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Original Paper

Simultaneous quantification of lignans in *Arabidopsis thaliana* by highly sensitive capillary liquid chromatography-electrospray ionization-ion trap mass spectrometry

Atsushi Okazawa[1,*], Katsuhito Hori[1], Ryohei Okumura[1], Yoshihiro Izumi[1,a], Naoki Hata[1], Takeshi Bamba[1], Eiichiro Fukusaki[1], Eiichiro Ono[2], Honoo Satake[3], Akio Kobayashi[1]

[1] Graduate School of Engineering, Osaka University, Suita, Osaka 565-0871, Japan; [2] R & D Planning Division, Suntory Holdings, Ltd., Mishima, Osaka 618-8503, Japan; [3] Suntory Insitutute of Bioorganic Research, Mishima, Osaka 618-8503, Japan
* E-mail: okazawa@bio.eng.osaka-u.ac.jp Tel & Fax: +81-6-6879-7425

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Abstract Lignans are phenylpropanoid dimers in which the monomers are linked by the central carbon (C8) atoms. Because many lignans have physiological effects, including antioxidant activity, they are now in demand as components in health foods. However, the lignan biosynthetic pathways in plants are only now being understood. Recently, lariciresinol was detected in *Arabidopsis thaliana.* This observation indicated the existence of common lignan biosynthetic pathways in *A. thaliana*, despite a low amount of lignans other than lariciresinol glycosides. In this study, we established a highly sensitive analytical method that enables quantification of both glycoside and aglycone forms of lignans in *A. thaliana* simultaneously using capillary liquid chromatography-electrospray ionization-ion trap mass spectrometry. Some lignans not previously detected in *A. thaliana* were quantified in extracts of both roots and shoots. Our method can be used for the comprehensive analysis of lignans in small samples from mutants and transformants. This method will be utilized to elucidate the metabolic pathways and physiological roles of lignans as well as the regulation of their biosynthesis in plants.

Key words: Capillary LC-ESI-IT-MS/MS, lignan, metabolic profiling.

Lignans are a class of secondary metabolites produced by oxidative dimerization of two phenylpropanoids through a C8-C8′ linkage (Pan et al. 2009; Umezawa 2003). Lignans are widely distributed in the plant kingdom and have been found in various organs, including roots, rhizomes, stems, leaves, seeds and fruits in species belonging to more than 70 families (Saleem et al. 2005; Umezawa 2003). Some lignans are used as medicines and dietary supplements because of their anticancer and antioxidative properties (Adlercreutz 2007; Saarinen et al. 2007). In spite of their extensive distribution and utilization, the biological roles of lignans in plants are still unclear, but it has been suggested that lignans play an important role in defense against various biological stresses such as pathogens and pests (Dixon et al. 2002).

Biosynthetic pathways of lignans have mainly been studied in lignan-rich *Forsythia* spp. (Davin and Lewis 2003; Suzuki and Umezawa 2007; Umezawa 2003). The first step of lignan biosynthesis is dimerization of two phenylpropanoids. Pinoresinol (Pin) is formed by the enantioselective dimerization of two coniferyl alcohol molecules with the aid of dirigent protein (Davin et al. 1997). Then secoisolariciresinol (Sec) is formed by a two-step reduction of pinoresinol via lariciresinol (Lar) catalyzed by pinoresinol/lariciresinol reductase (Dinkova-Kostova et al. 1996). Sec is oxidized to matairesinol (Mat) by secoisolariciresinol dehydrogenase (Xia et al. 2001). Since a wide range of plant species contain these lignans and related enzymes, this pathway is believed to be a common lignan biosynthetic pathway (Davin and Lewis 2003; Suzuki and Umezawa 2003; Umezawa 2003).

Recently, pinoresinol reductase was identified and the

Abbreviations: Arc, arctiin; Arg, arctigenin; Epi, epipinoresinol; Epi-Glc, epipinoresinol glucoside; Lar, lariciresinol; LC-ESI-IT-MS/MS, liquid chromatography-electrospray ionization-ion trap mass spectrometry; LC-ESI-MS, liquid chromatography-electrospray ionization-mass spectrometry; LOD, limit of detection; Mat, matairesinol; Me-Pin-Glc, pinoresinol methyl ether glucoside; MRM, multiple reaction monitoring; MS, Murashige and Skoog; Phg, phillygenin; Phr, phyllyrin; Pin, pinoresinol; Pin-Glc, pinoresinol glucoside; PrR, pinoresinol reductase; RSD, relative standard deviation; Rt, retention time; Sec, secoisolariciresinol

[a] Present address: Graduate School of Engineering, Kobe University, Kobe, Hyogo 657-8501, Japan

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presence of Lar was detected in *A. thaliana* roots (Nakatsubo et al. 2008), suggesting that *A. thaliana* possesses part of the common lignan biosynthetic pathway, though other typical lignans, such as Pin, Sec, and Mat, were not detected in either roots or shoots. Nakatsubo et al. (2008) showed that Lar exists mainly as a glucoside because it was not detected when root extracts were analyzed in the absence of β-glucosidase treatment. Development of a highly sensitive quantitative analytical method for lignans that can quantify their glycoside and aglycone forms simultaneously is expected to promote great advances in research on lignan biosynthesis in *A. thaliana* as well as agriculturally and industrially important plants.

Many methods of lignan detection and quantification have been established (Slanina and Glatz 2004; Willför et al. 2006). Recently, a combination of liquid chromatography and electrospray ionization mass spectrometry (LC-ESI-MS) has become a sensitive method for lignan analysis (Eklund et al. 2008; Smeds et al. 2007). Since the sensitivity of LC-ESI-MS can be increased by lowering the flow rate of the mobile phase, capillary LC-ESI-MS systems with narrow diameter LC columns are more sensitive than conventional LC-ESI-MS systems (Abian et al. 1999; Hopfgartner et al. 1993). Highly sensitive capillary LC-ESI-MS has been applied in peptide mapping (Yang et al. 2009) and in quantification of flavonoids (Bottcher et al. 2009) and plant hormones (Izumi et al. 2009), but has not been used for lignan analysis. Here we established a highly sensitive analytical system based on capillary liquid chromatography-electrospray ionization-ion trap mass spectrometry (LC-ESI-IT-MS/MS) that enables lignan profiling of *A. thaliana*. This is the first report on the detection of Pin, arctigenin (Arg), epipinoresinol (Epi), phillygenin (Phg), pinoresinol glucoside (Pin-Glc) and epipinoresinol glucoside (Epi-Glc) in *A. thaliana*.

Materials and methods

Chemicals

(+)-Pinoresinol (Pin), (+)-lariciresinol (Lar), (−)-secoisolariciresinol (Sec) and (−)-matairesinol (Mat) were purchased from ArboNova (Turku, Finland). (−)-Arctigenin (Arg) and bisphenol A (BisA) were purchased from Sigma Aldrich (St. Louis, MO, USA). (−)-Arctiin (Arc) was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Epipinoresinol (Epi), pinoresinol glucoside (Pin-Glc), epipinoresinol glucoside (Epi-Glc), pinoresinol methyl ether glucoside (Me-Pin-Glc), phillyrin (Phr), and phillygenin (Phg), were provided by Suntory Holdings (Osaka, Japan). The structures of these lignans are illustrated in Figure 1.

For all experiments, HPLC grade acetic acid, distilled water, acetonitrile and methanol from Kishida Chemical (Osaka, Japan) were used. Milli-Q water was made by a Milli-Q Gradient A10 system (Millipore, Billerica, MA, USA). Other chemicals were purchased from Wako Pure Chemical Industries unless otherwise noted.

Capillary liquid chromatography

A 300 mm i.d.×5 mm, 5 μm particle size Inertsil ODS-3 C18 column (Dionex, Sunnyvale, CA, USA) was used as a pre-column for sample trapping. The loading pump was run at 10 μl min^{-1} with 0.05% acetic acid in water and 1 μl was injected per sample. The sample was loaded over a 5-min period. The loaded sample was then switched on-line to a capillary LC column, 300 mm i.d.×150 mm, 3 μm particle size Inertsil ODS-3 C18 column (GL Sciences, Tokyo, Japan). The mobile phase was composed of (A) water/acetonitrile/acetic acid (95/5/0.05, v/v/v) and (B) water/acetonitrile/acetic acid (5/95/0.05, v/v/v). The flow rate was adjusted to 4 μl min^{-1} with a CAP-200 splitter (Dionex). Separations were performed using a gradient of increasing acetonitrile content as follows: 25% B for 5 min, 25–60% from 5 to 28 min, 60–90% from 28 to 30 min, and 90–95% from 30 to 35 min. After 3 min, the initial conditions were restored and the column was allowed to equilibrate for 10 min.

MS spectrometer

The mass spectrometer was an Esquire 3000 plus (Bruker Daltonics, Billerica, MA, USA). ESI-MS/MS analysis was performed in the negative ion mode. The operation parameters of the ESI ion source were as follows: drying gas (N_2) temperature, 250°C; drying gas flow, 4 l min^{-1}; nebulizer gas (N_2) pressure, 9.0 psi; and capillary voltage, 3.1 kV. Ion-trap parameters were as follows: accumulation time, 20 ms; spectral averages, 7; ion charge control target, 10,000; trap drive level, 50%; mass range, *m*/*z* 100–800; target mass, *m*/*z* 350; fragmentation amplitude, 1.00 V; and compound stability, 100%. All data were analyzed with 5.1 Bruker Daltonics Esquire Data Analysis software, version 3.1 (Bruker Daltonics).

Calibration curve

Standard solutions of authentic lignans (1, 10, 50, 100, 500, 1,000, 2,000 and 5,000 nM, each with 1,000 nM of an internal standard of BisA) prepared in 50% methanol were used to construct calibration plots. The calibration curves were generated from triplicate analyses of these standard solutions using the ratio of the chromatographic peak area for each analyte to that of the corresponding internal standard in the multiple reaction monitoring (MRM) mode.

Plant materials

Seeds of *A. thaliana* (L.) Heynh. ecotype Columbia were surface-sterilized with 70% ethanol and 5% sodium hypochlorite, followed by several rinses in distilled water. Then, about 50 sterile seeds were sown in a row at the upper side of 1/2 Murashige and Skoog (MS) medium (Murashige and Skoog 1962) solidified with 0.7% agar and supplemented with 1% sucrose, 0.05 mg l^{-1} pyridoxine hydrochloride, 0.3 mg l^{-1} thiamine hydrochloride, and 0.5 mg l^{-1} nicotinic acid in rectangular plates (100×140×10 mm) set perpendicularly. Following vernalization of the seeds at 4°C in the dark for 2 days, seedlings were grown in a growth chamber set at 23°C under a 16-h light/8-h dark photoperiod for 3 weeks. Shoots

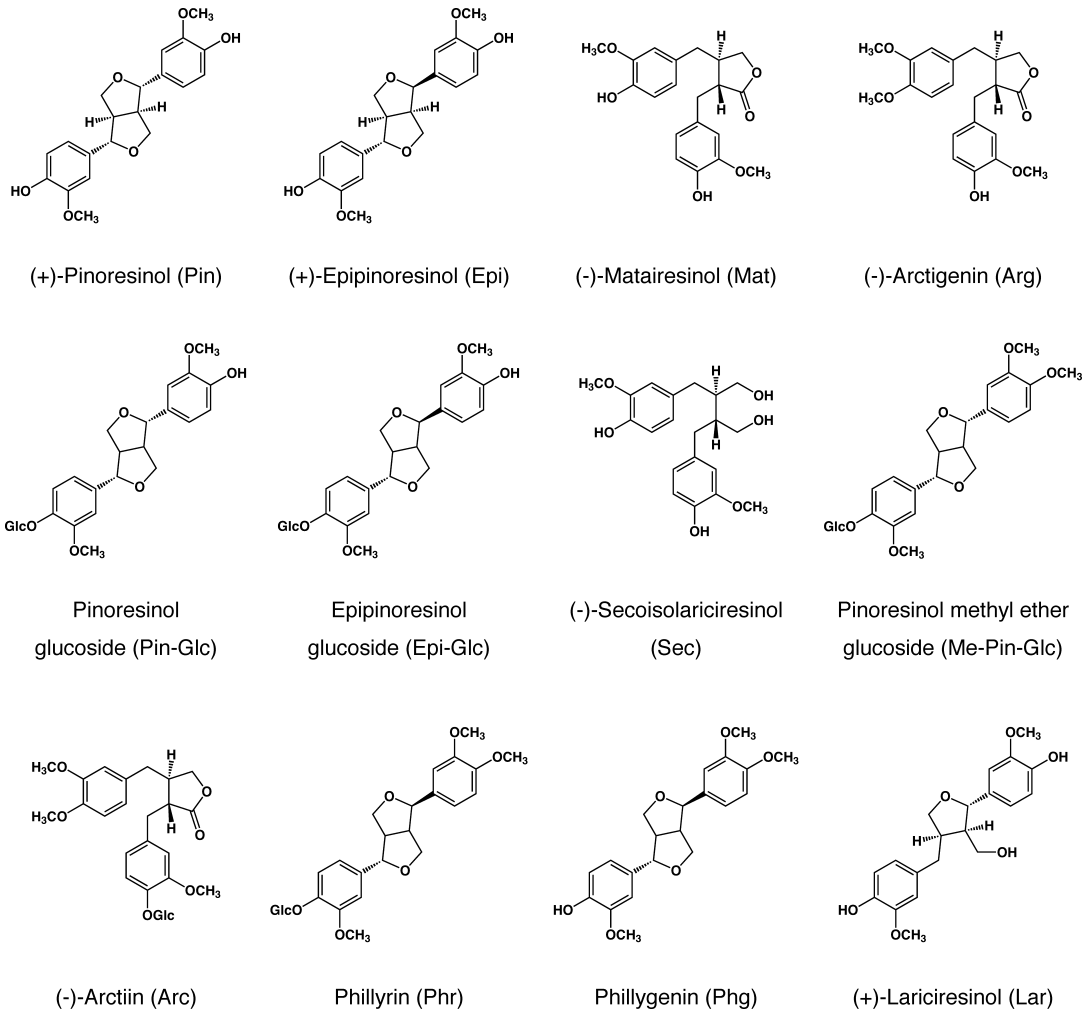

Figure 1. Chemical structures of the analyzed lignans.

and roots were harvested separately from all seedlings grown on six plates, immediately frozen in liquid nitrogen and stored at −80°C until extraction.

Sample extraction and purification

After freeze-drying, shoot and root samples were divided into three approximately equal volumes (ca. 50 and 20 mg dry wight (DW), respectively). Each sample in a 2-ml Eppendorf tube was ground with a ball-mill (Model MM301, Retsch, Haan, Germany) at 20 Hz for 2 min and mixed with 1 ml of 50% methanol. The mixture was shaken at 60°C for 1 h and centrifuged at 15,000×*g* for 3 min. An 800-μl aliquot of the supernatant was collected and the residue was reextracted with 800 μl of 50% methanol. The second extract was centrifuged at 15,000×*g* for 3 min, and then 800 μl of supernatant was combined with the first extract and dried with a centrifugal concentrator (VC-36S, TAITEC, Saitama, Japan). The residue was dissolved in 2 ml of water/methanol (97.5/2.5, v/v), and then purified on Oasis HLB 3 cc cartridges (Waters, Milford, MA, USA) that had been conditioned with 3 ml of methanol and equilibrated with 2 ml of Milli-Q water. Each sample was loaded on a cartridge, and then the cartridge was washed with 2 ml of Milli-Q water. Lignans were eluted with 2 ml of acetonitrile/water/acetic acid (75/25/0.05, v/v/v), and the eluates were dried by centrifugation under vacuum. The dried samples were dissolved in 100 μl of 50% methanol and the solution was passed through a 0.45-μm Chromatodisc syringe filter (GL Sciences, Tokyo, Japan). After filtration, the samples were analyzed by capillary LC-ESI-MS/MS as described above.

Results

Capillary LC-ESI-MS/MS optimization

An authentic standard (1 pmol) of each target compound (Figure 1) was injected directly into the mass spectrometer and the fragment pattern was analyzed. MRM was used to detect the target peaks selectively with high sensitivity. The conditions for the MRM1 method were determined as follows: the trap drive level was set at 10% from 0–12 min and 100% from 12–30 min; transition (*m*/*z*) was set at 357.0>150.6 and 357.0>209.0 (12–20 min), and 370.8>294.8 and 226.6>211.6 (20–30 min). The conditions for the MRM2 method were determined as follows: the trap drive level was set at 100%; transition (*m*/*z*) was set at 578.8>356.8, 518.7>

Table 1. Optimized MRM transitions and analytical validation of capillary LC-ESI-MS/MS for determination of target lignans

Analyte	Transition	Retention time		Linear range (fmol)	LOD (fmol)[b]	Calibration r^2
		min	RSD (%)[a]			
MRM method 1						
Pin	357.0>150.6	16.7	5.5	10–5000	8.7	0.9902
Epi	357.0>150.6	18.4	5.1	50–5000	11.3	0.9940
Mat	357.0>209.6	19.5	4.9	50–1000	11.6	0.9825
Arg	370.8>294.8	23.5	4.0	10–5000	4.5	0.9973
MRM method 2						
Pin-Glc	578.8>356.8	7.7	14.3	10–1000	2.7	0.9996
Epi-Glc	518.7>356.8	8.5	15.6	10–1000	2.1	0.9969
Sec	361.0>165.0	10.9	13.9	10–1000	4.4	0.9975
Arc	592.8>370.8	11.1	15.3	10–1000	1.6	0.9970
Me-Pin-Glc	592.8>370.8	12.2	14.4	10–1000	1.2	0.9995
Phr	592.8>370.8	12.7	13.5	10–1000	0.5	0.9967
Phg+Arg	370.8>355.8	23.1	6.3	10–1000	2.0	0.9954

[a] Relative standard deviation (RSD) was obtained by repeated injection of authentic lignan mixture (n=18).
[b] LOD was estimated based on S/N = 3.

356.8, 361.0>165.0 and 592.8>370.8 (0–16 min), and 370.8>355.8 and 226.6>211.6 (16–30 min) (Table 1).

Eleven out of 12 compounds could be separated; however, a clear MS/MS fragment pattern for Lar was not obtained. MRM chromatograms of the authentic lignans are shown in Figure 2. Phg could not be separated from Arg by the MRM2 method because the most abundant MS/MS fragments (m/z 356) and retention time did not differ for Phg and Arg; however, Arg could be selectively detected by the MRM1 method with the fragment at m/z 295. Thus, Phg content can be calculated from the amount of Arg+Phg obtained by the MRM2 method and the amount of Arg obtained by the MRM1 method.

Method validation

The reproducibility of the retention time of each lignan under the optimized capillary LC-ESI-MS/MS conditions was investigated by repeated injections (n=18) of a mixture of authentic lignans. The relative standard deviation (RSD) for each authentic standard was below 15.6% (Table 1). Based on a signal-to-noise ratio (S/N) of 3, the limit of detection (LOD) ranged from 0.5 fmol (Phr) to 11.6 fmol (Mat) (Table 1). Satisfactory linearity of the peak area was found over the investigated calibration range. The coefficients of correlation (r^2) of this method were above 0.9825.

Lignan analysis in A. thaliana

Under the optimum capillary LC-ESI-MS/MS conditions, some lignans were detected in extracts of shoots (ca. 50 mg) and roots (ca. 20 mg). Representative MRM chromatograms of lignans obtained from the *A. thaliana* extracts are shown in Figure 3. Three major peaks were detected in shoot extracts. By comparison with the MRM chromatograms of authentic lignans, peaks at retention time (Rt) 8.2, 9.1, and 24.4 min were assigned to Pin-Glc, Epi-Glc, and Arg+Phg, respectively

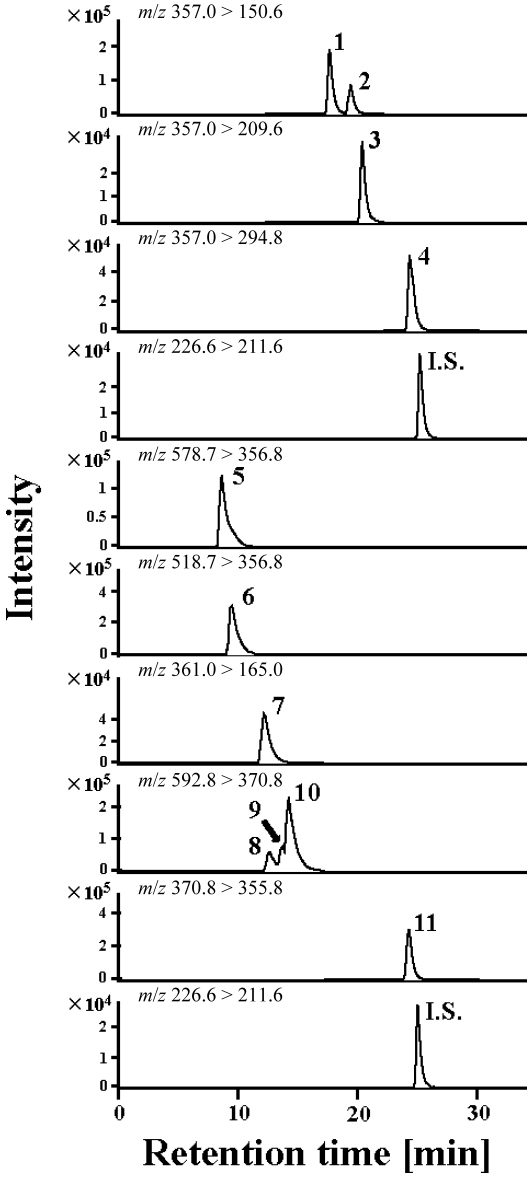

Figure 2. MRM chromatograms of authentic lignans. 1, Pin; 2, Epi; 3, Mat; 4, Arg; 5, Pin-Glc; 6, Epi-Glc; 7, Sec; 8, Arc; 9, Me-Pin-Glc; 10, Phr; 11, Phg+Arg and I.S., internal standard (bisphenol A).

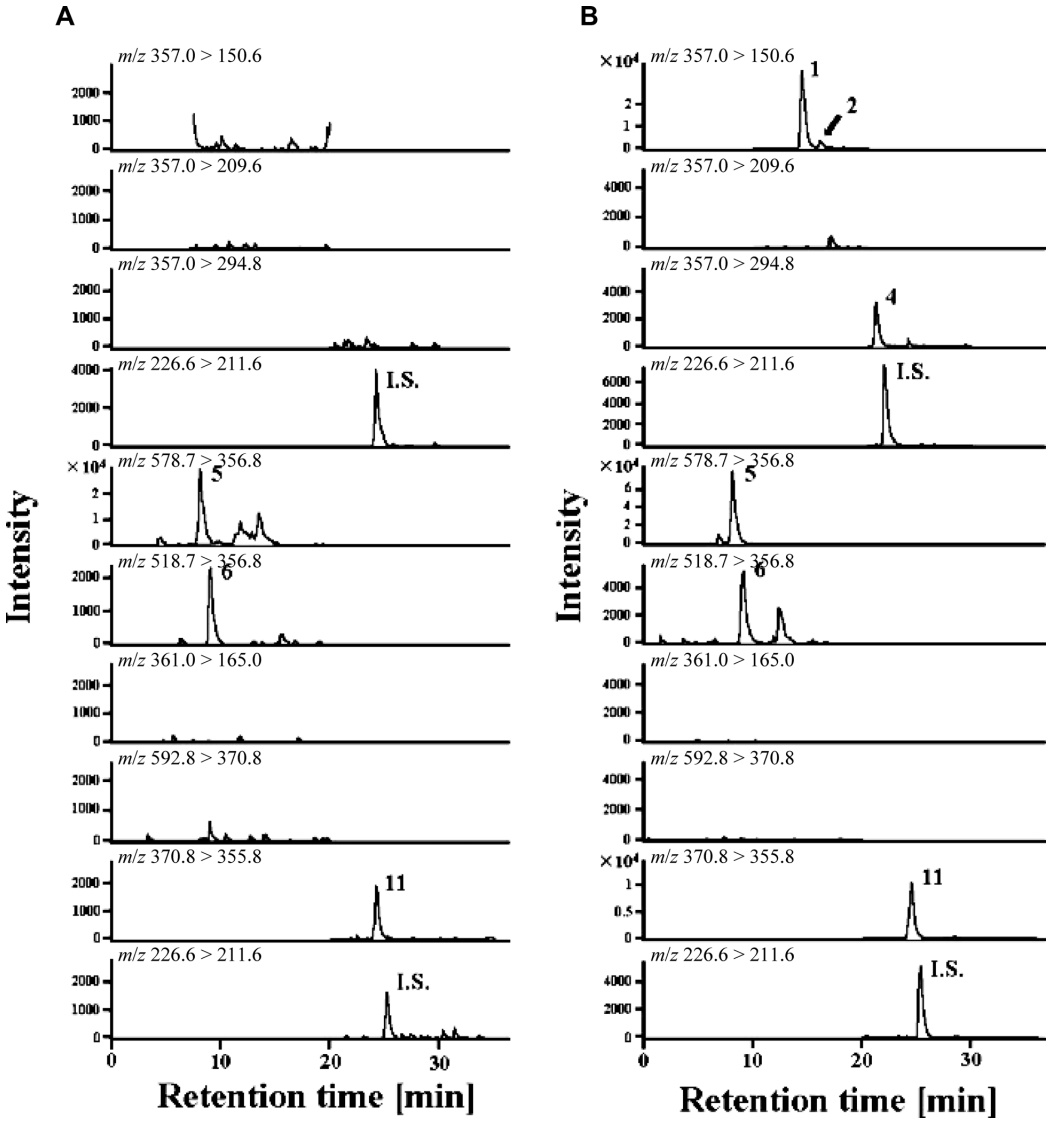

Figure 3. MRM chromatograms of lignans in shoots (A) and roots (B). 1, Pin; 2, Epi; 4, Arg; 5, Pin-Glc; 6, Epi-Glc; 11, Phg+Arg and I.S., internal standard (bisphenol A).

(Figure 3A). Since Arg was not detected in the shoots by the MRM 1 method, the peak at Rt 24.4 min was identified as Phg. Six major peaks were observed in root extracts. The peaks at Rt 8.3, 9.2, 15.5, 17.4, 22.7, and 24.5 min were assigned to Pin-Glc, Epi-Glc, Pin, Epi, Arg, and Arg+Phg, respectively (Figure 3B).

The quantitative data on lignans detected in shoots and roots are summarized in Table 2. The amounts of detected lignans ranged from 0.370 to 46.4 ng mg^{-1} DW. In the shoots, the amount of Pin-Glc (29.0 ng mg^{-1} DW) was highest of the lignans detected. The amount of Epi-Glc (2.05 ng mg^{-1} DW) was about 1/15 of the amount of Pin-Glc. A very small amount of Arg+Phg was also detected (0.370 ng mg^{-1} DW). Since Arg was not detected by the MRM 1 method, the value was attributed to Phg. In the roots, Pin, Epi, and Arg were detected in addition to Pin-Glc, Epi-Glc, and Arg+Phg. The amount of Pin-Glc (46.4 ng mg^{-1} DW) was again highest. The amount of Epi-Glc (4.39 ng mg^{-1} DW) was about 1/10 of the amount of Pin-Glc. The amounts of Pin-Glc and Epi-Glc were approximately 2 times higher than that of their aglycones, Pin (20.8 ng mg^{-1} DW) and Epi (2.40 ng mg^{-1} DW). There was no difference between the amounts of Arg (1.4 ng mg^{-1} DW) and Arg+Phg (0.8 ng mg^{-1} DW).

Table 2. The amounts of detected lignans in *A. thaliana*

Compound	Amount (ng mg^{-1} DW)[a]	
	Shoots	Roots
Pin	ND[b]	20.8 ± 1.3
Epi	ND[b]	2.40 ± 0.38
Arg	ND[b]	1.37 ± 2.0
Pin-Glc	29.0 ± 7.0	46.4 ± 2.8
Epi-Glc	2.05 ± 2.2	4.39 ± 0.050
Phg+Arg	0.370 ± 0.070	0.820 ± 1.2

[a] Values are means±SD, n=3. DW, dry weight.
[b] Not detected.

Thus, the roots might not contain Phg, which differed from the results for the shoots. The amounts of Pin-Glc and Epi-Glc were about 2 times higher in the roots than in the shoots. Other target lignans, Sec, Mata, Phr, Arc, Me-Pin-Glc, were not detected in either the shoots or the roots.

Discussion

Initially, the MS/MS conditions were optimized by analyzing the fragment patterns of authentic lignans. Out of 12 target lignans, all but Lar could be separated (Figure 2).

In the MS spectra of both Pin and Epi, which is a epimer of Pin, the most abundant ion peaks were detected at *m/z* 151. On the other hand, the ion peak at *m/z* 342 was detected only in the Epi MS spectrum. The ion peak at *m/z* 342 seemed to be a methyl radical-detached product ion of Epi, which enabled us to quantify Pin and Epi separately.

The validation data obtained from the analysis of lignans by capillary LC-ESI-MS/MS are summarized in Table 1. Our method has enabled simultaneous quantification of lignan glucosides and aglycones with high sensitivity. The LOD of our analytical method is about 20–300 times lower than that of the HPLC-DAD method, which can also quantify lignan glucosides and aglycones simultaneously (Guo et al. 2007).

A. thaliana, as a model plant, is used in various experiments in many research fields but has not been used for research on lignans until quite recently (Nakatsubo et al. 2008). In preliminary experiments, lignans in *A. thaliana* roots could not be detected by HPLC-ESI-MS. However, our capillary LC-ESI-MS/MS method not only enabled quantification of several lignans in roots, but unexpectedly also allowed quantification of lignans in shoots (Figure 3, Table 2). Nakatsubo et al. (2008) reported that no lignans were detectable in the aerial part of *A. thaliana* and significant amounts (0.36 $\mu g\,mg^{-1}$ DW) of Lar in roots could be quantified only after β-glucosidase treatment. In our study, all the lignans quantified without β-glucosidase treatment were very low, with values ranging from 0.370 to 46.4 $ng\,mg^{-1}$ DW (Table 2). These results indicated that *A. thaliana* is a plant specifically accumulating Lar glucosides, whereas the amounts of other lignans, at least those analyzed in our study, were very low.

In the roots, Sec and Mat were not detected but Pin (20.8 $ng\,mg^{-1}$ DW) and Arg (1.37 $ng\,mg^{-1}$ DW) were (Table 2). Arg is produced by the reduction of Mat, and Mat is synthesized from Pin by pinoresinol/lariciresinol reductase and secoisolariciresinol dehydrogenase via two intermediates, Lar and Sec. The presence of Arg suggests that the common pathway of lignan biosynthesis, i.e. from Pin to Mat or Arg is also existed in *A. thaliana* as in other plant species (Davin and Lewis 2003; Suzuki and Umezawa 2007; Umezawa 2003).

In spite of many studies on lignans, there is little information about the biosynthesis of Epi and other lignans with the *epi* structure. Our study indicated that lignans with the *epi* structure exist in *A. thaliana*: Epi-Glc (2.05 $ng\,mg^{-1}$ DW) and Phg (0.370 $ng\,mg^{-1}$ DW) were detected in shoots, and Epi-Glc (4.39 $ng\,mg^{-1}$ DW) and Epi (2.40 $ng\,mg^{-1}$ DW) were detected in roots (Table 2). It is expected that the biosynthesis of lignans with the *epi* structure will be better understood using *A. thaliana* as a resource.

A large number of lignan glycosides have been discovered in many plant species, which indicates that glycosylation is a common modification in lignan biosynthesis as it is for other secondary metabolites (Jones and Vogt 2001). In our study, the amounts of the glucosides Pin-Glc and Epi-Glc were higher than the amounts of their aglycones Pin and Epi (Table 2). Our results are consistent with a previous report that most Lar exists in glucoside form in *A. thaliana* roots (Nakatsubo et al. 2008). From these observations, lignans are expected to exist largely as glycosides in *A. thaliana*. Unlike Pin-Glc and Epi-Glc, no Arc or Phr, which are glucosides of Arg and Phg, respectively, were detected (Table 2). Studies with the model plant *A. thaliana* may also enable elucidation of the mechanism controlling glucosylation of lignans.

The amounts of lignans in the roots were higher than in the shoots (Table 2). Similarly, Nakatsubo et al. (2008) reported that significant amounts of Lar glucosides were detected in the roots, but no lignans were detected in the aerial part of *A. thaliana*. Localization of lignans in the roots of *A. thaliana* seems not to be originated by translocation of lignans from shoots (leaves) to roots, but to be derived from lignan biosynthesis in the root itself, because expression of pinoresinol reductase genes *AtPrR1* and *AtPrR2*, both involved in the biosynthesis of Lar, were higher in the roots than in the shoots (Nakatsubo et al. 2008).

Previously, the amounts of podophyllotoxin, a aryltetralin lignan used for semisynthesis of anticancer drugs, in the organs of some plant species were investigated. Podophyllotoxin and other related lignans were localized mostly in the leaves of *Diphylleia cymosa*, while *D. grayi* roots contained higher amount of lignans than its leaves (Broomhead and Dewick 1990a). The leaves of *Linum album* contained higher amount of phodophyllotoxin-related lignans than its rhizomes (Mohagheghzadeh et al. 2006). On the other hand, *Linum flavum* contained high amount of lignans in the roots not in the leaves (Broomhead and Dewick 1990b). *Phyllanthus amarus*, which has been used as traditional medicine in tropical countries, contained significantly higher amounts of lignans in the leaves than in the roots

(Khan et al. 2010). Hence, no general tendency in lignan localization was derived from these analyses until now. Since secondary metabolites usually accumulate in specific tissues of cells to exhibit their function, the roles of lignans in these plant species should be clarified to explain their diverse pattern of localization.

As described above, our method can be used for the comprehensive analysis of lignans in small quantities sampled from mutants and transformants of *A. thaliana*. This method will be utilized to elucidate the metabolic pathways and physiological roles of lignans as well as the regulation of their biosynthesis in plants.

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