissect the physiological roles of CA.

196 Using our newly developed method, we quantified the levels of *trans*- and *cis*-CA in different
197 plant species. Although we detected both *trans*- and *cis*-CA in all plants analyzed, we found that the
198 ratios of *trans*/*cis*-CA differed between plant species. Between two different rice cultivars,
199 Nipponbare contained much more *trans*-CA; thus, the *cis*-CA ratio in total CA was much lower
200 compared with that of Shiokari. Such differences between cultivars of the same plant species may be
201 useful for identifying the factors involved in CA isomerization.
202
203

204 **MATERIALS AND METHODS**

205 **Plant materials**

206 We used *Arabidopsis thaliana* (Col-0), rice (Nipponbare and Shiokari), tobacco (*Nicotiana*
207 *benthamiana*), tomato (*Solanum lycopersicum*, moneymaker), liverwort (*Marchantia polymorpha*,
208 TAK-1).
209

210 **Chemicals**

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211 Unlabeled *cis*-CA was chemically synthesized from *trans*-CA as we previously reported [4].
212 *Trans*-CA is commercially available (TCI).
213

214 **Chemical synthesis of d_5-*trans*- and *cis*-CA**

215 d_5-*trans*-ethyl-cinnamate: THF (11 mL) and triethyl phosphonoacetate (1.4 mL, 7.06 mmol) were
216 added to NaH (170 mg, 7.05 mmol) under an argon atmosphere and stirred on ice for 10 min.
217 *d*5-benzaldehyde (379 mg, 3.41 mmol) in THF (5 mL) was added to the mixture, and the entire
218 mixture was stirred at room temperature for 5 h. The reaction was quenched by adding 1 N HCl. The
219 sample was extracted with EtOAc (3×30 mL) and washed with brine (3×90 mL). The organic layer
220 was dried over Na_2SO_4 and concentrated *in vacuo*. The crude sample was purified by silica gel
221 column chromatography (*n*-hexane/EtOAc:9/1) to obtain *d*5-*trans*-ethyl-cinnamate (quant). ^{1}H NMR
222 (300 MHz, $CDCl_3$): δ 7.69 (d, *J*=16.2 Hz, 1H), 6.44 (d, *J*=15.9 Hz, 1H), 4.26 (dd, *J*=6.9, 7.2 Hz, 2H),
223 and 1.33 (t, *J*=6.9, 3H).

224 *d*5-*trans*-CA: *d*5-*trans*-ethyl-cinnamate (657.9 mg, 3.63 mmol) was dissolved in EtOH (6.10 mL),
225 and 5 M NaOH (6.2 mL) was added to the solution. The mixture was stirred at room temperature
226 overnight. After the reaction, the solution was diluted to 30 mL with distilled water. The sample was
227 extracted with EtOAc (3×30 mL), and the aqueous layer was collected. The pH of the aqueous layer
228 was adjusted to 1 using 1 N HCl, and the sample was extracted with EtOAc (3×30 mL). The organic
229 layer was dried over Na_2SO_4 and concentrated *in vacuo* to obtain *d*5-*trans*-CA (438.9 mg, 2.87 mmol,
230 79% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.81 (d, *J*=16.2 Hz, 1H) and 6.46 (d, *J*=16.2 Hz, 1H).
231 HRMS [ESI- (*m*/*z*)] calculated for $(C_9H_3\,{}^2H_5O_2\ \text{-H})^-$ 152.0765, found 152.0767.
232

233 d_5-*cis*-CA: *d*5-*trans*-CA (438.9 mg, 2.87 mmol) was dissolved in EtOH (50 mL). The solution was
234 placed under a UV lamp (254 nm) overnight. The solvent was evaporated *in vacuo*, and the mixture
235 of d_5-*trans*- and *cis*-CA was suspended in 10 mL of distilled water. The sample was sonicated for 5
236 min and then centrifuged at 18,000 g for 30 min at 4°C. The supernatant was filtered and diluted to
237 30 mL with distilled water. The pH was adjusted using 1 N HCl, and the sample was extracted with
238 EtOAc (3×20 mL). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The crude
239 sample was purified by reverse-phase HPLC (ODS SP-100, MeOH/H_2O:6/4). A portion of the
240 sample was purified by HPLC to give *d*5-*cis*-CA (7.1 mg, 0.046 mmol, 0.14% yield). ^{1}H NMR (300
241 MHz, $CDCl_3$): δ 7.08 (d, *J*=12.6 Hz, 1H) and 5.99 (d, *J*=12.6 Hz, 1H). HRMS [ESI- (*m*/*z*)] calculated
242 for $(C_9H_3\,{}^2H_5O_2\ \text{-H})^-$ 152.0765, found 152.0771.
243
244

245 **Analysis of endogenous levels of *cis*- and *trans*-CA**

246 Plants were grown on half-strength Murashige and Skoog medium with 1% agar for 1–2 weeks under either
247 fluorescent light (HITACHI; FLR20S) or LED light (NK system: Plantfleck) (light/dark: 16 h/8 h). The light
248 spectra for each light condition was measured by LA-105 (Nippon Medical & Chemical Instruments CO., Ltd,

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249 Osaka, Japan, Fig. S2). The aboveground parts and the roots were separated, and the fresh weight of each was
250 measured. The samples were added to acetone for extraction, and the mixed solution of 100 pg/µL d_5-*trans*-CA and
251 10 pg/µL d_5-*cis*-CA were added as the internal standards. The extracts were filtered, concentrated, and dissolved in
252 H_2O containing 1% AcOH (1 mL). The solutions were loaded onto an HLB cartridge column (1 cc, 30 mg, Waters),
253 washed with H_2O containing 1% AcOH (1 mL), and eluted with 80% MeCN containing 1% AcOH (3 mL). H_2O
254 containing 1% AcOH (500 µL) and evaporated MeCN was added to the HLB-eluted fraction. These solutions were
255 loaded onto a WAX cartridge column (1 cc, 30 mg, Waters), washed with H_2O containing 1% AcOH (1 mL) and
256 80% MeCN (2 mL), and eluted with 80% MeCN containing 1% AcOH (2 mL). The eluates were concentrated and
257 dissolved in 50% MeCN. The samples were subjected to LC–MS/MS to quantify the amount of endogenous *cis*-
258 and *trans*-CA. LC–MS/MS analysis of CA and d_5-CA was conducted using a quadrupole/time-of-flight tandem
259 mass spectrometer (X500R, AB SCIEX) and an ultrahigh performance liquid chromatography (Nexera, Shimadzu)
260 equipped with a reverse-phase column (CORTECS UPLC phenyl, 1.6 µm, ϕ 2.1×75 mm; Waters). For CA and
261 d_5-CA analysis, the elution of the samples was carried out with H_2O containing 0.05% AcOH (solvent A) and
262 MeCN containing 0.05% AcOH (solvent B), and the mobile phase was changed from 10% B to 40% and 100% at 5
263 and 7 min after the injection, respectively, at a flow rate of 0.3 mL/min. The MS/MS analysis conditions were as
264 follows: negative-ion mode; declustering potential, −80 V; collision energy, −15 V; and parent ions (m/z) of 147.10
265 for unlabeled CA and 152.10 for d_5-CA.
266
267

268 **d_5-*trans*-CA feeding experiment**

269 *A. thaliana* plants, which were grown up for 2 weeks under either fluorescent light (+UV) or LED (−UV), were
270 transferred to 24-well plates. Each well also contained 10 µM d_5-*trans*-CA in 0.1% acetone. The plates were
271 incubated in a growth chamber under fluorescent or LED light for 24 h (light/dark: 16 h/8 h). After the 24 h
272 incubation, the plants were washed with sterile water and extracted with acetone. The extracts were filtered,
273 concentrated, and H_2O containing 1% AcOH (1 mL) was added. These samples were purified on HLB and WAX
274 cartridge columns. The eluted WAX fraction was concentrated and dissolved in 50% MeCN. LC–MS/MS was
275 conducted on the sample, and it was analyzed for d_5-*cis*- and *trans*-CA. Purification using HLB and WAX cartridge
276 columns and LC–MS/MS analysis of the d_5-CAs were conducted using the same system as that used in the analysis
277 of endogenous CA levels.
278
279

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284

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308

309

310

311 **Figure legends**

312

313 **Fig. 1.** Synthesis of d_5-*trans*/*cis*-cinnamic acid. (a) triethyl phosphonoacetate, NaH, THF, room
314 temperature, quantitative; (b) EtOH, 5 M NaOH, room temperature, 79%; (c) MeOH, UV(254 nm),
315 overnight.

316

317 **Fig. 2.** LC–MS/MS analysis of *trans*/*cis*-CA after sample preparation.

318

319 **Fig. 3**. LC–MS/MS analysis of endogenous CA and d_5-CA from Arabidopsis. Selected reaction
320 monitoring (left) and full-scan spectra of fragmented ions (right) of the unlabeled authentic standard,
321 d_5-CA purified from extracts, and endogenous CA.

322

323 **Fig. 4.** LC–MS/MS analysis of the endogenous levels of *trans*/*cis*-CA in Arabidopsis grown under

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324 fluorescent (FL) or LED light. Data are means ± SD (n=3). n.s. indicates not significant (p>0.05).

325

326 **Fig. 5** Analysis of *trans*-to-*cis* conversion of CA in Arabidopsis using d_5-*trans*-CA. LC–MS/MS
327 analysis was performed after feeding d_5-*trans*-CA to Arabidopsis seedlings under either fluorescent
328 (top three) or LED (bottom three) lights for 24 h (light/dark: 16 h/8 h).

329 **Table 1**. Quantitative analysis of CA in *O. sativa* (Nipponbare), *O. sativa* (Shiokari), *N. benthamiana*,
330 *S. lycopersicum*, *M. polymorpha.* Data are means ± SD (n = 3).

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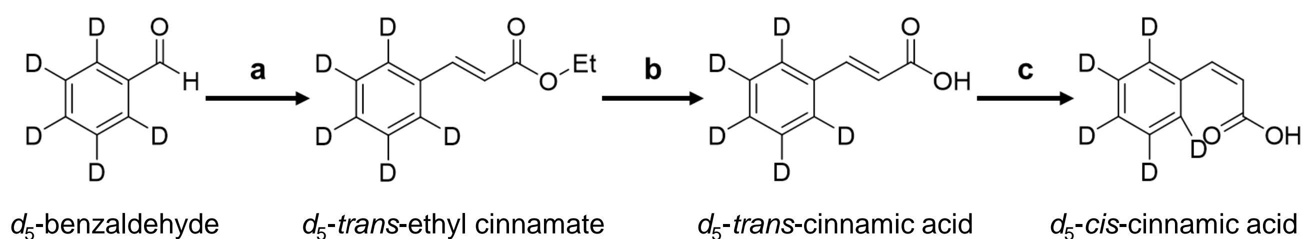

Fig. 1. Synthesis of d_5-*trans*/*cis*-cinnamic acid. (a) triethyl phosphonoacetate, NaH, THF, room temperature, quantitative; (b) EtOH, 5 M NaOH, room temperature, 79%; (c) MeOH, UV(254 nm), overnight.

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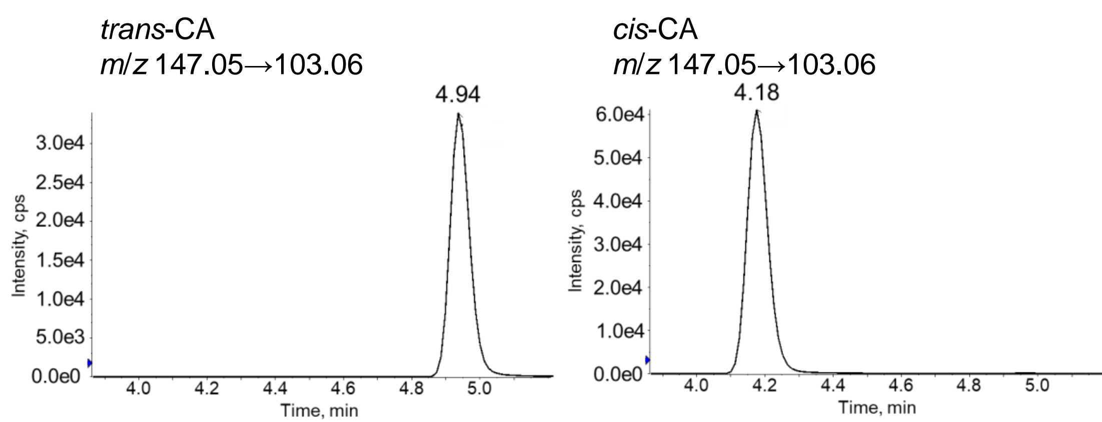

Fig. 2. LC–MS/MS analysis of *trans*/*cis*-CA after sample preparation.

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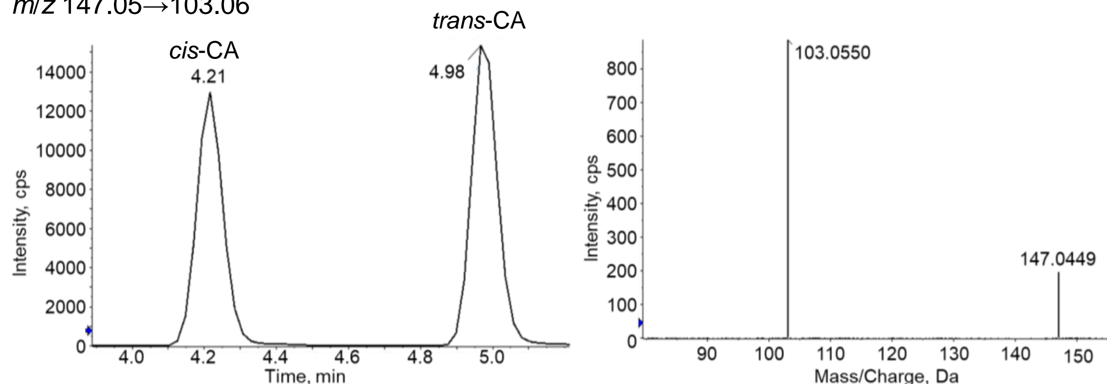
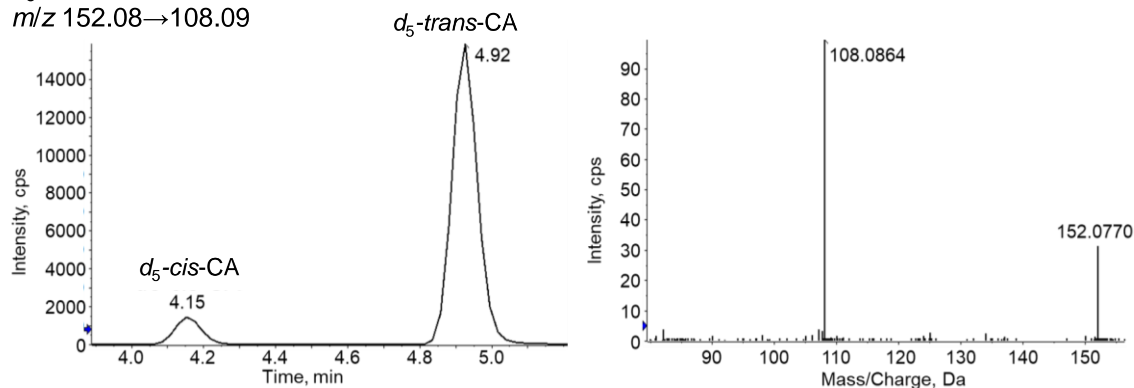
Unlabeled *trans*/*cis*-CA standard
m/*z* 147.05→103.06



d_5-*trans*/*cis*-CA (internal standards)
m/*z* 152.08→108.09



endogenous *trans*/*cis*-CA
m/*z* 147.05→103.06

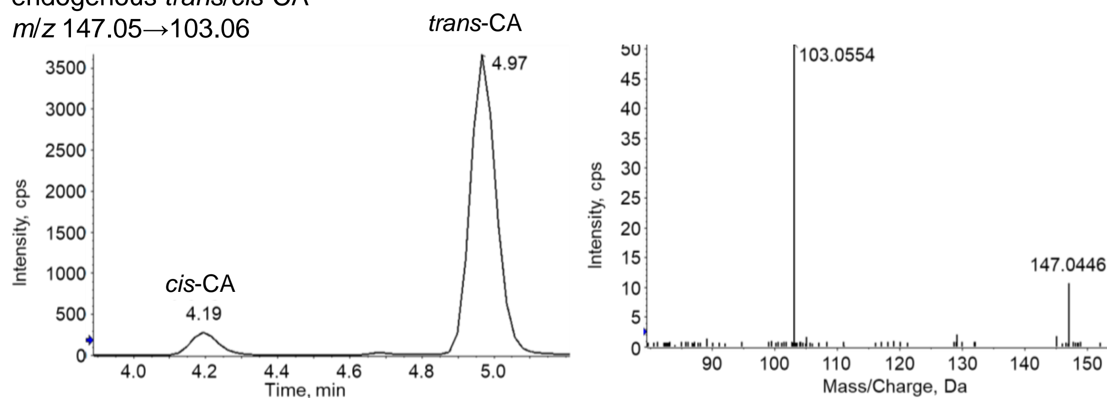


Fig. 3. LC–MS/MS analysis of endogenous CA and d_5-CA from Arabidopsis. Selected reaction monitoring (left) and full-scan spectra of fragmented ions (right) of the unlabeled authentic standard, d_5-CA purified from extracts, and endogenous CA.

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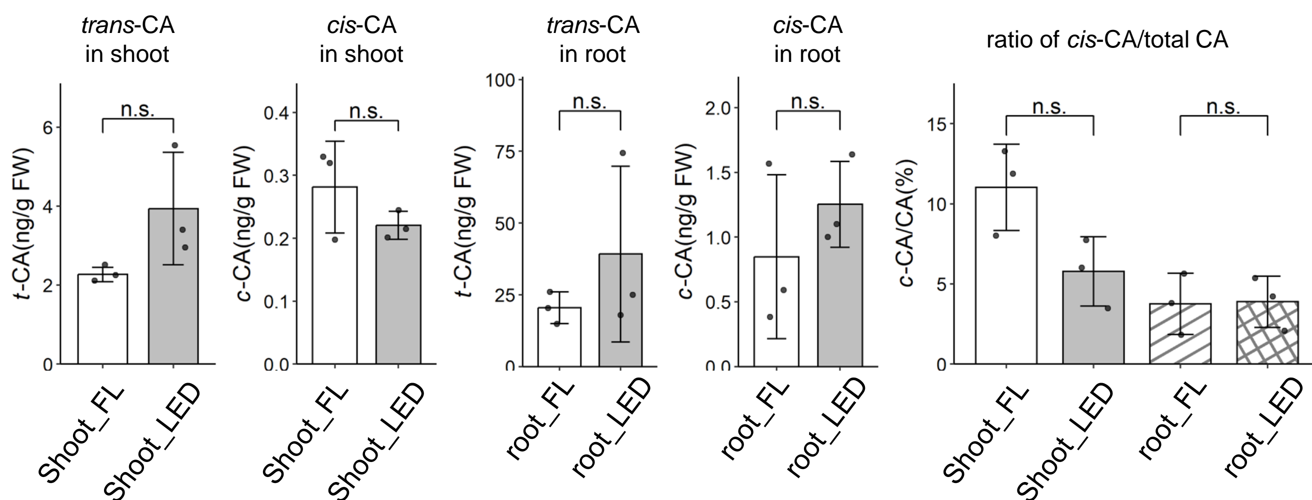

Fig 4. (**A**) **.** LC–MS/MS analysis of the endogenous levels of *trans*/*cis*-CA in Arabidopsis grown under fluorescent (FL) or LED lights. Data are means ± SD (n=3). n.s. indicates not significant (p>0.05).

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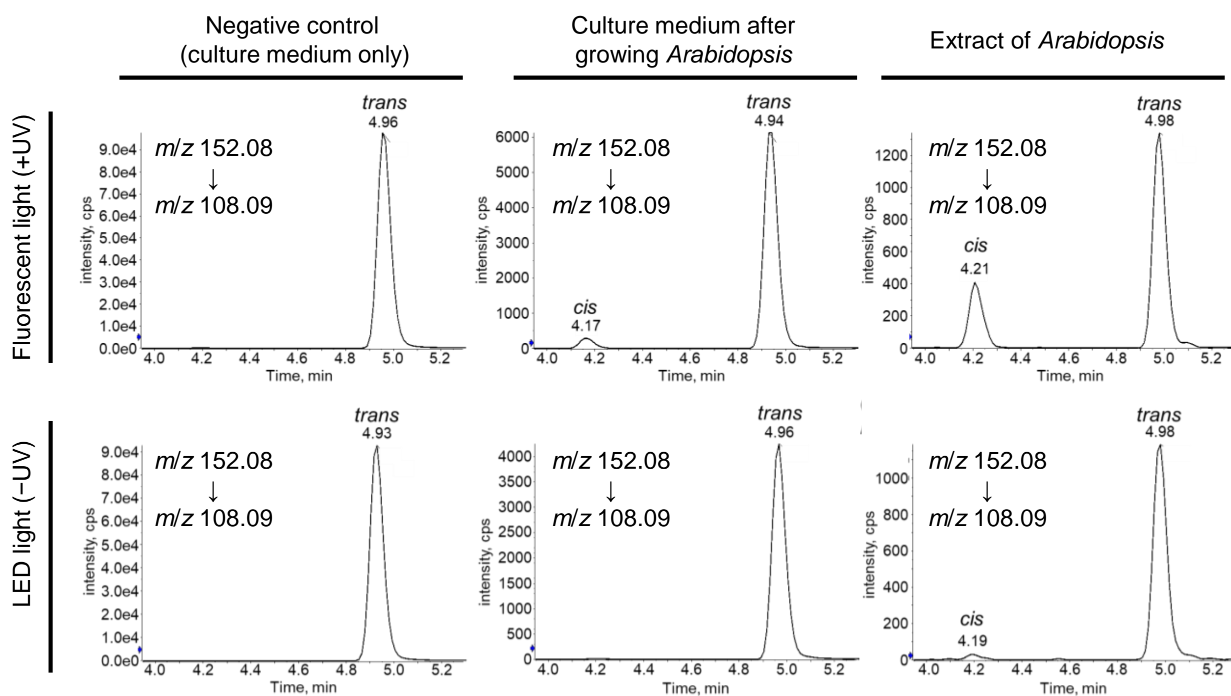

Fig. 5. Analysis of *trans*-to-*cis* conversion of CA in Arabidopsis using d_5-*trans*-CA. LC–MS/MS analysis was performed after feeding d_5-*trans*-CA to Arabidopsis seedlings under either fluorescent (top three) or LED (bottom three) lights for 24 h (light/dark: 16 h/8 h).

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Table 1. Quantitative analysis of CA in *O. sativa* (Nipponbare), *O. sativa* (Shiokari), *N. benthamiana*, *S. lycopersicum*, *M. polymorpha.* Data are means ± SD (n = 3).

		***trans*-CA (ng/g FW)**	***cis*-CA (ng/g FW)**	***cis*-CA/CA (%)**
***Oryza sativa* (Nipponbare)**	**shoot**	117.43 ± 6.38	0.523 ± 0.114	0.442 ± 0.0819
	root	15.25 ± 4.04	0.285 ± 0.0463	1.91 ± 0.472
***Oryza sativa* (Shiokari)**	**shoot**	31.38 ± 6.06	0.690 ± 0.158	2.20 ± 0.685
	root	18.81 ± 2.00	0.540 ± 0.0875	2.81 ± 0.565
Nicotiana benthamiana	**shoot**	1.31 ± 0.209	0.217 ± 0.0448	14.2 ± 1.92
	root	7.94 ± 1.24	1.50 ± 0.108	16.0 ± 1.18
Solanum lycopersicum	**shoot**	2.50 ± 0.353	0.248 ± 0.0398	9.16 ± 2.17
	root	5.82 ± 1.56	2.84 ± 0.873	32.6 ± 1.39
Marchantia polymorpha		1.24 ± 0.0971	0.624 ± 0.0272	33.4 ± 0.839