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Targeted Metabolomics of the Phenylpropanoid Pathway in *Arabidopsis thaliana* using Reversed Phase Liquid Chromatography Coupled with Tandem Mass Spectrometry

Rohit Jaini,[a] Peng Wang,[b] Natalia Dudareva,[b,c] Clint Chapple[b] and John A. Morgan[a,b]*

ABSTRACT:

Introduction – The phenylpropanoid pathway is a source of a diverse group of compounds derived from phenylalanine, many of which are involved in lignin biosynthesis and serve as precursors for the production of valuable compounds, such as coumarins, flavonoids, and lignans. Consequently, recent efforts have been invested in mechanistically understanding monolignol biosynthesis, making the quantification of these metabolites vital.

Objective – To develop an improved and comprehensive analytical method for (i) extensively profiling, and (ii) accurately quantifiying intermediates of the monolignol biosynthetic network, using *Arabidopsis thaliana* as a model system.

Method – A liquid chromatography-tandem mass spectrometry with electrospray ionization was developed to quantify phenylpropanoid metabolites in Arabidopsis wildtype and cinnamoyl CoA reductase1 (CCR1) deficient lines (*ccr1*).

Results – Vortexing at high temperatures (65°C) enhanced release of phenylpropanoids, specifically the more hydrophobic compounds. A pH of 5.3 and ammonium acetate buffer concentration of 2.5 mM resulted in an optimal analyte response across standards. Ion suppression was estimated using standard spike recovery studies for accurate quantitation. The optimized method was used to profile Arabidopsis wildtype and *ccr1* stems. An increase in hydroxycinnamic acid derivatives and a decrease in the hydroxycinnamyl aldehydes and alcohols in *ccr1* lines, supports a shift of flux from lignin synthesis to other secondary metabolites and phenylpropanoid derivatives.

Conclusions – Compared to existing targeted profiling techniques, our method is capable of quantifying a wider range of intermediates (15 out of 22 in WT Arabidopsis stems) at low *in vivo* concentrations (~50 pmol/g-FW for certain compounds), while requiring minimal sample preparation.

Supporting information can be found in the online version of this article.

Keywords: liquid chromatography; tandem mass spectrometry; phenylpropanoid pathway; plant metabolomics; matrix effects

Introduction

Lignin is an aromatic heteropolymer synthesised by radical polymerisation of hydroxycinnamyl alcohol monomers – also known as monolignols – the end products of the phenylpropanoid pathway (Fig. 1) (Vanholme *et al.*, 2008). This three-dimensional polymer imparts rigidity and strength to plant cell walls, enabling upright growth, and provides mechanical support and hydrophobicity to plant vasculature, facilitating transport of water and nutrients. While essential to plant viability, lignin impedes degradation of plant cell wall polysaccharides into simple sugars during their fermentation, making biofuel production from lignocellulosic feedstock a cost intensive process (Chapple *et al.*, 2007; Weng *et al.*, 2008). Therefore, the past two decades have witnessed several genetic engineering efforts targeting the phenylpropanoid pathway, especially in *Arabidopsis thaliana*, to alter lignin amount and composition (Wang *et al.*, 2015; Hood, 2016). Despite the progress that has been made, several questions pertaining to control and regulation of carbon flux in this pathway remain unanswered. Recent research efforts have hence been directed towards gaining a mechanistic understanding of lignin synthesis (Bonawitz and Chapple, 2010; Lee and Voit, 2010; Shi *et al.*, 2010). These systems biology driven studies call for accurate quantification of metabolites of the pathway (Moseley, 2013; Noack and Wiechert, 2014). In addition, many phenylpropanoids are industrially relevant products such as tannins, flavonoids, coumarins, and hydroxycinnamic

* Correspondence to: John A. Morgan, School of Chemical Engineering, Purdue University, West Lafayette, IN 47907, USA. Email: jamorgan@purdue.edu

[a] School of Chemical Engineering, Purdue University, West Lafayette, IN, 47907, USA

[b] Department of Biochemistry, Purdue University, West Lafayette, IN, 47907, USA

[c] Department of Horticulture & Landscape Architecture, West Lafayette, IN, 47907, USA

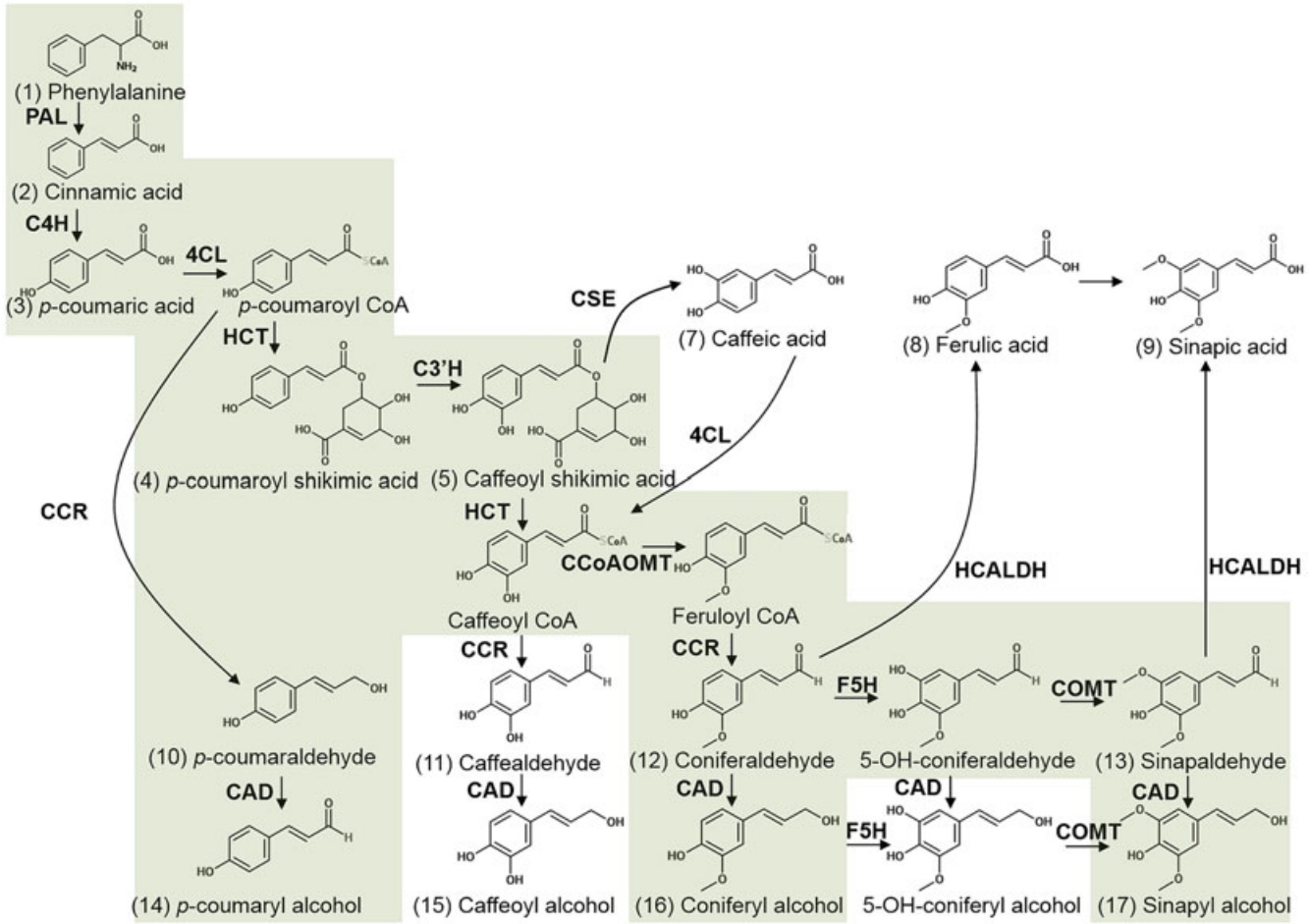

Figure 1. Schematic of the phenylpropanoid pathway leading to monolignol synthesis. The highlighted part of the pathway is considered the major route for lignin biosynthesis. Intermediates and enzymes currently known in lignin formation are indicated. 4CL, 4-(hydroxy)cinnamoyl CoA ligase; C3′H, pcoumarate 3′-hydroxylase; C4H, cinnamate 4-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; CCoAOMT, caffeoyl CoA O-methyltransferase; CCR, cinnamoyl CoA reductase; COMT, caffeic acid/5-hydroxyferulic acid O-methyltransferase; F5H, ferulate 5-hydroxylase; HCALDH, hydroxycinnamaldehyde dehydrogenase; HCT, hydroxycinnamoyl CoA:shikimate hydroxycinnamoyltransferase; PAL, phenylalanine ammonia-lyase. [Colour figure can be viewed at wileyonlinelibrary.com]

acid conjugates (Fraser and Chapple, 2011), further justifying their quantitation.

Mass spectrometry (MS)-based detection associated with various separation techniques [gas chromatography (GC), liquid chromatography (LC), capillary electrophoresis, etc.] has been widely employed in quantitative metabolic profiling, specifically in analysing plant metabolomes (Kueger *et al.*, 2012; Sumner *et al.*, 2015). Reversed phase high-performance liquid chromatography (RP-HPLC) coupled to electrospray ionisation mass spectrometry (ESI-MS) is known for achieving high selectivities and sensitivities (Kueger *et al.*, 2012). Furthermore, instruments equipped with a triple quadrupole (QqQ) allow for fast measurements, improved sensitivities and precise quantitations by multiple reaction monitoring (MRM) (Arrivault *et al.*, 2009). Although there is no dearth of analytical techniques in profiling intracellular intermediates, often the data reported is plagued with inaccuracies, which arise from sample handling starting from extract preparations to extract analysis on a detector. Accurate and reliable data can be obtained by addressing and improving various avenues in LC–MS based analytical methods such as (i) chromatography, (ii) sample preparation and extraction protocols, and (iii) matrix effects. The last decade has seen many studies on profiling phenolics – particularly the ones associated with the phenylpropanoid pathway (Meyermans *et al.*, 2000; Chen *et al.*, 2003; Damiani *et al.*, 2005; Lin *et al.*, 2009; Callipo *et al.*, 2010; Frolov *et al.*, 2013; Reuben *et al.*, 2013; Ferreres *et al.*, 2014; Eudes *et al.*, 2015; Šibul *et al.*, 2016). Nevertheless, these analytical methods have scope for improvement in one or more of the following: (i) canvassing a larger range of intermediates, (ii) accounting for signal suppression because of matrix effects, (iii) simplifying cumbersome extraction procedures.

In an effort to address the aforementioned issues and taking into consideration the sources of error that occur during sample handling and analysis; we developed a rapid, sensitive, reproducible, and an accessible analytical method for the accurate quantitation of the metabolites of the monolignol synthesis pathway using *A. thaliana* as our model system. The effects of chromatographic conditions, such as optimal pH, buffer concentration, column temperature, etc. on analyte responses were investigated. Sample preparation and extraction protocols were tuned to efficiently extract soluble intermediates from *A. thaliana* stem tissue. Ion suppression caused by matrix effects was evaluated by spiking plant extracts with a known concentration of standards. Finally, the analytical method was applied to profile stems from *A. thaliana CCR1* T-DNA insertional lines (*ccr1*) and wild-type (WT) plants.

Materials and methods

Chemicals

L-Phenylalanine (>99%), *trans*-cinnamic acid (>99%), *p*-coumaric acid (>98%), caffeic acid (>98%), ferulic acid (>99%), sinapic acid (>99%), shikimic acid (>99%), coniferyl aldehyde (>98%), sinapaldehyde (98%), coniferyl alcohol (98%), sinapyl alcohol (80%), *p*-fluoro-DL-phenylalanine (>98%), ammonium acetate (>98%) and HPLC grade acetonitrile (ACN) were purchased from Sigma Aldrich (St Louis, MO). *p*-Coumaraldehyde, *p*-coumaryl alcohol, caffeoyl alcohol and caffealdehyde were synthesised at Discovery Park, Purdue University (West Lafayette, IN). *p*-Coumaroyl shikimate and caffeoyl shikimate esters were acquired from Prof. John Ralph at the University of Wisconsin-Madison (Madison, WI). Glacial acetic acid (>99.7%) was purchased from Mallinckrodt Chemicals (Phillipsburg, NJ) while HPLC-grade methanol was purchased at Macron Fine Chemicals (Center Valley, PA). Water used for making mobile phase solutions was purified using a Barnstead Nanopure Infinity ultrapure water system. All chemicals were used without further processing or purification.

Plant material

Columbia-0 and the *ccr1* mutant *Arabidopsis* plants were grown in growth chambers (West Lafayette, IN) at 23°C under 16/8 hour day/night conditions and light intensity of 100 μE/m^2 s. Stems used for analysis were harvested from five-week old plants. The T-DNA mutant *ccr1* (SALK_123689) was obtained from the Arabidopsis Biological Resource Centre. Homozygous *ccr1* mutant was confirmed by polymerase chain reaction (PCR) with primers cc2550 (5′-GTG TCG TAG AGG CTT TGC TTG-3′), cc2551 (5′-TTG TGG AAA TAT TTC CGG TTG-3′), and cc2449 (5′-ATT TTG CCG ATT TCG GAA C-3′).

Standard solutions

The core monolignol biosynthetic pathway has 22 compounds (Fig. 1) of which standards for 17 of them were available and considered in the current study. Stock solutions of standards for calibration and determination of limits of detection (LODs) were prepared at a concentration of 0.5 mg/mL in methanol. In the case of shikimic acid, 50/50 (%*v*/*v*) methanol/water solution was used owing to its immiscibility in methanol at that concentration. Standard mixtures containing all 17 available compounds were prepared at six different concentrations, approximately ranging from 50 nM to 500 μM, for calibration. *p*-fluoro-DL-phenylalanine was used as an internal standard (IS). All extraction solvents used for the study were prepared with a known concentration of the IS.

Extraction and concentration of soluble metabolites

The basal 0–2 cm fragments of *A. thaliana* stems were harvested and frozen in 2 mL eppendorf tubes using liquid nitrogen. Each biological replicate contained four *Arabidopsis* stems, which allowed (i) to obtain higher yields of the secondary metabolites and (ii) to reduce biological variation. To determine the most suitable extraction solvent composition, different concentrations of methanol in water were employed keeping the sample preparation procedure the same.

Stem tissue was pulverised using a pestle in a 2 mL eppendorf tube and 10 μL of solvent was added for every milligram of fresh weight (FW) of tissue harvested. The extraction solvents were prepared with the IS at a concentration of 0.001 mg/mL to account for extraction recoveries. The samples were then vortexed for 30, 60 or 120 min, using a Midwest Scientific Benchmark Multi-Therm shaker (Valley Park, MO) followed by centrifugation at 18000 rpm for 15 min. The supernatants from each sample were dried under a vacuum at 30°C using a LABCONCO centrifugal evaporator (Kansas City, MO). The residues were re-dissolved in 60 μL of the extraction solvent and transferred to a standard HPLC vial. Subsequently, 10 μL was injected into the HPLC-MS/MS system for analysis.

Ion suppression

The extent of ion suppression was quantified using the standard spike recovery method. The dried residues (obtained after extraction from biomass) were redissolved in 60 μL of the extraction solvent and divided into two parts. One was spiked with 50% (*v*/*v*) methanol solution and the other was spiked with a stock solution containing a known concentration of available standards. The study was conducted at three different concentrations of standard compounds in the stock, namely two-, three- and five-fold of the concentrations observed in *A. thaliana* stem extracts before accounting for matrix effects. The sample spiked with the extraction solvent (**S**), sample spiked with the stock solution (**SSt**), and the standard stock solution (**Std)** were individually injected into the HPLC-MS/MS system for analysis. The recovery factor (f_i) for each metabolite was computed using Equation (1), where A_{SSt} is the integrated area of a compound in the samples spiked with the stock solution, A_S is the area of a compound in the sample spiked with the extraction solvent, A_{std} is the area of a compound in the standard mixture, *i* indicates a specific intermediate and 2 is the dilution factor. The analysis was done in triplicate. It should be noted that the recovery factors were estimated after taking into account the recovery of the IS added prior to extraction.

$$f_i = 2\times \frac{A_{SSt_i} - A_{S_i}}{A_{Std_i}} \quad (1)$$

Metabolomics using LC-MS/MS

Chromatography was performed on an HPLC-20 AD system from Shimadzu (Columbia, MD) comprising of a quaternary pump, an autosampler, a thermostat controlled column compartment, and a photo diode array detector. Chromatographic separations were performed on a Zorbax Eclipse C8 column (150 mm × 4.6 mm, 5 μm, Agilent Technologies, Santa Clara, CA) at a column temperature of 30°C and a flow rate of 1 mL/min. The injection volume was set to 10 μL. A linear gradient of aqueous solvent A (2.5 mM ammonium acetate in water, adjusted to pH 5.3 using glacial acetic acid) and organic solvent B (98% acetonitrile, 2% water and 0.02% formic acid) was used as follows: 10% B (*v*/*v*) for 1 min, 10–20% B over 3 min, 20–20.8% B over 9 min, 20.8–50% B over 1 min, 50–70% B over 1 min, hold at 70% B for 3 min, return to 10% B over 1 min, and equilibrate for 4 min at 10% B resulting in a total run time of 23 min per sample. The gradient was unchanged for all aqueous mobile phases considered for the study.

Metabolite profiling was performed using a QTrap 5500 triple quadrupole mass spectrometer from AB Sciex (Redwood City, CA), operating in the negative ion mode. The mass spectrometer

Table 1. Retention time (RT), mass transition Q1/Q3 (*m/z*), and limits of quantification (LOQs) data for the phenylpropanoid pathway intermediates[a]

Number[b]	Metabolite	RT (min)	Q1 $[M-H]^-$	Q3 $[M-H]^-$	R^{2c}	LOQ[d] (μM)
1	Phenylalanine	2.53	164.0	147.0	0.99	0.04
2	Cinnamic acid	16.2	147.0	103.0	0.98	26.5
3	*p*-Coumaric acid	7.13	163.0	119.1	0.99	0.10
4	*p*-Coumaroyl shikimate	7.64	319.2	163.1	0.99	0.05
5	Caffeoyl shikimate	6.02	335.2	179.1	0.99	0.01
6	Shikimic acid	1.54	173.0	93.0	0.99	0.18
7	Caffeic acid	5.16	179.0	135.0	0.98	0.02
8	Ferulic acid	7.87	193.1	178.1	0.99	0.26
9	Sinapic acid	8.49	223.1	208.1	0.99	0.15
10	*p*-Coumaraldehyde	11.0	147.0	129.0	0.99	0.01
11	Caffealdehyde	7.41	163.0	145.0	0.97	0.30
12	Coniferaldehyde	12.1	177.1	162.0	0.99	0.12
13	Sinapaldehyde	11.6	207.1	192.1	0.98	0.03
14	*p*-Coumaryl alcohol	6.87	149.1	131.0	0.99	2.66
15	Caffeoyl alcohol	5.25	165.1	147.0	0.98	0.17
16	Coniferyl alcohol	7.48	179.1	146.0	0.99	0.03
17	Sinapyl alcohol	7.23	209.1	194.1	0.99	0.68

[a]Analysis was performed using an AbSciex QTrap 5500 mass spectrometer coupled to Shimadzu RP-HPLC system.
[b]Metabolite annotation as represented in Fig. 1.
[c]Correlation coefficients from linear fits of standard calibration curves covering a concentration range of ~50 nM to 500 μM.
[d]The LOQs are reported as values at which a signal-to-noise ratio (*S/N*) of 10 was obtained.

is equipped with an ESI-Turbolon-spray interface and all data analysis was conducted using Analyst 1.5.1 software. A low pressure of 1.5×10^{-5} torr was maintained in the QTrap 5500 vacuum manifold as indicated by the pressure gauge. The source parameters for the MS were set as follows: curtain gas flow rate, 25 l/h; collision gas, low; ion source voltage, -4.5 kV; desolvation temperature, 700 K; ion source gas 1, 60 l/h; ion source gas 2, 40 l/h. ESI parameters for every standard, such as declustering potential (DP), entrance potential (EP), collision energy (CE), and cell exit potential (CXP) were manually tuned (Supporting Information Table S1). Metabolite recoveries were recorded by subjecting standard mixtures to the extraction protocol (Table S2).

Linearity and sensitivity

Linearity of standard responses was expressed in terms of the correlation coefficient obtained as a result of a linear fit of the peak areas against the concentrations of the metabolites used for the study.

The measure of sensitivity of the analytical technique was reported as LODs and limits of quantification (LOQs). These are designated as the concentration of analyte injected that would result in a signal-to-noise ratio (*S/N*) of 3 and 10, respectively (ICH, 2005). The LOQs along with the correlation coefficients for all the 17 standards are presented in Table 1. Other metabolites, such as the hydroxycinnamic acid derivatives were profiled using ESI parameters of the corresponding hydroxycinnamic acid as standards were not available. The putative retention times and confirmed mass transitions for these compounds have been reported in Table S1.

Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA) for independent samples using the online calculator on vassarstats.net/ (Vassar College, Poughkeepsie, NY). A p-value <0.05 was considered as a significant difference. Tukey's HSD test was employed as a *post hoc* test to determine the differences between means. Standard Student's *t*-test was applied to analyse differences between individual metabolite concentrations of *Arabidopsis ccr1* and WT stems. The *p*-values of 0.003 have been used after applying the Bonferroni correction to establish a significant difference.

Results and discussion

Improving analyte responses by manipulating chromatography

Chromatographic conditions, such as mobile phases, buffer pH, buffer concentration, solvent flow rate, and column temperature, in addition to achieving metabolite separation also affect metabolite responses when associated with ESI-MS (Bajad *et al.*, 2006). A flow rate of 1 mL/min is suggested given the column dimension used for the study and ACN was used as the organic buffer due to its high eluotropic nature and low viscosity (Giorgianni *et al.*, 2004). As a result, in this study we focussed on the effects of buffer pH and buffer concentration on analyte responses. The solvent gradient, as described in the methods section, was optimised for resolution enough to prevent co-elution of multiple compounds in order to minimise their contribution to ion suppression (Fig. 2). Using MRM mode does not require strict baseline separation of standards as long as the *m/z* ratios of the parent (Q1) and fragment (Q3) ions are distinct. As a result, all the optimisation strategies considered in the following sections were motivated to obtain higher analyte responses instead of improving resolution. Standard mixtures containing all 17 compounds at a concentration of 0.01 mg/mL – which is within the linear range of calibration – were used for the optimisation studies.

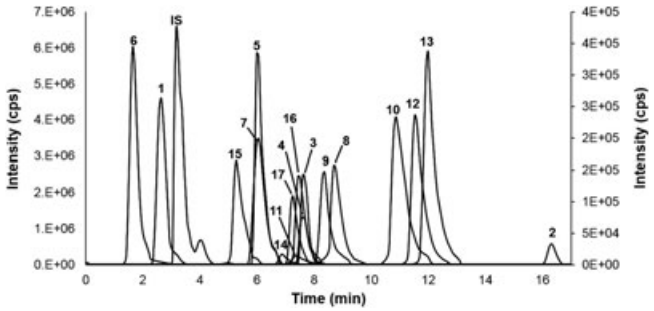

Figure 2. Chromatogram obtained by HPLC-MS/MS profiling of a standard mixture of 17 phenylpropanoid metabolites (0.01 mg/mL each). Separation was performed on a Zorbax-Eclipse C8 column (150 mm × 4.6 mm, 5 μm) using 2.5 mM ammonium acetate in water (pH of 5.3) as solvent A and ACN/H_2O/HCOOH (98/2/0.02% – *v*/*v*) as solvent B. Two different *y*-axes were used to accommodate metabolites with very high responses. Compound intensities are reported in counts per second (cps). Metabolites are marked according to Table 1.

Effect of mobile phase pH. Mobile phase pH plays an essential role in the dissociation/deprotonation of analytes and also the ionisation of the silanol groups of the column support (Hashem *et al.*, 2011), thereby altering the selectivity and retention on the column (Liigand *et al.*, 2014). The buffers in this study were prepared at a pH of 5, 5.3 and 5.6, which fall within 1 unit of the pK_a of acetate thus ensuring effective buffering capacity (Snyder *et al.*, 2010). The largest change in retention times was observed for the hydroxycinnamic acids in the pathway when their pK_a values (~4.5) were close to the buffer pH. Changing the buffer pH from 5 to 5.6 can cause a four-fold increase in the degree of ionisation, as per the Henderson–Hasselbalch relation, thereby altering the retention time. As expected, no significant change in retention time was noted for the extremely polar (phenylalanine and shikimic acid) or hydrophobic compounds (hydroxycinnamyl aldehydes and alcohols). In other words, the pK_a values for the former are too low and the latter are too high compared to the buffer pH such that they either are entirely ionised or neutral. Interestingly, higher responses were observed across all metabolites at the lower pH values of 5 and 5.3 (Fig. S1). A buffer pH of 5.3 resulted in statistically higher intensities for 11 out of 17 standard compounds relative to pH 5.6 and 6 out of 17 compounds relative to pH 5 (Fig. S1). Such a relation between mobile phase pH and analyte response in the negative ion mode, termed as wrong-way-around (Hua *et al.*, 2012), was previously observed. An acidic mobile phase provides excess protons that are reduced to hydrogen gas during ESI. The excess negative charges thus accumulate and increase the local pH value eventually aiding in the deprotonation of analytes. All further experiments were performed using a buffer of pH 5.3.

Effect of buffer concentration. The competition between the analyte ion and the buffer anion has also been known to affect the retention factor (Hashem *et al.*, 2011) and sensitivity of the analyte (Constantopoulos *et al.*, 1999). In order to test the effect of electrolyte concentration on analyte response, standard mixtures were analysed using buffer solutions consisting of 2.5, 5 and 10 mM ammonium acetate that were adjusted to a pH of 5.3. Higher salt concentration in the mobile phase led to a decrease in retention times and hence lowering the resolution of the analytes. Retention times were not significantly affected but a considerable signal enhancement was observed at buffer concentrations of 2.5 and 5 mM relative to 10 mM across all metabolites (Fig. S2). A signal enhancement of at least 1.5-fold for all metabolites and up to three-fold in the case of sinapic acid was obtained at a buffer concentration of 2.5 mM (Fig. S3). The reduction in signal response at a higher concentration may be due to the competition between the analyte ions and the buffer counter anion during ESI (Constantopoulos *et al.*, 1999). Buffer concentrations below 2.5 mM were not considered due to a risk of lowering the effective buffering capacity (Snyder *et al.*, 2010) of the mobile phase and increasing the method run time. For all further studies, buffers with salt concentration of 2.5 mM and a pH 5.3 were employed.

Effect of column temperature. A higher column temperature offers several advantages such as (i) faster method runs, (ii) reduced pressure drop, (iii) improved peak shapes (Yang *et al.*, 2002), and (iv) increased resolution. We performed studies at column temperatures of 30°C and 40°C to observe the effects on analyte resolution and response. No statistical difference was observed in the signal response across all metabolites except sinapaldehyde (**13**, Fig. S3). The 10°C temperature rise marginally reduced the retention times of most metabolites, while cinnamic acid still eluted at around 16 min (data not shown). Since no significant advantages were incurred using a higher column temperature, all experiments were performed at 30°C.

Improving extraction of soluble phenylpropanoids from *A. thaliana* stem tissue

Efficient solid–liquid extraction of soluble metabolites is governed by many factors, such as extraction technique used, solid-to-liquid ratio, tissue size, solvent composition, temperature, extraction duration, number of repeated extractions (Dent *et al.*, 2013; Khoddami *et al.*, 2013). This necessitates optimisation of extraction conditions to achieve complete extraction of desired metabolites. As part of our preliminary studies, we tested several extraction techniques such as vortexing, bullet blending and ultrasonication on pulverised *Arabidopsis* stems at room temperature for a fixed duration and observed no statistical differences (data not shown). Vortexing has been used in all the previously discussed experiments as it is gentle on metabolites and ensures constant suspension (mixing) of plant tissue. A solvent-to-tissue ratio of 10 μL/mg was used for extraction, which is sufficient for standard sample preparation in targeted metabolomics (Römisch-Margl *et al.*, 2012). As a result, we focussed mainly on optimising the extraction solvent composition, temperature, and duration of extraction.

Effect of extraction solvent composition. Composition of an extraction solvent has an inevitable intrinsic bias towards certain metabolite classes given the vast chemical diversity of the plant metabolome (Hall, 2006). Consequently, it is necessary to use a solvent system that maximises the number and amount of metabolites extracted. Previous studies have shown that methanol/water solutions best meet the demands of a chemically heterogeneous system such as the phenylpropanoid pathway (De Vos *et al.*, 2007; t'Kindt *et al.*, 2008). We therefore investigated the effects of methanol concentration on metabolite extraction using 50% (*v*/*v*) methanol in water (Control), 75% methanol in water (M75), or double extraction with pure methanol followed by a wash in 50% (*v*/*v*) methanol in water (MD). Extraction was carried out for 60 min at room temperature (25°C). The one-way ANOVA analysis on four replicates in each case resulted in no significant effect of methanol concentration on the extraction of phenylpropanoid metabolites

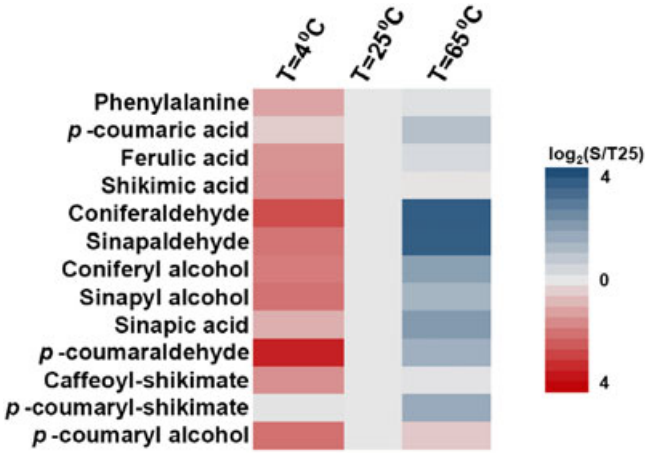

Figure 3. Heat map depicting metabolite fold changes as a result of extraction temperature. Data presented as $\log_2$(abundance in sample/abundances at 25°C). Data are fold changes from biological replicates ($n = 4$). [Colour figure can be viewed at wileyonlinelibrary.com]

(Table S3). Only nine metabolites were detected above their LOQs as a result of the extraction (Fig. S4). Although no statistical variations were observed, 75% (*v/v*) methanol in water was chosen as the solvent for all subsequent experiments to ensure deactivation of plant enzymes since fresh tissue is used for extraction.

Effect of extraction temperature. Higher temperatures favour solute dissolution into the extraction solvent and increase solvent accessibility to plant tissue due to a reduced viscosity (Dent *et al.*, 2013) but there is a trade-off due to certain metabolites being labile to high temperatures. Thus, to test the effect of temperature on metabolite extraction, samples were vortexed at 4, 25 and 65°C for 60 min. One-way ANOVA analysis indicated a significant effect of temperature on metabolite extraction (Table S3), with higher temperatures favouring metabolite extraction. Extraction at 65°C resulted in almost a 10-fold improvement in coniferaldehyde and sinapaldehyde pool sizes, while coniferyl and sinapyl alcohols, sinapic acid and *p*-coumaraldehyde showed two- to four-fold improvements compared to that at room temperature (Fig. 3, Fig. S5). It should be noted that the more hydrophobic metabolites of the pathway showed the most improvement at higher temperatures. The compounds being more hydrophobic may preferably partition into the cell membrane than into methanol/water solution. Higher temperatures may improve analyte solubilities in methanol/water mixture as well as disintegrate cellular membranes enhancing their release into the solvent. In light of these findings, extraction in all further experiments was carried out at 65°C.

Effect of extraction duration. The amount of analyte released may also be a function of extraction time if its extraction is kinetically limited (Gonzales *et al.*, 2014). To investigate this, stem tissue was vortexed at 65°C for 30 min (ED30), 60 min (ED60), and 120 min (ED120). Our results revealed no statistical effect of duration of extraction on increasing metabolite yields (Fig. S6, Table S3). This indicates that the extraction of phenylpropanoids is not limited by its dissolution kinetics in the solvent when vortexing at a high temperature, as 30 min suffices complete extraction of metabolites. Accordingly, all further sample preparations were conducted by vortexing tissue at 65°C for 30 min. It should be noted that there might be interaction effects considering the number of parameters optimised in this study. Capturing such interactions may lead to an improved response in the form of LODs and LOQs. These are the subjects of future work.

Ion suppression due to matrix effects

Tandem MS, albeit offering crucial advantages for compound quantitation like high selectivity, sensitivity, and throughput, finds its Achilles heel in matrix effects (Taylor, 2005). The alteration in ionisation efficiency of an analyte at the electrospray interface due to a co-eluted or co-extracted compound(s) is termed a matrix effect. This phenomenon causes analyte signal suppression

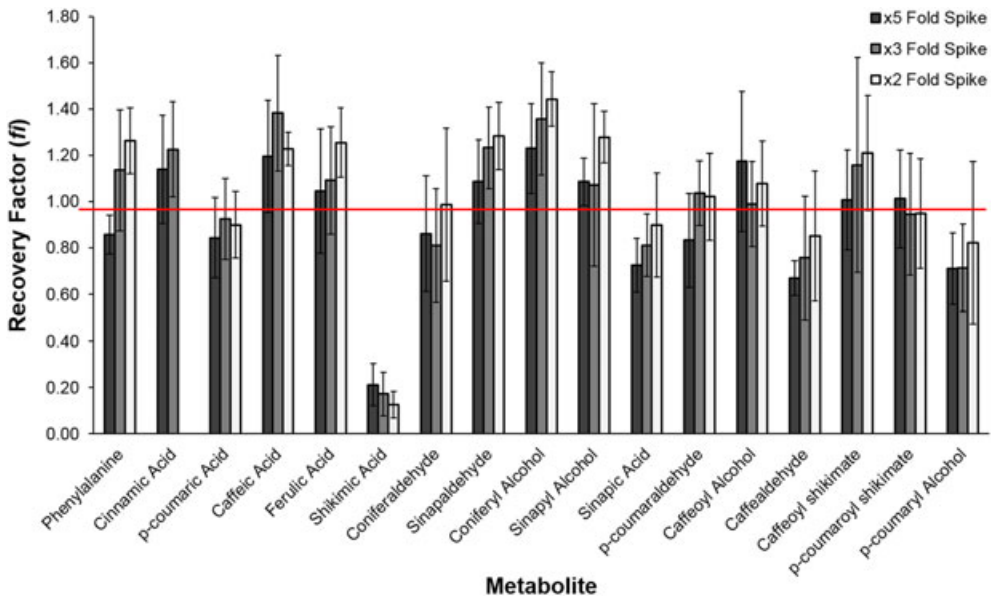

Figure 4. Ion-suppression recovery factors obtained by spiking the tissue extract with stock solution containing all standards at two-, three- and five-fold of their endogenous concentrations. Data are means ± standard deviation (s.d.) ($n = 4$ biological replicates). *$p < 0.05$ and **$p < 0.001$ obtained by Tukey's HSD post ANOVA test. [Colour figure can be viewed at wileyonlinelibrary.com]

leading to incorrect quantitation of compounds of interest. Although the exact mechanism of matrix effects is still debated, it is largely believed to originate because of a competition between analyte and co-eluting matrix components during ESI. These matrix components may be endogenous species (extracted from biomass) or mobile phase additives (Gosetti *et al.*, 2010). Various approaches have been proposed to minimise ion suppression or account for its effects (Yaroshenko and Kartsova, 2014). One is reduction of injected sample volume or the dilution of the samples, but this hinders the ability to detect certain metabolites. Another approach is reduction of ion suppression by choosing an appropriate sample preparation procedure such as protein precipitation, solid phase extraction, liquid phase extraction, etc. Often these methods lead to a decrease or an increase in matrix effects, loss of analytes during extraction and hinder high throughput analysis of biological samples. Addition of an internal standard

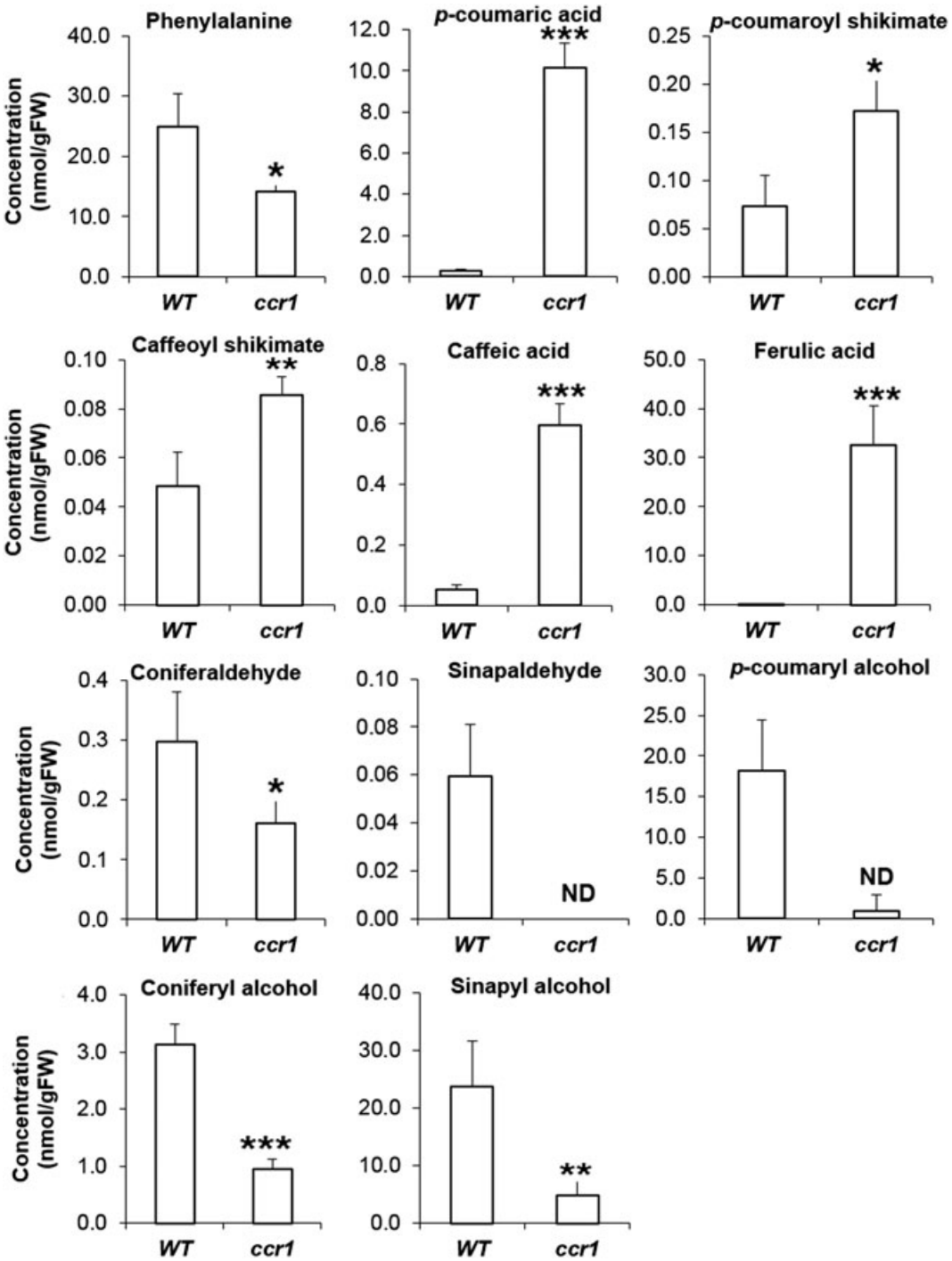

Figure 5. Pool sizes of phenylpropanoid pathway intermediates in WT and *ccr1* lines of *A. thaliana* stem tissue. Data of metabolites presented as means ± s. d. ($n = 4$ replicates). Analyte responses normalized to fresh weight of tissue. *$p < 0.05$, **$p < 0.001$, and ***$p < 0.0001$ obtained using the standard Student's *t*-test. The *p*-value established after the Bonferroni correction is 0.003, indicating that metabolites marked as ** and *** are significantly different. Only metabolites with significant differences between the two lines have been reported in the figure.

(Berg and Strand, 2011), either structurally similar to an analyte or labelled with stable isotope, that co-elutes with the analyte can account for losses due to signal suppression. However, this may fail or prove to be expensive when profiling multiple compounds spanning a wide range of chemical properties. Taking all factors into consideration we studied the matrix effects by a standard spike recovery method (Mathias *et al.*, 2014), also known as post-extraction addition, as described in the methods section. The experiments were conducted with three different spike solution concentrations, namely ×2, ×3, and ×5 fold of the endogenous concentrations of metabolites.

The study was done in four replicates and recovery factors (f_i) for all metabolites were determined accordingly. A lower f_i value is indicative of a significant suppression in signal due to matrix effects, while a value close to one indicates almost complete recovery of the spiked metabolite. Data from *Arabidopsis* WT extracts showed no statistical trend of the recovery factors of phenylpropanoid metabolites across different concentrations of the spike solution (Fig. 4). Shikimic acid suffered the highest signal suppression (f_i = 0.2), while sinapic acid, caffealdehyde, and *p*-coumaryl alcohol resulted in recoveries between 70 and 80% (Fig. 4). Reliable recovery factors could not be estimated in case of cinnamic acid due to the spike solution concentrations being close to its LOD, and hence have not been reported. The remaining pathway intermediates were almost fully recovered. Shikimic acid, due to its highly polar nature, elutes first from the column at 1.64 min (Table 1), close to the residence time of un-retained metabolites. This causes it to elute with a host of other endogenous polar metabolites extracted from the plant that may compete for ionisation at the electrospray interface. As a result, only 20% of the added shikimic acid was recovered from the spiked sample implying that the true shikimic acid concentrations may be five-fold higher. Improved chromatographic conditions, individually optimised ESI parameters and use of negative ion mode (Antignac *et al.*, 2005) have been conducive in obtaining close to no signal suppression for a majority of the metabolites analysed.

Metabolite profiling of *Arabidopsis* WT and *ccr1* lines

CCR (EC 1.2.1.44) catalyses the NADPH-dependent conversion of hydroxycinnamoyl-CoA esters to their respective aldehydes, a crucial step in monolignol biosynthesis (Fig. 1) (Fraser and Chapple, 2011). Two *Arabidopsis* enzymes, CCR1 and CCR2, have been kinetically characterised showing high affinity to feruloyl-CoA and lower affinities for sinapoyl- and caffeoyl-CoA (Lauvergeat *et al.*, 2001; Baltas *et al.*, 2005). It has been previously shown that CCR1 has a greater catalytic efficiency in converting feruloyl-CoA to coniferaldehyde and is primarily involved in lignin synthesis due to its high expression in stem tissue. CCR2, however, is barely detectable under normal growth conditions and is hypothesised to be involved in pathogen induced lignification (Lauvergeat *et al.*, 2001). CCR1 knockout plants exhibit a dwarf phenotype, with collapsed xylem vessels, significant reduction in total lignin content and change in composition (Mir Derikvand *et al.*, 2008; Ruel *et al.*, 2009), and altered cell wall cohesion leading to improved saccharification efficiency (Van Acker *et al.*, 2013). Given the drastic phenotype invoked as a result of the T-DNA insertion and that CCR1 is required for the production of monolignols, we proposed to profile *ccr1* to visualise changes in the metabolite abundances within the phenylpropanoid pathway. The CCR1 deficient lines were analysed using the optimised analytical technique and compared with WT *Arabidopsis* plants.

Our analysis of stem tissue from *ccr1* lines revealed a significant increase in pools of the hydroxycinnamic acids, *p*-coumaric acid (~35-fold), caffeic acid (~12-fold), sinapic acid (~two-fold), with the highest increase observed in ferulic acid (~200 fold, Fig. 5, Table S4) with respect to the WT stems. Simultaneously, a marked reduction in pools of the hydroxycinnamyl aldehydes and alcohols was detected, with *p*-coumaraldehyde and *p*-coumaryl alcohol approaching their LODs (Fig. 5, Table S4). In addition, we saw an increase in hydroxycinnamic acid derived esters, such as sinapoyl glucose and feruloyl glucose, in agreement with previous attempts in profiling *ccr1* mutant lines (Mir Derikvand *et al.*, 2008; Vanholme *et al.*, 2012). Although ferulic and sinapic acid synthesis occurs via the action of aldehyde dehydrogenases on their respective aldehydes (Fig. 1), it was interesting to see high levels of these acids when their aldehyde precursors have been depleted. This may be reconciled given that a large increase in the caffeic acid pool can invoke ferulic acid synthesis via COMT by outcompeting (K_m = 24.2 μM; Moinuddin *et al.*, 2010) the other substrates. In addition, the feruloyl-CoA esters, accumulated as a result of the knockout, can be hydrolysed to ferulic acid by the action of putative thioesterases (Mir Derikvand *et al.*, 2008). A part of the ferulic acid so formed can be further hydroxylated by F5H (K_m = 1 mM; Humphreys *et al.*, 1999) to 5-hydroxyferulic acid followed by its methylation via COMT (K_m = 31.6 μM; Moinuddin *et al.*, 2010) to sinapic acid (Fig. 1). Given the concentration of ferulic acid observed (Table S4), it is likely that the synthesis of sinapic acid via the suggested route may occur in spite of ferulic acid's low binding affinity to F5H. Apart from the hydroxycinnamic acid derived esters, other phenylpropanoid derivatives, such as certain kaempferol glucosides were also previously shown to accumulate in *ccr1* (Mir Derikvand *et al.*, 2008). This is reasonable given the large accumulation of *p*-coumaric acid, a precursor to flavonoids. Overall, these results strongly suggest a shift away from lignin synthesis to that of hydroxycinnamic acid-esters and other secondary metabolite derivatives of the phenylpropanoid pathway, in stems of *ccr1*.

Summary

We developed a novel and comprehensive LC-MS/MS based analytical method for quantifying intermediates of the phenylpropanoid pathway. In this study, we presented a systematic strategy that optimised every unit operation starting from sample preparation to compound detection by MS. Manipulating chromatographic conditions resulted in a 1.5–5-fold increase in analyte responses across standards considered for the study with a buffer pH of 5.3 and buffer concentration of 2.5 mM being the optimal. Extraction studies showed that vortexing at high temperature results in higher yields of analytes. Although no significant effect of the solvent composition or the extraction duration have been observed, this is highly dependent on the metabolites of interest as well as the model system. Quantifying signal suppression due to matrix effects indicate a considerable loss of the shikimate signal due to its co-elution with a host of other polar endogenous metabolites. Applicability of our method was tested by quantifying phenylpropanoid intermediates in WT and *ccr1* lines of *A. thaliana*. Our findings were congruent with previous studies profiling WT stems, and to our knowledge, this is the first study presenting absolute concentrations of phenylpropanoid pathway metabolites in *ccr1* lines of *Arabidopsis*. Of the 17 metabolites in the core metabolite network that have been considered, this study quantifies *in vivo* concentrations of 15 compounds in *Arabidopsis* that

is higher compared to previous reports of seven (Reuben *et al.*, 2013) or eight (Eudes *et al.*, 2015) metabolites. In addition, this method is able to detect hydroxycinnamic acid derivatives like sinapoyl glucose, sinapoyl malate, feruloyl glucose, and feruloyl malate, and allows for conducting stable isotope labelling experiments demonstrating its potential application in detecting products of enzyme assays, hydrolysis of cell-wall bound phenolics, lignin degradation, and systems biology efforts in profiling genetically engineered plants.

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Author information

R.J. and J.A.M. conceived the project and designed experiments; R. J. performed the experiments and wrote the manuscript; P.W. grew all the *Arabidopsis* lines used in this study. R.J., P.W., N.D., C.C., and J. A.M. analysed all data. All authors read and edited the manuscript.

Declaration of interest statement

The authors declare no competing financial interests.

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