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Metabolite profiling of Arabidopsis seedlings in response to exogenous sinalbin and sulfur deficiency

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ABSTRACT

In order to determine how plant uptake of a sulfur-rich secondary metabolite, sinalbin, affects the metabolic profile of sulfur-deficient plants, gas chromatography time-of-flight mass spectrometry (GC–TOF-MS), in combination with liquid chromatography–mass spectrometry (LC–MS), was used to survey the metabolome of Arabidopsis seedlings grown in nutrient media under different sulfur conditions. The growth media had either sufficient inorganic sulfur for normal plant growth or insufficient inorganic sulfur in the presence or absence of supplementation with organic sulfur in the form of sinalbin (*p*-hydroxybenzylglucosinolate). A total of 90 metabolites were identified by GC–TOF-MS and their levels were compared across the three treatments. Of the identified compounds, 21 showed similar responses in plants that were either sulfur deficient or sinalbin supplemented compared to sulfur-sufficient plants, while 12 metabolites differed in abundance only in sulfur-deficient plants. Twelve metabolites accumulated to higher levels in sinalbin-supplemented than in the sulfur-sufficient plants. Secondary metabolites such as flavonol conjugates, sinapinic acid esters and glucosinolates, were identified by LC–MS and their corresponding mass fragmentation patterns were determined. Under sinalbin-supplemented conditions, sinalbin was taken up by Arabidopsis and contributed to the endogenous formation of glucosinolates. Additionally, levels of flavonol glycosides and sinapinic acid esters increased while levels of flavonol diglycosides with glucose attached to the 3-position were reduced. The exogenously administered sinalbin resulted in inhibition of root and hypocotyl growth and markedly influenced metabolite profiles, compared to control and sulfur-deficient plants. These results indicate that, under sulfur deficient conditions, glucosinolates can be a sulfur source for plants. This investigation defines an opportunity to elucidate the mechanism of glucosinolate degradation in vivo.

1. Introduction

Sulfur is one of the essential macroelements in plants and is assimilated by the roots as sulfate from the soil (Leustek et al., 2000). In planta, sulfate is first reduced to sulfide and then converted into cysteine, with the latter serving as the principal substrate for the biosynthesis of various other sulfur-containing organic compounds which, in crucifer plants, include glucosinolates. One approach to understand sulfur metabolism has been to compare plant metabolite differences between sulfur-depleted and normal plants (Nikiforova et al., 2005). Under sulfur-deficient conditions, it was anticipated that plants employ adaptive mechanisms that compensate for nutrient deficiency. Interestingly, integrative metabolomic and transcriptomic approaches have identified aspects of the global responses of Arabidopsis plants to sulfur deficiencies that include changes in glucosinolate metabolism (Hirai et al., 2004; Nikiforova et al., 2005).

Glucosinolates are plant secondary metabolites whose basic skeleton consists of a β-thioglucose, an *N*-hydroxyiminosulfate moiety, and a variable side-chain (R) (Halkier and Gershenzon, 2006) (Fig. 1). Glucosinolates are biochemically stable and non-toxic compounds that accumulate in crucifers, yet they function as chemical defense compounds. Tissue damage associated with plant pest activity releases glucosinolates, which are hydrolyzed by myrosinase yielding glucose, sulfate, and a variety of other potentially cytotoxic products such as isothiocyanates, nitriles and thiocyanates (Halkier and Gershenzon, 2006). Sulfatase is the only other enzyme known to use all glucosinolates as substrates converting them to their desulfated derivatives (desulfoglucosinolates) (Fig. 1). The enzyme is found in the guts of *Plutella xylostella* larvae and the snail *Helix pomatia*, and modifies the ingested glucosinolates resulting in the inactivation of the glucosinolate–myrosinase defense system (Ratzka et al., 2002). Sinalbin (**1**)

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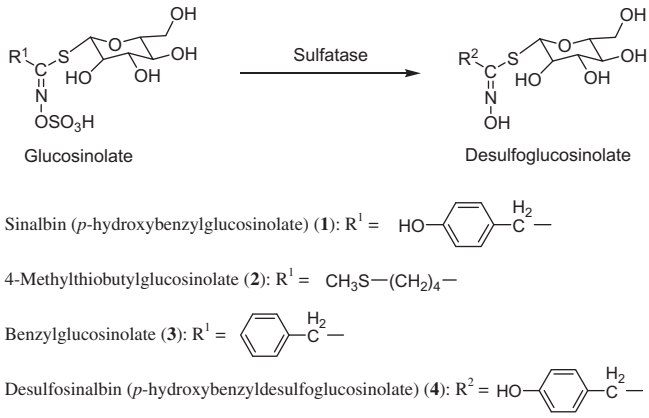


Fig. 1. Structures of glucosinolates and desulfoglucosinolates.

(*p*-hydroxybenzylglucosinolate) is found in seeds of white mustard (Thies, 1988). The variable side-chain of the sinalbin structure (**1**) is a *p*-hydroxybenzyl moiety derived from the amino acid tyrosine (Fig. 1). Because glucosinolates contain at least two sulfur atoms in their structure, they potentially have an important role in sulfur storage. Maruyama-Nakashita et al. (2003) regard the glucosinolates accumulating in root tissues as an alternative source for sulfur assimilation under sulfur-deficient conditions. Glucosinolates, such as sinalbin (**1**), that have been purified and administered to plants, are taken up, transported, and have been observed in the phloem exudates of Arabidopsis petioles (Chen et al., 2001). However, the effect of exogenous glucosinolate on the metabolite profile of sulfur-deficient plants is still unknown, as in the case when glucosinolates are broken down in intact plants through the activity of myrosinase or other unknown factors.

It is a great challenge to obtain the whole metabolome using a single analytical technique. GC–MS gives possibilities of analyzing different classes of compounds, including organic and amino acids, sugars, sugar alcohols, sterols, phosphorylated and lipophilic compounds (Wagner et al., 2003; Lisec et al., 2006). However, LC–MS is also a powerful technology to separate and analyze semi-polar secondary metabolites that can comprise large and unique groups of compounds in plants (Brown et al., 2003; von Roepenack-Lahaye et al., 2004; Stobiecki et al., 2006; de Vos et al., 2007). Therefore, the combination of GC–MS and LC–MS allows for a broad view of metabolic differences when comparing plants grown on different nutrient media. In the present study, GC–TOF-MS and LC–MS were used as profiling methods to study whether exogenously administered glucosinolate, such as sinalbin (**1**), could be metabolized in sulfur-deficient plants as a sulfur source and how such sulfur-containing organic compounds affect the metabolic profiles under sulfur-deficient conditions. Also, the effect of sinalbin (**1**) on the content of endogenous glucosinolate formation was studied in detail.

2. Results and discussion

2.1. Comparison of symptoms

In contrast to the morphology of 13-day-old Arabidopsis plants grown under control conditions (+S medium) (Fig. 2A), plants grown on inorganic sulfur-deficient (−S) medium supplemented with 846 μM sinalbin (**1**) were characterized by root and hypocotyl growth inhibition (Fig. 2B). The culturing experiments were replicated six times with consistent results. Arabidopsis seedlings grown in the presence of either other intact or desulfated glucosinolates, such as 4-methylthiobutylglucosinolate (**2**), benzylglucosinolate (**3**) and *p*-hydroxybenzyldesulfoglucosinolate (**4**) gave the same results as plants supplemented with sinalbin (**1**) (data not shown). Up to now, however little was known about the influence of exogenous glucosinolates on plant morphology. A report by Gijzen et al. (1989) found that the addition of 2-propenylglucosinolate to a growth medium containing rapeseed embryos resulted in a decline in embryo fr. wt. In the present study, Arabidopsis plants grown on sulfur-deficient media showed typical symptoms of sulfur deficiency such as stunted growth, leaf chlorosis and enhanced root growth (Fig. 2C). Enhanced root growth is a developmental response of plants to nutrient deficiency (Forde and Lorenzo, 2001; Kutz et al., 2002).

2.2. Comparison of metabolite profiles obtained with GC–TOF-MS

To investigate differences in the primary metabolite levels of samples prepared from plants grown on different nutrient media, whole seedlings (eight biological replicates) were harvested and extracted, and the extract samples derivatized and analyzed. The average relative concentration of a metabolite was compared by calculating the response ratio ($R_{-S/+S}$ or $R_{+sinalbin/+s}$) of the average relative concentration on −S medium or +sinalbin medium to that on +S medium as a control. When the relative ratio ($R_{-S/+S}$ or $R_{+sinalbin/+s}$) was more than 2.0 or less than 0.5 with $P < 0.05$, the relative concentration of metabolites was considered to be significantly altered, which was similar to the criterion designated by Nikiforova et al. (2005). The differences in the metabolite levels obtained with GC–TOF-MS are listed in Table 1. A total of 90 metabolites (**1, 5–92**) were identified, but the levels of some sulfur-containing compounds (e.g., cysteine and methionine) were below limits of detection.

Twenty-one metabolites (**5–25**) had similar responses (increased or decreased abundance) to both sulfur-deficient and sinalbin-supplemented nutrient conditions relative to the sulfur-sufficient

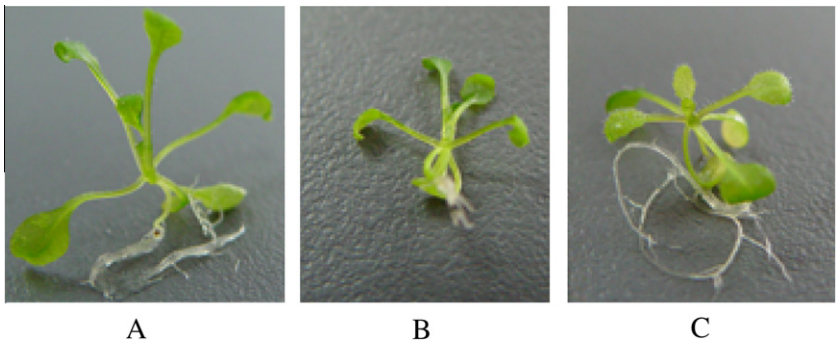


Fig. 2. The morphological appearance of 13-day-old Arabidopsis grown on Murashige and Skoog media with the sole sulfur source as (A) standard 846 μM sulfate (+S medium, control), (B) 846 μM sinalbin (**1**) (+sinalbin medium) or (C) no sulfur (−S medium).

Table 1
Differences in metabolite levels obtained by GC–TOF-MS of extracts from Arabidopsis seedlings grown on different sulfur-containing media.

No.	Name	Substance class	+S Avg[a]	+Sinalbin Avg[a]	-S Avg[a]	$R_{+Sinalbin/+S}$[b]	+Sinalbin/+S *P* value	$R_{-S/+S}$[b]	-S/+S *P* value
1	Sinalbin	Glucosinolate	-	303.97	.-	-	-	-	-
5	Ornithine	Amino acid	104.51	27.19	26.18	0.26	0.0446	0.25	0.0392
6	β-Alanine	Amino acid	32.21	77.43	94.31	2.40	7.54E-06	2.93	8.42E-07
7	Putrescine	Amine	10.36	24.72	31.15	2.39	8.01E-06	3.01	0.0042
8	Raffinose	Trisaccharide	115.01	392.00	1301.70	3.41	0.0005	11.32	0.0005
9	Mannose	Monosaccharide	269.34	754.26	822.63	2.80	0.0016	3.05	0.0010
10	Trehalose	Disaccharide	61.37	216.19	126.81	3.52	1.29E-06	2.07	0.0025
11	Xylose	Monosaccharide	4.89	10.54	22.49	2.15	0.0174	4.60	0.0129
12	*myo*-Inositol	Sugar alcohol	55.25	156.33	114.23	2.83	3.61E-09	2.07	0.0009
13	L(+)-ascorbate	Organic acid	3.10	27.68	21.65	8.93	0.0036	6.99	0.0044
14	Threonate	Organic acid	39.71	192.36	80.66	4.84	3.04E-05	2.03	0.0002
15	Glycerate	Organic acid	24.75	97.06	143.54	3.92	0.0002	5.80	1.31E-05
16	α-Ketoglutarate	Organic acid	36.14	129.49	135.36	3.58	0.0229	3.75	0.0009
17	*O*-Acetyl-L-serine	Amino acid	12.60	50.86	1407.47	4.04	0.0097	111.67	0.0006
18	*N*-Acetyl-L-serine	Amino acid	4.81	15.29	185.41	3.18	0.0229	38.53	0.0167
19	L-Serine	Amino acid	78.13	221.05	500.41	2.83	0.0025	6.41	0.0002
20	L-Threonine	Amino acid	29.96	79.12	190.03	2.64	0.0174	6.34	7.62E-05
21	L-Isoleucine	Amino acid	51.24	909.00	1804.86	17.74	0.0041	35.22	0.0054
22	5-Oxoproline	Organic acid	415.57	874.14	1122.28	2.10	1.91E-05	2.70	9.05E-05
23	Glutamine	Amino acid	22.79	152.37	183.59	6.69	0.0133	8.06	0.0035
24	1-Ethylglucopyranoside	Monosaccharide	4.62	31.25	10.69	6.76	0.0014	2.31	0.0150
25	Glucopyranose	Monosaccharide	298.01	1007.69	1142.04	3.38	1.34E-05	3.83	0.0004
26	L-Proline	Amino acid	53.31	144.96	394.10	2.72	0.0817	7.39	0.0010
27	Glycine	Amino acid	67.04	128.83	222.43	1.92	2.04E-05	3.32	2.26E-06
28	L-Tryptophan	Amino acid	9.42	12.35	87.13	1.31	0.6676	9.25	0.0009
29	L-Tyrosine	Amino acid	49.56	55.33	213.24	1.12	0.6962	4.30	0.0029
30	L-Valine	Amino acid	26.55	47.26	210.52	1.78	0.1280	7.93	0.0016
31	L-Asparagine	Amino acid	44.78	67.16	132.68	1.50	0.0432	2.96	0.0006
32	4-Aminobutyrate	Organic acid	172.31	173.49	452.07	1.01	0.9634	2.62	2.84E-06
33	Citrate	Organic acid	13.23	14.10	34.38	1.07	0.5432	2.60	7.47E-05
34	Fumarate	Organic acid	210.42	276.18	490.91	1.31	0.0320	2.33	0.0051
cis -35.	*cis*-Sinapinate	Organic acid	8.75	17.10	19.76	1.96	0.0012	2.26	0.0096
trans -35	*trans*-Sinapinate	Organic acid	214.59	530.37	355.90	2.47	0.0024	1.66	0.1282
36	Erythronate	Organic acid	3.46	4.93	23.97	1.43	0.2762	6.93	2.65E-05
37	Maleate	Organic acid	30.48	43.90	76.95	1.44	0.1376	2.52	0.0015
38	Fructose	Monosaccharide	142.38	121.94	70.84	0.86	0.1052	0.50	3.85E-06
39	2-(4-Hydroxyphenyl)acetonitrile	Nitrile	-	122.79	-	-	-	-	-
40	L-Homoserine	Amino acid	14.48	44.69	10.90	3.09	0.0018	0.75	0.5123
41	L-Phenylalanine	Amino acid	2.65	10.47	4.18	3.94	0.0108	1.58	0.1134
42	L-Alanine	Amino acid	32.49	66.77	61.00	2.06	0.0089	1.88	0.0037
43	Sucrose	Disaccharide	57.25	193.11	87.46	3.37	7.55E-09	1.53	0.0212
44	Maltose	Disaccharide	1.19	3.03	1.15	2.55	0.0197	0.97	0.9351
45	Galactinol	Sugar alcohol	28.42	58.38	39.11	2.05	9.95E-07	1.38	0.0897
46	Sorbitol	Sugar alcohol	2.09	6.00	2.66	2.86	4.19E-05	1.27	0.3838
47	Fructose-6-phosphate	Phosphorylated compound	5.09	10.18	6.05	2.00	0.0003	1.19	0.2502
48	L-Glycerol-3-phosphate	Phosphorylated compound	16.56	57.57	26.84	3.48	0.0003	1.62	0.0262
49	Glucose-6-phosphate	Phosphorylated compound	16.50	24.17	12.02	1.47	0.1815	0.73	0.3970
50	*myo*-Inositol-1-phosphate	Phosphorylated compound	4.38	4.21	5.00	0.96	0.7484	1.14	0.4323
51	Gluconate	Organic acid	12.91	13.10	12.16	1.01	0.9113	0.94	0.7287
52	Glucose	Monosaccharide	165.33	153.57	116.09	0.93	0.7336	0.70	0.1987
53	Dehydroascorbate	Organic acid	41.10	80.04	37.03	1.95	5.50E-05	0.90	0.4859
54	Erythritol	Sugar alcohol	7.27	2.48	0.60	0.34	0.4278	0.08	0.2668
55	Shikimate	Organic acid	2.56	4.57	2.81	1.78	1.56E-07	1.10	0.2236
56	L-Leucine	Amino acid	77.11	77.03	331.79	1.00	0.9983	4.30	0.1198
57	Malate	Organic acid	330.15	370.76	439.07	1.12	0.2446	1.33	0.0418
58	L-Asparate	Amino acid	372.58	536.74	569.65	1.44	0.2321	1.53	0.1471
59	L-Lysine	Amino acid	19.23	16.13	27.36	0.84	0.6710	1.42	0.4005
60	L-Glutamate	Amino acid	413.94	544.91	726.63	1.32	0.4442	1.76	0.1042
61	Ornithine; arginine	Amino acid	5.25	4.90	3.72	0.93	0.8355	0.71	0.1203
62	Spermidine	Amine	5.84	5.96	2.12	1.02	0.9803	0.36	0.3290
63	Dotriacontanol	Alcohol	17.61	24.16	17.01	1.37	0.0923	0.97	0.8819
64	Phytol	Alcohol	141.25	105.06	97.20	0.74	0.1080	0.69	0.0665
65	Triacontanol	Alcohol	19.56	22.64	23.10	1.16	0.3610	1.18	0.4744
66	9,12-(Z,Z)-Octadecadienoate	Fatty acid	133.01	142.36	124.26	1.07	0.6700	0.93	0.7557

(*continued on next page*)

Table 1 (*continued*)

No.	Name	Substance class	+S Avg[a]	+Sinalbin Avg[a]	-S Avg[a]	$R_{+Sinalbin/+S}$[b]	+Sinalbin/+S *P* value	$R_{-S/+S}$[b]	-S/+S *P* value
67	9,12,15-(Z,Z,Z)-Octadecatrienoate	Fatty acid	42.85	50.96	45.49	1.19	0.2549	1.06	0.7928
68	Hexadecanoate	Fatty acid	126.13	121.55	123.82	0.96	0.7507	0.98	0.9160
69	Octadecanoate	Fatty acid	16.43	22.14	21.65	1.35	0.0640	1.32	0.2404
70	Oleate	Fatty acid	5.70	8.16	4.18	1.43	0.0147	0.73	0.0720
71	1-Monohexadecanoylglycerol	Glyceride	164.42	178.57	120.65	1.09	0.7071	0.73	0.1834
72	Phosphorate	Inorganic acid	122.35	78.51	235.55	0.64	0.0071	1.93	0.0166
73	1,6-Anhydroglucose	Monosaccharide	9.48	6.21	6.98	0.66	0.0973	0.74	0.1823
74	Ribose	Monosaccharide	9.09	9.57	6.08	1.05	0.9485	0.67	0.6671
75	4-Hydroxybenzoate	Organic acid	8.44	19.63	11.04	2.33	0.0625	1.31	0.6933
76	Glycolate	Organic acid	32.29	66.40	30.17	2.06	0.0539	0.93	0.8906
77	Lactate	Organic acid	368.41	519.67	552.64	1.41	0.1799	1.50	0.1419
78	Glutarate	Organic acid	1.71	0.85	0.63	0.49	0.4522	0.37	0.3571
79	Ribonate	Organic acid	24.98	35.67	31.77	1.43	0.0001	1.27	0.0375
80	*trans*-Threonic acid-1,4-lactone	Organic acid	44.50	80.64	70.75	1.81	0.1852	1.59	0.2539
81	Sorbitol-6-phosphate	Phosphorylated compound	9.03	17.66	38.28	1.96	0.4852	4.24	0.3578
82	α-Tocopherol	Sterol	89.56	123.98	156.95	1.38	0.0754	1.75	0.0363
83	β-Sitosterol	Sterol	59.62	68.60	71.63	1.15	0.1083	1.20	0.3360
84	β-Tocopherol	Sterol	29.63	47.66	40.55	1.61	0.0015	1.37	0.2244
85	Campesterol	Sterol	26.21	26.13	30.04	1.00	0.9760	1.15	0.4526
86	Cholesterol	Sterol	6.75	7.60	7.41	1.13	0.3440	1.10	0.6009
87	Stigmasterol	Sterol	25.65	17.72	27.05	0.69	0.0074	1.05	0.7867
88	Octacosanol	Sugar alcohol	9.04	9.89	13.15	1.09	0.5167	1.45	0.1015
89	Threitol	Sugar alcohol	3.00	2.79	0.20	0.93	0.9129	0.07	0.1422
90	Xylitol	Sugar alcohol	7.00	4.19	6.72	0.60	0.1758	0.96	0.9017
91	2,4,6-Tri-*tert*-butylbenzenethiol	Thiol	38.21	35.29	72.12	0.92	0.6630	1.89	0.1060
92.	Urea	Urea	778.16	0.71	2.10	0.08	0.3363	0.01	261.6600

[a] Raw GC–TOF-MS data were corrected for variations in sample loading by dividing by the peak area for ribitol, added as an internal control during sample extraction. Values for each biological sample were normalized with respect to the mean values of technical replicates run in each batch of samples.

[b] *R*, ratio of average relative concentration at depleted sulfur or supplemented sinalbin to average relative concentration at normal sulfur ($n = 8$).

media. Only ornithine (**5**) showed decreased accumulation levels under both conditions. Twenty metabolites (**6–25**) exhibited increased accumulation levels and included not only those with important roles in plant stress resistance such as β-alanine (**6**), putrescine (**7**), and sugars such as raffinose (**8**), mannose (**9**), trehalose (**10**) and xylose (**11**) but also the sugar alcohol inositol (**12**), plus organic acids such as L-ascorbate (**13**), threonate (**14**), glycerate (**15**) and α-ketoglutarate (**16**). In addition, *O*-acetyl-L-serine (**17**) and *N*-acetyl-L-serine **(18)** showed increases of 111- and 38-fold, respectively, under sulfur-deficient conditions, while exhibiting only ~4.0- and ~3.2-fold increases under sinalbin-supplemented conditions. Other metabolites demonstrated an increasing trend under both conditions, such as serine (**19**) (a metabolic precursor of cysteine), threonine (**20**) and isoleucine (**21**), which are metabolites derived from homoserine (**40**), a precursor in common with methionine biosynthesis, and 5-oxoproline (**22**), a metabolite of proline (**26**).

Although 21 metabolites showed similar trends under both sulfur-deficient and sinalbin-supplemented conditions, other metabolites showed significant differences under only one or the other sulfur-deficient condition relative to the sulfur-sufficient condition (Table 1, Fig. 3). In response solely to inorganic sulfur deficiency, 12 metabolites (**26–34, *cis*-35, 36–37**) exhibited increased levels whereas fructose (**38**) showed a slightly decreased level. These profile differences are similar to those observed by Nikiforova et al. (2005). The amino acids which exhibited increased levels only in response to sulfur deficiency included proline (**26**), glycine (**27**), tryptophan (**28**), tyrosine (**29**), valine (**30**), asparagine (**31**) and 4-aminobutyrate (**32**). In addition, the TCA cycle intermediates citrate (**33**) and fumarate (**34**) and the phenylpropanoid pathway intermediate *cis*-sinapinic acid (*cis*-**35**) showed increased levels.

Twelve metabolites (**1, *trans*-35, 39–48**) exhibited increased levels in response only to sinalbin (**1**) supplementation including two metabolites normally found in white mustard seed but not in Arabidopsis, and which appeared only in sinalbin-supplemented Arabidopsis viz., sinalbin (**1**) and 2-(4-hydroxyphenyl)acetonitrile (**39**). The metabolites showing unique responses to sinalbin (**1**) supplementation included the amino acids homoserine (**40**), phenylalanine (**41**), and alanine (**42**), the organic acid *trans*-sinapinic acid (*trans*-**35**), the sugars sucrose (**43**) and maltose (**44**), the sugar alcohols galactinol (**45**) and sorbitol (**46**), and the phosphorylated intermediates fructose-6-phosphate (**47**) and glycerol-3-phosphate (**48**). The two metabolites unique to Arabidopsis, sinalbin (**1**) and 2-(4-hydroxyphenyl)acetonitrile (**40**), which is a breakdown product of sinalbin (**1**) hydrolysis catalyzed by myrosinase, were identified by accurate mass and mass fragment determinations, and sinalbin (**1**) was further confirmed by its retention time and mass spectra relative to an authentic standard.

To determine whether 2-(4-hydroxyphenyl)acetonitrile (**39**) was produced either as an artifact of tissue extraction or under normal metabolism in vivo, the sinalbin (**1**)-supplemented plants were processed at room temperature to allow for either post-harvest myrosinase-mediated hydrolysis of glucosinolates or by a boiling water extraction which inhibits post-harvest hydrolysis. The results showed that 2-(4-hydroxyphenyl)acetonitrile (**39**) was not observed in boiling water extracts, whereas in the room temperature extracts not only 2-(4-hydroxyphenyl)acetonitrile (**39**) was observed but also other glucosinolate-derived nitriles, including 4-(methylthio)butanenitrile, 5-(methylthio)pentanenitrile, 8-(methylthio)octanenitrile, 2-(1H-indol-3-yl)acetonitrile, and 2-(4-methoxy-1H-indol-3-yl)acetonitrile (data not shown). Alternate glucosinolate breakdown products such as isothiocyanates were not detected in extracts prepared at either room temperature or with boiling water. Therefore, the 2-(4-hydroxyphenyl)acetonitrile (**39**) detected was likely a product of limited post-harvest hydrolysis of sinalbin by myrosinase.

Many of the 20 metabolites that showed increased levels in response to both sulfur deficiency and sinalbin supplementation

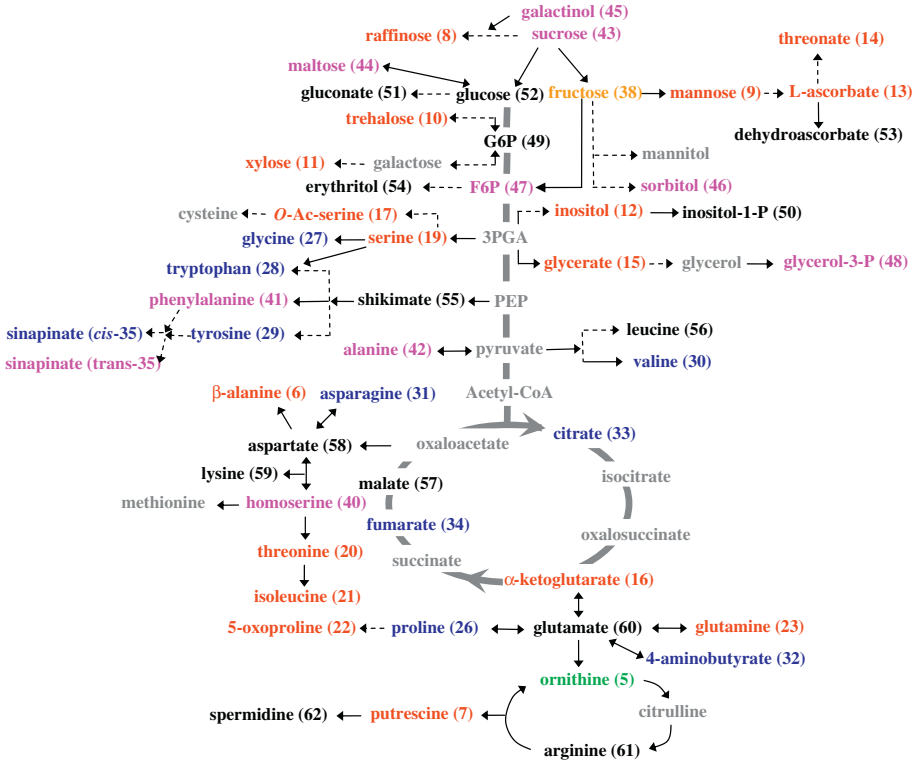
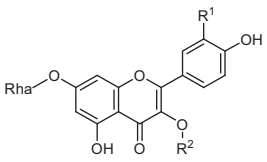


Fig. 3. Changes in the mean metabolite levels of Arabidopsis seedlings in response to sulfur deficiency and sinalbin (**1**) supplementation (data from Table 1) arrayed by pathways of central metabolism. Consistent increases or decreases in metabolite concentrations (with $R > 2.0$, $P < 0.05$ or $R < 0.5$, $P < 0.05$) of both sulfur-deficient and sinalbin (**1**)-supplemented plants relative to control plants are indicated with red or green letters, respectively. The metabolites showing increases only under sulfur deficiency or sinalbin (**1**) supplementation are depicted in blue or pink letters, respectively, and decreases only under sulfur deficiency in orange letters. Black letters indicate no significant change, and grey letters denote metabolites that were not measured. Abbreviations are as follows: 3PGA, 3-Phosphoglycerate; PEP, Phosphoenolpyruvate; *O*-Ac-serine, *O*-acetyl-L-serine (**17**); F6P, Fructose-6-phosphate (**47**); glycerol-3-P, Glycerol-3-phosphate (**48**); G6P, Glucose-6-phosphate (**49**); inositol-1-P, Inositol-1-phosphate (**50**). Adapted from Messerli et al. (2007).

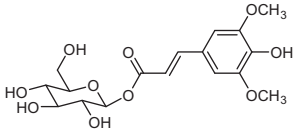
have similar responses to other environmental stresses. Raffinose (**8**) and galactinol (**45**), which accumulate in drought-, high salinity- and cold-treated Arabidopsis plants (Taji et al., 2002), are involved in stress tolerance by plants exposed to abiotic stress. Herein, however, it was demonstrated that the levels of galactinol (**45**) were elevated in response to sinalbin (**1**) supplementation only. *O*-Acetyl-L-serine (**17**) is a general regulator of gene expression associated with sulfur nutrition (Hirai et al., 2003). The contents of *O*-acetyl-L-serine (**17**) increased significantly in sulfur-deficient Arabidopsis seedlings, consistent with earlier reports (Nikiforova et al., 2003, 2005). Under short-term sulfur deficiency or with exogenous supplementation of *O*-acetyl-L-serine (**17**), the endogenous content of *O*-acetyl-L-serine (**17**) increased significantly in both leaves and roots of Arabidopsis (Hirai et al., 2003). Under sulfur deficiency, the decreased levels of cysteine resulted in the accumulation of its upstream metabolites, namely *O*-acetyl-L-serine (**17**), serine (**19**) and glycine (**27**) (Nikiforova et al., 2005). In Arabidopsis seedlings, sinalbin (**1**) treatment led to increased *O*-acetyl-L-serine (**17**) levels, but the content was far lower than that found in sulfur-deficient plants. This intermediate level of *O*-acetyl-L-serine (**17**) indicates that the sulfur deficiency was milder under sinalbin (**1**)-supplemented conditions and that the exogenously applied sinalbin (**1**) provided organic sulfur for the plant thus partially alleviating sulfur deficiency.

2.3. Identification of secondary metabolites obtained with LC–ESI-MS-MS

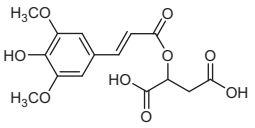
Secondary metabolites play important roles in plant development and in plant defense against biotic and abiotic stress (Kutchan, 2001). In extracts of Arabidopsis roots and leaves, ca. 2000 different mass signals were detected by CapLC-QqTOF-MS, and a majority of which originate from secondary metabolites (Von Roepenack-Lahaye et al., 2004). However, many of these secondary metabolites of Arabidopsis are as yet unidentified. Some metabolites were identified from a comprehensive analysis of mass fragmentation patterns and by comparison with published literature (Graham, 1998; Von Roepenack-Lahaye et al., 2004; Tohge et al., 2005; Kerhoas et al., 2006; Routaboul et al., 2006; Stobiecki et al., 2006; Besseau et al., 2007; Stracke et al., 2007). Additionally, compounds were identified by molecular weight determinations from protonated $[M+H]^+$ and deprotonated $[M-H]^-$ molecular ions in the first order mass spectra and then MS/MS spectra where sufficient collision energy was applied to obtain maximum structural information. The most abundant secondary metabolites identified were flavonoid glycosides (**93**–**100**) and sinapinic acid esters (**101**–**102**) (Fig. 4, Table 2, and Supplementary Fig. I). For flavonol conjugates, identification was facilitated by the analysis of fragmentation pathways of $[M-H]^-$ ions in the negative ion mode and the observation that glycosidic bonds (to rhamnosyl 146u and glucosyl 162u) were cleaved sequentially and generated characteristic aglycone fragments. The major fragment ions, fragmentation pathways and retention times of secondary metabolites identified in extracts of Arabidopsis seedlings are listed in Table 2 and Supplementary Fig. II. In total, eight flavonol conjugates were identified including five diglycosides, namely, kaempferol 3-*O*-glucoside 7-*O*-rhamnoside (**93**), kaempferol 3-*O*-rhamnoside 7-*O*-rhamnoside (**94**), quercetin 3-*O*-glucoside 7-*O*-rhamnoside (**95**), quercetin 3-*O*-rhamnoside 7-*O*-rhamnoside (**96**), and isorhametin 3-*O*-glucoside 7-*O*-rhamnoside (**97**), and three triglycosides,



Kaempferol 3-*O*-glucoside 7-*O*-rhamnoside (**93**): R^1 = H, R^2 = Glc
Kaempferol 3-*O*-rhamnoside 7-*O*-rhamnoside (**94**): R^1 = H, R^2 = Rha
Quercetin 3-*O*-glucoside 7-*O*-rhamnoside (**95**): R^1 = OH, R^2 = Glc
Quercetin 3-*O*-rhamnoside 7-*O*-rhamnoside (**96**): R^1 = OH, R^2 = Rha
Isorhametin 3-*O*-glucoside 7-*O*-rhamnoside (**97**): R^1 = OCH_3, R^2 = Glc
Kaempferol 3-*O*-rhamnosyl-glucoside 7-*O*-rhamnoside (**98**): R^1 = H, R^2 = Glc-Rha
Kaempferol 3-*O*-glucosyl-glucoside 7-*O*-rhamnoside (**99**): R^1 = H, R^2 = Glc-Glc
Quercetin 3-*O*-glucosyl-glucoside 7-*O*-rhamnoside (**100**): R^1 = OH, R^2 = Glc-Glc



Sinapoyl glucose (**101**)



Sinapoyl malate (**102**)

Fig. 4. Structures of flavonoid glycosides (**93–100**) and sinapinic acid esters (**101–102**) identified in Arabidopsis seedlings.

namely, kaempferol 3-*O*-rhamnosyl-glucoside 7-*O*-rhamnoside (**98**), kaempferol 3-*O*-glucosyl-glucoside 7-*O*-rhamnoside (**99**), and quercetin 3-*O*-glucosyl-glucoside 7-*O*-rhamnoside (**100**). Characteristic fragment ions of sinapoyl glucose (**101**) and sinapoyl malate (**102**) at 223 *m/z* corresponding to deprotonated sinapinic acid were found in the product ion spectra of both compounds (**101**-**102**). In analyses of the secondary metabolites, sinalbin (**1**) was also identified by comparing its CID spectrum and retention time with those of an authentic sinalbin (**1**) standard. The desulfated form of sinalbin (**1**), *p*-hydroxybenzyldesulfoglucosinolate (**4**) was not detected. From the above, it can be deduced that after exogenous intact sinalbin (**1**) is assimilated, it exists in an intact rather than a desulfated form in the plant. In sinalbin (**1**)-supplemented Arabidopsis, the presence of sinalbin (**1**), the absence of desulfosinalbin (**4**), and unchanged tyrosine (**29**) levels were noted. These metabolite levels indicate that the high levels of sinalbin (**1**) did not arise by de novo biosynthesis from tyrosine (**29**), but rather by uptake from the culture medium as described by Chen et al. (2001). In addition, significant changes in the concentration of many other metabolites were also found in sulfur-deficient or sinalbin (**1**)-supplemented plants, but the metabolites remain unidentified. In a future study, LC–ESI-MS-MS and other techniques could be combined to identify those metabolites in sinalbin-supplemented Arabidopsis seedlings (Supplementary Fig. I).

2.4. Comparison of metabolite profiles obtained with LC–MS

In comparison with control plants, sinalbin (**1**)-supplemented plants showed significantly decreased levels of flavonol diglycosides with a glucose residue substituted at C3 such as kaempferol 3-*O*-glucoside 7-*O*-rhamnoside (**93**), quercetin 3-*O*-glucoside 7-*O*-rhamnoside (**95**) and isorhametin 3-*O*-glucoside 7-*O*-rhamnoside (**97**), while flavonol diglycosides with a rhamnose residue attached to the 3-position such as kaempferol 3-*O*-rhamnoside 7-*O*-rhamnoside (**94**) and quercetin 3-*O*-rhamnoside 7-*O*-rhamnoside (**96**) clearly increased. Furthermore, levels of all flavonol triglycosides (**98–100**) increased while those of the sinapinic acid esters (**101–102**) were only slightly elevated (Table 3).

In sulfur-deficient plants, all flavonol glycosides and sinapinic acid esters were found in greater amounts compared to control plants, but the flavonol diglycosides with glucose substituted at C3 including kaempferol 3-*O*-glucoside 7-*O*-rhamnoside (**93**), quercetin 3-*O*-glucoside 7-*O*-rhamnoside (**95**) and isorhametin 3-*O*-glucoside 7-*O*-rhamnoside (**97**) and sinapinic acid esters (**101–102**) were only slightly higher than those in control plants. The patterns of accumulation of flavonol diglycosides with rhamnose attached at the 3-position and the flavonol triglycosides in the sulfur-deficient plants were identical to those in the sinalbin (**1**)-supplemented plants, but the flavonol diglycosides with glucose attached at the 3-position had opposite patterns of accumulation under the two conditions.

Flavonoids and sinapinic acid esters, compounds derived from the phenylpropanoid pathway, fulfill important functions of the plant in development and interactions with the environment (Milkowski et al., 2004; Taylor and Grotewold, 2005). Flavonoids have crucial roles in cellular protection against UV light irradiation, as well as in maintaining cellular redox balance (Laundry et al., 1995; Pietta, 2000). Flavonoids also serve as phytoalexins to protect against damage caused by pathogen attack (Van Etten et al., 1994). Sinapoyl glucose (**101**) and sinapoyl malate (**102**) serve as UV protectants and constitute the major sinapate esters in *Arabidopsis thaliana* leaves (Milkowski et al., 2004). Because each glucosinolate contains a β-D-glucopyranose residue, the uptake of exogenous sinalbin (**1**) is expected to have a significant effect on the levels of the secondary metabolites, especially flavonol glycosides. To illustrate the changes in flavonoid glycoside abundance, a simplified scheme of the biosynthetic pathway of flavonoid glycosides and sinapinic acid esters in the *A. thaliana* seedling is shown in Fig. 5. Tyrosine (**29**) and phenylalanine (**41**) serve as

Table 2
The major fragmentation ions of secondary metabolites identified in extracts of Arabidopsis seedlings.

No.	Name[a]	RT (min)	Major fragmentation ions
1	Sinalbin	7.58	$[M+H]^+$ 426 $[M-H]^-$ 424 $[M-SO_3]^+$ 344 $[M-SO_3+2H]^+$ 346 $[M-G\ SO_3+3H]^+$ 184 $[M-S-Glc-SO_3+3H]^+$ 150
93	K 3-*O*-glc 7-*O*-rha	39.87	$[M+H]^+$ 595 $[M-H]^-$ 593 $[M-Rha-H]^-$ 447 $[M-Glc-H]^-$ 431 $[M-Glc-2H]^-$ 430 $[M-Rha-Glc-H]^-$ 285 $[M-Rha-Glc-3H]^-$ 283
94	K 3-*O*-rha 7-*O*-rha	45.87	$[M+H]^+$ 579 $[M-H]^-$ 577 $[M-Rha-H]^-$ 431 $[M-2Rha-H]^-$ 285 $[M-2Rha-3H]^-$ 283
95	Q 3-*O*-glc 7-*O*-rha	35.37	$[M+H]^+$ 611 $[M-H]^-$ 609 $[M-Rha-H]^-$ 463 $[M-Glc-H]^-$ 447 $[M-Glc-2H]^-$ 446 $[M-Rha-Glc-3H]^-$ 299
96	Q 3-*O*-rha 7-*O*-rha	40.78	$[M+H]^+$ 595 $[M-H]^-$ 593 $[M-Rha-H]^-$ 447 $[M-Rha-2H]^-$ 446 $[M-2Rha-H]^-$ 301 $[M-2Rha-3H]^-$ 299
97	I 3-*O*-glc 7-*O*-rha	41.25	$[M+H]^+$ 625 $[M-H]^-$ 623 $[M-Rha-H]^-$ 477 $[M-Glc-H]^-$ 461 $[M-Glc-Rha-H]^-$ 315
98	K 3-*O*-glc-rha 7-*O*-rha	31.10	$[M+H]^+$ 741 $[M-H]^-$ 739 $[M-Rha-H]^-$ 593 $[M-Rha-Glc-2H]^-$ 430 $[M-2Rha-Glc-2H]^-$ 284 $[M-2Rha-Glc-3H]^-$ 283
99	K 3-*O*-glc-glc 7-*O*-rha	35.83	$[M+H]^+$ 757 $[M-H]^-$ 755 $[M-Rha-H]^-$ 609 $[M-Rha-Glc-H]^-$ 447 $[M-Rha-2Glc-H]^-$ 285
100	Q 3-*O*-glc-glc 7-*O*-rha	31.36	$[M+H]^+$ 773 $[M-H]^-$ 771 $[M-Rha-H]^-$ 625 $[M-2Glc-3H]^-$ 445 $[M-Rha-2Glc-3H]^-$ 301
101	Sinapoyl glucose	26.35	$[M+H]^+$ 387 $[M-H]^-$ 385 $[M-Glc]^-$ 223 $[M-O-Glc-H]^-$ 206 $[M-O-Glc-2H]^-$ 205
102	Sinapoyl malate	44.14	$[M+H]^+$ 341 $[M-H]^-$ 339 $[M-Mal+H]^+$ 225 $[M-Mal]^-$ 223

[a] Abbreviations are as follows: K 3-*O*-glc 7-*O*-rha, kaempferol 3-*O*-glucoside 7-*O*-rhamnoside (**93**); K 3-*O*-rha 7-*O*-rha, kaempferol 3-*O*-rhamnoside 7-*O*-rhamnoside (**94**); Q 3-*O*-glc 7-*O*-rha, quercetin 3-*O*-glucoside 7-*O*-rhamnoside (**95**); Q 3-*O*-rha 7-*O*-rha, quercetin 3-*O*-rhamnoside 7-*O*-rhamnoside (**96**); I 3-*O*-glc 7-*O*-rha, isorhametin 3-*O*-glucoside 7-*O*-rhamnoside (**97**); K 3-*O*-glc-rha 7-*O*-rha, kaempferol 3-*O*-rhamnosyl-glucoside 7-*O*-rhamnoside (**98**); K 3-*O*-glc-glc 7-*O*-rha, kaempferol 3-*O*-glucosyl-glucoside 7-*O*-rhamnoside (**99**); Q 3-*O*-glc-glc 7-*O*-rha, quercetin 3-*O*-glucosyl-glucoside 7-*O*-rhamnoside (**100**).

Table 3
Differences in secondary metabolite levels identified in extracts of Arabidopsis seedlings grown on different sulfur-containing media ($n = 8$).

No.	Name	+S Avg	+Sinalbin Avg	−S Avg	$R_{+sinalbin/+S}$	+sinalbin/+S *P* value	$R_{-S/+S}$	−S/+S *P* value
93	K 3-*O*-glc 7-*O*-rha	29.09	19.39	56.90	0.67	0.0172	1.96	6.13E-05
94	K 3-*O*-rha 7-*O*-rha	14.89	52.89	53.40	3.55	8.21E-05	3.59	0.0022
95	Q 3-*O*-glc 7-*O*-rha	67.32	12.82	94.10	0.19	1.49E-07	1.40	0.0027
96	Q 3-*O*-rha 7-*O*-rha	2.35	5.18	9.25	2.20	4.94E-05	3.94	0.0289
97	I 3-*O*-glc 7-*O*-rha	14.37	2.62	18.80	0.18	5.51E-08	1.31	0.0067
98	K 3-*O*-glc-rha 7-*O*-rha	10.21	101.80	27.30	9.97	1.33E-09	2.67	0.0007
99	K 3-*O*-glc-glc 7-*O*-rha	3.91	11.64	11.10	2.98	1.84E-07	2.84	0.0039
100	Q 3-*O*-glc-glc 7-*O*-rha	1.75	7.27	4.06	4.15	2.21E-06	2.31	0.0065
101	Sinapoyl glucose	12.26	20.56	16.50	1.68	0.0029	1.35	0.2794
102	Sinapoyl malate	33.50	65.25	62.00	1.95	0.0003	1.85	0.0039

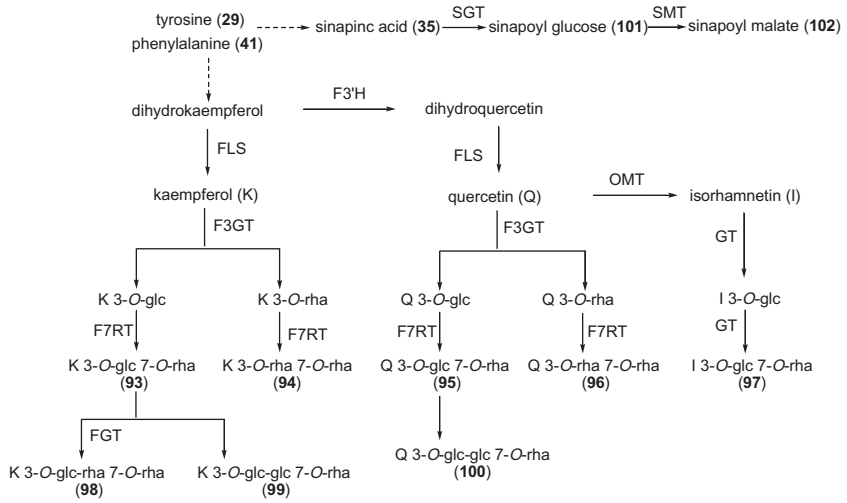


Fig. 5. Simplified schema of the biosynthesis of flavonoid glycosides (**93–100**) and sinapinic acid esters (**101–102**) in Arabidopsis seedlings. Enzymes are indicated by capital letters. Abbreviations are as follows: F3′H, flavanone 3-hydroxylase; FLS, flavonol synthase; GT, glycosyltransferase; F3GT, flavonol 3-*O*-glycosyltransferase; F7RT, flavonol 7-O-rhamnosyltransferase; SGT, sinapate UDP-glucose sinapoyltransferase; SMT, sinapoylglucose malate sinapoyltransferase. K, kaempferol; Q, quercetin; I, isorhamnetin; K 3-*O*-glc, kaempferol 3-*O*-glucoside; K 3-*O*-rha, kaempferol 3-*O*-rhamnoside; Q 3-*O*-glc, quercetin 3-*O*-glucoside; Q 3-*O*-rha, quercetin 3-*O*-rhamnoside; I 3-*O*-glc, Isorhamnetin 3-*O*-glucoside; K 3-*O*-glc 7-*O*-rha, kaempferol 3-*O*-glucoside 7-*O*-rhamnoside (**93**); K 3-*O*-rha 7-*O*-rha, kaempferol 3-*O*-rhamnoside 7-*O*-rhamnoside (**94**); Q 3-*O*-glc 7-*O*-rha, quercetin 3-*O*-glucoside 7-*O*-rhamnoside (**95**); Q 3-*O*-rha 7-*O*-rha, quercetin 3-*O*-rhamnoside 7-*O*-rhamnoside (**96**); I 3-*O*-glc 7-*O*-rha, isorhametin 3-*O*-glucoside 7-*O*-rhamnoside (**97**); K 3-*O*-glc-rha 7-*O*-rha, kaempferol 3-*O*-rhamnosyl-glucoside 7-*O*-rhamnoside (**98**); K 3-*O*-glc-glc 7-*O*-rha, kaempferol 3-*O*-glucosyl-glucoside 7-*O*-rhamnoside (**99**); Q 3-*O*-glc-glc 7-*O*-rha, quercetin 3-*O*-glucosyl-glucoside 7-*O*-rhamnoside (**100**). Adapted from Jones et al. (2003), Besseau et al. (2007), Stracke et al. (2007).

precursors of flavonol glycosides and sinapinic acid esters. Tyrosine (**29**) levels were almost constant and phenylalanine (**41**) levels were elevated ~3.9-fold in the sinalbin (**1**)-supplemented plants in comparison with those levels in control plants. In sulfur-deficient plants, tyrosine (**29**) levels were increased 4.3-fold and phenylalanine (**41**) was almost unchanged. The accumulation patterns of flavonol diglycosides (**93–97**) were similar to those described for the *ugt78d2* mutant, which lacks the ability to glucosylate flavonols at the 3-position as UDP-glucose: flavonoid 3-*O*-glucosyltransferase (Tohge et al., 2005). In the sinalbin (**1**)-supplemented plants, the decreased content of flavonol diglycosides with glucose attached at the 3-position (**93**, **95**, **97**) was correlated with increased levels of their downstream products, the flavonol triglycosides (**98–100**). The result indicates that UGT78D2 is the rate-limiting enzyme in the formation of flavonol triglycosides under sinalbin-supplementation conditions. Consequently, when the activity of enzymes of the competing branch pathway and downstream of UGT78D2 increased relative to UGT78D2 activity, the levels of the flavonol diglycosides (**93**, **95**, **97**) with a glucose attached at the 3-position were markedly reduced and the flavonol diglycosides (**94**, **96**) with rhamnose attached at the 3-position and flavonol triglycosides with glucose attached at the 3-position were significantly increased in abundance. Under sulfur-deficient conditions, genes of the R2R3-MYB family of transcriptional factors, which participate in flavonol accumulation in the *A. thaliana* seedling (Nikiforova et al., 2003; Stracke et al., 2007), are especially upregulated and could account for the increases of these flavonol glycosides. However, flavonol diglycosides (**93**, **95**, **97**) with a glucose attached at the 3-position did not significantly increase in amount. For further explanation of the levels of flavonol glycosides, it will be necessary to identify and quantify the expression levels of genes encoding flavonoid glycosyltransferases.

2.5. Principal component analysis

In addition to investigating differences in levels of individual metabolites, principal component analysis (PCA) was performed to compare differences in metabolic profiles of Arabidopsis seedlings grown on different media (Fig. 6). For the primary metabolite profiles analyzed by GC–TOF-MS, PCA was applied to all peaks. The samples clearly separated into three groups according to the culture media (Fig. 6A), indicating that exogenous sinalbin (**1**) had a great effect on metabolite profiles of Arabidopsis seedlings obtained by GC–TOF-MS. Replicate analyses of samples of each individual treatment gave very similar profiles, which validated the high reproducibility of the experimental procedure from plant harvest to data analysis. When only metabolites identified by LC–MS

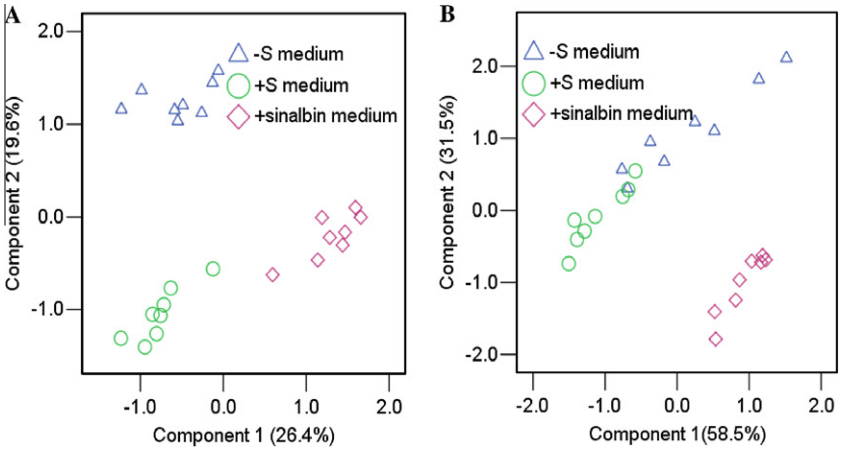


Fig. 6. Principal component analysis of the metabolic profiles from Arabidopsis. Seedlings were grown on Murashige and Skoog media containing sulfur as 846 μM sulfate (blue triangle, +S medium), 846 μM sinalbin (**1**) (green circle, +sinalbin medium), or lacking sulfur (pink diamond, −S medium). The metabolites were extracted and analyzed by (A) GC–TOF-MS and (B) HPLC and HPLC–MS.

were considered, the samples formed three clusters that were dependent on the culture media. However, the metabolite profiles of plants grown in +S and −S media showed slightly similar patterns (Fig. 6B). Altogether, these results indicate that the culture media markedly affects the metabolite profile of Arabidopsis seedlings.

2.6. Identification of desulfoglucosinolates

Desulfoglucosinolates (**4**, **103**–**115**) were identified by diode-array UV spectra and mass fragmentation based on published reports (Hogge et al., 1988; Griffiths et al., 2000; Kiddle et al., 2001). Glucosinolates have maximal UV absorptions at 229 nm, these being the characteristic absorptions of S-C = N structures. Indole glucosinolates have an additional absorbance maximum near 265 nm due to the characteristic absorbance of the conjugated structure of the indole ring. The major fragmentation ions and structures of desulfoglucosinolates (**4**, **103**–**115**) are listed in Table 4 and Supplementary Fig. III. In the absence of collision-induced dissociation (CID), the base peak in the MS of each desulfoglucosinolate was the $[M+H]^+$ ion. Each spectrum also contained a diagnostic ion corresponding to $[M+H-162]^+$ or $[M+2H-162]^+$, which was attributed to the loss of glucosyl moiety from the parent ion (Matthäus and Luftmann, 2000). Desulfoglucosinolates (**4**, **103**–**115**) also contained prominent diagnostic ions derived from the side-chain R, which correspond to $[RCNOH]^+$ or $[RCNOH+H]^+$, $[RCN]^+$ or $[RCN+H]^+$. The $[R]^+$ ion was also observed in each indole desulfoglucosinolate. In addition, *m/z* 130 was used to distinguish 4-methoxy-indol-3-yl-methyldesulfoglucosinolate (**103**) from 1-methoxy-indol-3-yl-methyldesulfoglucosinolate (**104**) as shown in Table 4. When CH_3O- was substituted at the 1-position, an ion was formed corresponding to *m/z* 130. However, fragment ion *m/z* 130 was not detected in the CID spectra of 4-methoxy-indol-3-yl-methyldesulfoglucosinolate (**103**). For short chain aliphatic desulfoglucosinolates, additional fragment ions were observed such as *m/z* 118 of 3-methylthiopropyldesulfoglucosinolate (**105**) and 3-methylsulfinylpropyldesulfoglucosinolate (**106**), *m/z* 132 of 4-methylthiobutyldesulfoglucosinolate (**107**) and 4-methylsulfinylbutyldesulfoglucosinolate (**108**), which are consistent with the loss of CH_3S- or CH_3SO- and glucosyl residues from the parent ions.

2.7. Comparison of glucosinolate and sulfate levels

Glucosinolates (**1**–**2**, **116**–**127**) were quantified with respect to an added internal standard using relative response factors derived from pure glucosinolate standards (Brown et al., 2003). The abundance of individual glucosinolates (**1**, **2**, **116**–**127**) in Arabidopsis seedlings cultured in different media are summarized in Table 5. Seedlings of Arabidopsis grown in sulfur-depleted media showed a marked reduction of total glucosinolates from 26.36 μmol/g dry wt at the normal nutrient condition to 1.62 μmol/g dry wt at the

Table 4
The major fragmentation ions of desulfoglucosinolates extracted from *Arabidopsis thaliana*.

No.	Desulfoglucosinolate	Major fragmentation ions (*m/z*)[a]
4	*p*-Hydroxybenzyl	346$[M+H]^+$ 184$[M+2H-162]^+$ 150$[RCNOH]^+$
103	4-Methoxy-indol-3-yl-methyl	399$[M+H]^+$ 237$[M+2H-162]^+$ 204$[RCNOH+H]^+$ 186$[RCN]^+$ 160$[R]^+$
104	1-Methoxy-indol-3-yl-methyl	399$[M+H]^+$ 237$[M+2H-162]^+$ 203$[RCNOH]^+$ 186$[RCN]^+$ 160$[R]^+$ 130$[R\text{-}OCH_3]^+$
105	3-Methylthiopropyl	328$[M+H]^+$ 166$[M+H-162]^+$ 118$[M-162\ -CH_3S+H]^+$
106	3-Methylsulfinylpropyl	344$[M+H]^+$ 182$[M+H-162]^+$ 118$[M-162\ -CH_3SO+H]^+$
107	4-Methylthiobutyl	342$[M+H]^+$ 180$[M+H-162]^+$ 132$[M-162\ -CH_3S+H]^+$
108	4-Methylsulfinylbutyl	358$[M+H]^+$ 196$[M+H-162]^+$ 132$[M-162\ -CH_3SO+H]^+$
109	5-Methylsulfinylpentyl	372$[M+H]^+$ 210$[M+2H-162]^+$
110	6-Methylsulfinylhexyl	386$[M+H]^+$ 224$[M+2H-162]^+$ 174$[RCN+H]^+$
111	7-Methylthioheptyl	384$[M+H]^+$ 222$[M+2H-162]^+$ 188$[RCNOH]^+$ 173$[RCN+H]^+$
112	7-Methylsulfinylheptyl	400$[M+H]^+$ 238$[M+H-162]^+$ 188$[RCN+H]^+$
113	8-Methylthiooctyl	398$[M+H]^+$ 236$[M+2H-162]^+$ 202$[RCNOH]^+$ 186$[RCN+H]^+$
114	8-Methylsulfinyloctyl	414$[M+H]^+$ 252$[M+2H-162]^+$ 218$[RCNOH]^+$ 202$[RCN+H]^+$ 175$[R]^+$
115	Indol-3-yl-methyl	369$[M+H]^+$ 207$[M+2H-162]^+$ 174$[RCNOH+H]^+$ 156$[RCN]^+$ 130$[R]^+$

[a] R: side-chain.

Table 5
Glucosinolate contents (μmol/g dry wt) of Arabidopsis seedlings grown on Murashige and Skoog media with different sulfur content.

No.	Glucosinolate	Glucosinolate content (μmol/g)[a]		
		+S	−S	+Sinalbin
1	*p*-Hydroxybenzyl	n.d.	n.d.	35.14 ± 6.25
2	4-Methylthiobutyl	0.09 ± 0.01	n.d.	0.10 ± 0.02
116	5-Methylsulfinylpentyl	0.04 ± 0.03	n.d.	n.d.
117	6-Methylsulfinylhexyl	0.01 ± 0.01	n.d.	0.09 ± 0.05
118	3-Methylthiopropyl	0.32 ± 0.10	0.01 ± 0.01	0.05 ± 0.03
119	3-Methylsulfinylpropyl	1.37 ± 0.29	0.01 ± 0.004	0.22 ± 0.16
120	4-Methylsulfinylbutyl	0.25 ± 0.06	0.01 ± 0.005	0.08 ± 0.01
121	7-Methylthioheptyl	0.08 ± 0.03	0.01 ± 0.01	0.79 ± 0.16
122	7-Methylsulfinylheptyl	0.14 ± 0.09	0.03 ± 0.01	0.19 ± 0.06
123	8-Methylthiooctyl	0.57 ± 0.17	0.01 ± 0.01	0.11 ± 0.01
124	8-Methylsulfinyloctyl	1.04 ± 0.29	0.01 ± 0.002	0.15 ± 0.11
125	Indol-3-yl-methyl	7.98 ± 1.25	0.16 ± 0.10	2.85 ± 0.52
126	4-Methoxy-indol-3-yl-methyl	2.91 ± 0.47	0.30 ± 0.10	1.64 ± 0.29
127	1-Methoxy-indol-3-yl-methyl	11.56 ± 5.17	1.06 ± 0.65	4.00 ± 0.25
	Total	26.36 ± 5.26	1.62 ± 0.85	45.43 ± 6.84

[a] Mean values ± SD (n = 3). n.d. Not detected.

depleted sulfur condition. Under these conditions, the content of individual glucosinolates (**1**–**2**, **116**–**127**) decreased to ~6–19% of control levels and several such as 4-methylthiobutylglucosinolate (**2**), 5-methylsulfinylpentylglucosinolate (**116**), and 6-methylsulfinylhexylglucosinolate (**117**) decreased below the limits of detection. In addition, substantial amounts of sinalbin (35.10 μmol/g dry wt) (**1**) accounting for 77% of the total glucosinolates, accumulated in Arabidopsis seedlings grown in the sulfur-deficient media containing exogenous sinalbin (**1**). Compared to control conditions, the levels of endogenous glucosinolates were reduced in sinalbin-supplemented media, but still higher than those measured under sulfur starvation conditions.

Sulfate contents of Arabidopsis seedlings grown on different conditions were analyzed and the results are shown in Fig. 7. Compared to control conditions, sulfate contents under sulfur-deficient and sinalbin (**1**)-supplemented conditions decreased significantly. Sulfate contents in sinalbin (**1**)-supplemented plants relative to sulfur-depleted plants were also reduced.

Relative to sulfur starvation conditions, high levels of endogenous glucosinolates in sulfur-deficient conditions indicated that exogenous sinalbin (**1**) could be catabolized in vivo to provide a sulfur source for glucosinolate biosynthesis. Although the application of sinalbin (**1**) might lead to a stress-induced activation of the sulfur assimilation pathway, the increase in assimilated sulfur generally accounts for only a minor proportion of the total sulfur content in the plant (Kopriva, 2006; Hawkesford and de Kok, 2006). Moreover, as there is no sulfate available from the media, the sinalbin (**1**)-supplemented plants cannot assimilate sulfate from the culture media. In Arabidopsis seedlings, sinalbin (**1**) treatment led to increased *O*-acetyl-serine (**17**) levels compared to control plants, but the content was far lower than that found in sulfur-deficient plants. This indicates that the state of sulfur deficiency was alleviated, yet the sulfate content was decreased relative to sulfur-depleted plants. These results suggest that plants can use sinalbin (**1**) as a sulfur source and under sulfur-deficient conditions glucosinolate can be catabolized to release sulfur to support primary metabolic processes, such as protein synthesis. Glucosinolate catabolism is evident in the fate of radiolabelled sinalbin (**1**) taken up by germinating Arabidopsis seeds (Petersen et al., 2002). In seedlings at the cotyledon stage, approximately 30% of radiolabelled glucosinolate was lost and in young plants with rosettes of six to eight leaves, radiolabelled glucosinolate could not be detected. If glucosinolate catabolism occurs, this could be accelerated under an inorganic sulfur shortage. In sulfur-depleted seedlings, glucosinolates decreased significantly, which further demonstrated that glucosinolate catabolism occurs in Arabidopsis. However, the mechanism of glucosinolate catabolism is still unknown. Increased turnover of sinalbin (**1**) was found to coincide with elevated levels in activity of the glucosinolate-degrading enzyme myrosinase, which indicated the possibility that there was a continuous turnover of glucosinolates mediated by myrosinase during development and not only upon tissue disruption (Petersen et al., 2002). However, in extracts of sinalbin (**1**)-supplemented plants, although the nitrile breakdown product of sinalbin was detected and shown to be an artifact of extraction, other nitriles or isothiocyanates generated by in vivo myrosinase activity were not detected and the contents of SO_4^{2-} relative to sulfur-deficient plants decrease. Furthermore, Barth and Jander (2006) found that decreased levels of glucosinolates during senescence and germination are independent of TGG1 and TGG2 myrosinases, which are expressed in above-ground tissues. Therefore, sinalbin (**1**) breakdown via myrosinase activity does not provide sulfur for plants.

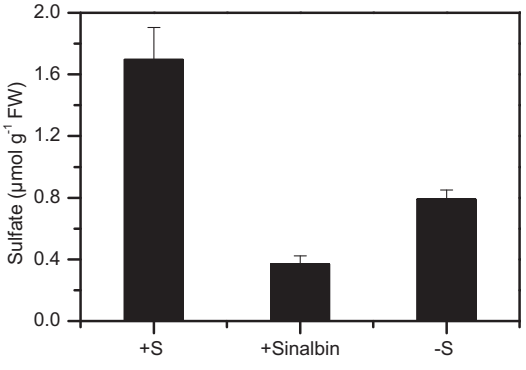


Fig. 7. Sulfate content (μmol g^{-1} fr. wt) of Arabidopsis seedlings grown on Murashige and Skoog media with different sulfur content. Error bars represent ± SD (n = 4).

3. Conclusions

In conclusion, using GC–TOF-MS and LC–MS as the methods of metabolic profiling, the metabolites in *Arabidopsis thaliania* growing on sulfur-deficient, sinalbin (**1**)-supplemented and control conditions were identified and their levels compared. The comparison of individual metabolite levels of Arabidopsis seedlings in different

media demonstrates that the exogenously supplied glucosinolate sinalbin (**1**) had large effects on metabolite profiles and Arabidopsis could utilize exogenous sinalbin (**1**) as a sulfur source. Under sulfur-deficient conditions, glucosinolates were catabolized to release sulfur for essential functions. The preliminary findings here, to some extent, may lead to new and important opportunities to elucidate glucosinolate catabolism in vivo.

4. Experimental

4.1. Plant material and growth conditions

Arabidopsis thaliana ecotype Wassilewskja (seeds provided by Prof. Qi Xie, Institute of Genetics and Developmental Biology Chinese Academy of Sciences, Beijing, China) was grown in a growth chamber on a solidified agarose medium (0.5 × Murashige-Skoog salts for control sulfur-sufficient medium) containing 846 μM sulfate, where they were subjected to a photosynthetic photon flux of 100 μmol m^{-2} s^{-1}, 65% relative humidity, and a photoperiod of, 16-h day at 22 °C/8-h night at 18 °C. For sulfur-deficient (0 μM) experiments, all sulfate in the sulfur-sufficient medium was replaced with chloride as described previously (Nikiforova et al., 2003). To investigate the use of the exogenously provided glucosinolate sinalbin (**1**) as a sulfur source for plant nutrition under sulfur-deficient conditions, the sulfate was replaced by an equimolar concentration of sinalbin (**1**). After 13 days of growth, plant material was harvested and immediately frozen in liq. N_2 and stored at −80 °C until extraction.

4.2. Extraction and GC–TOF-MS analysis of metabolites

To investigate the change in primary metabolite levels, eight plants were harvested from each of the three different media growth conditions. Individual Arabidopsis seedlings (100–150 mg) were homogenized under liq. N_2 and extracted with MeOH–$CHCl_3$–H_2O (2 mL, 2.5:1:1, v/v/v). Ribitol was added as an internal standard. The extracts were shaken for 15 min and centrifuged for 5 min at 23,000*g*. The supernatant was then concentrated to an aqueous phase under N_2 and lyophilized. The dried samples were derivatized with methoxyamine hydrochloride in pyridine (50 μL, 20 mg/mL), and subsequently with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) (80 μL) (Fiehn et al., 2000; Wagner et al., 2003; Weckwerth et al., 2004; Lisec et al., 2006). A mixture of C_{11} to C_{36} *n*-alkanes was used for the determination of retention time indices. The GC–TOF-MS system consisted of a gas chromatograph (Agilent 6890) and a time of flight mass spectrometer (Waters Micromass GCT Premier™). The GC was operated at a constant flow of 1 mL/min helium and an injector temperature of 250 °C. The samples were injected with a split ratio of 10:1 onto a 60 m DB5 column (0.25 mm i.d., 0.25 μm film thickness) using the following temperature program: 80 °C (held for 2 min), 80–180 °C (5 °C/min), 180–250 °C (3 °C/min), 250–300 °C (5 °C/min, held for 20 min). The ion source temperature of the TOF mass spectrometer was 200 °C and the transfer line temperature was set at 250 °C. Data were acquired by EI^+ mode in the mass range of *m/z* 30–800.

4.3. Automated identification and quantification of primary metabolites obtained with GC–TOF-MS

The GC–TOF-MS raw data files were converted to NetCDF format with Databridge (Waters). The mass spectra were automatically refined by AMDIS (Automated Mass Spectral Deconvolution and Identification Software; http://www.amdis.net). Then the target compounds were identified by matching the refined mass spectra against the Golm Metabolome database (Kopka et al., 2005) and NIST library. In addition, the retention time indices were calibrated using the *n*-alkane standards, which were also helpful for metabolite identification. Following identification of metabolites, the mass spectrometry data for metabolite profiles were automatically compared using XCMS software, which incorporates nonlinear retention time alignment, matched filtration, peak detection, and peak matching (Smith et al., 2006). The parameters for XCMS were default settings (http://www.metlin.scripps.edu/download/; Nordström et al., 2006; Smith et al., 2006). The relative concentrations of metabolites were determined by normalizing the area integration of a characteristic fragment ion trace to the fresh weight of the plant material extracted and the area of the internal standard ribitol (*m/z* 319). The average relative concentration was compared by calculating the response ratios ($R_{-S/+S}$ or $R_{+sinalbin/+s}$) of the average relative concentration on −S medium or +sinalbin medium to that on +S medium as a control. When the relative ratio ($R_{-S/+S}$ or $R_{+sinalbin/+s}$) was more than 2.0 or less than 0.5 with $P < 0.05$, the relative concentration of metabolites was considered to be significantly altered, a criterion similar to that applied by Nikiforova et al. (2005).

4.4. Data analysis

Statistical significance of the differences in relative concentrations of metabolites was estimated using statistical analyses including principal component analysis (PCA) and *t*-test. Missing data were replaced with 0 for PCA. R_i was the changes between relative concentrations in plants grown in normal medium as a control and plants grown in different media. Statistical significance of the differences in relative concentrations was analyzed with the *t* test (Microsoft Excel). Changes in relative concentrations with $R > 2.0$, $P < 0.05$ or $R < 0.5$, $P < 0.05$ were considered to be significantly different.

4.5. Extraction and LC–MS analysis of secondary metabolites

For LC–MS analysis of metabolites, MeOH-H_2O (1.4 mL, 80:20, v/v, precooled at −20 °C) containing 0.5 mM genistein (30 μL) as internal standard was added to freshly ground Arabidopsis seedlings (ca 300 mg). The extracts were sonicated at 4 °C for 15 min and centrifuged at 4 °C and 45,000*g* for 5 min. The supernatant was concentrated to the aqueous phase under N_2, lyophilized, and then redissolved in H_2O (1.4 mL) for either LC–ESI-MS or LC-PDA analysis. The apparatus and operation conditions were the same as mentioned above, except for the following parameters: mass scan: *m/z* 100–1100; scan mode: positive and negative ESI mode; gradient conditions (solvent A: 0.1% v/v HCOOH/H_2O, solvent B: CH_3CN): 0–10 min, 5–10% B; 10–40 min, 10–20% B; 40–50 min, 20–30% B; 50–65 min, 30–50% B; 65–80 min, 50–95% B; UV detection wavelength: 254 nm; collision energy (CE): 15∼35 eV. UV/VIS absorption spectra were taken in the range of 200–600 nm. Known metabolites were identified by their mass spectrometric fragmentation patterns and relevant data reported in the literature (Graham, 1998; von Roepenack-Lahaye et al., 2004; Tohge et al., 2005; Kerhoas et al., 2006; Routaboul et al., 2006; Besseau et al., 2007; Stracke et al., 2007). The secondary metabolites identified by LC–MS were quantified by integrating the peak areas monitored at 254 nm using the CSASS (Complex Sample Analysis Software System) software and by normalizing them to the fresh wt of the extracted plant material and the peak areas of the internal standard genistein. The CSASS software is tailored to accurately quantify complex samples especially overlapping peaks obtained by GC or LC using an automatic curve-fitting method (Xue, 2004).

4.6. Extraction and analysis of sinalbin

Sinalbin (**1**) was extracted and purified from white mustard seeds (*Sinapis alba* (L.), a generous gift from Dr. M. Reichelt, Max Planck Institute for Chemical Ecology, Jena, Germany) using a modification of the method described by Thies (1988). White mustard seed (ca. 50 g) was dried in an oven at 120 °C for 2 h to inactivate myrosinase. Dried samples were then ground to a powder and extracted with MeOH (2 × 100 mL). After 30 min gentle shaking at room temperature (25 °C), samples were centrifuged at 1500*g* for 5 min and the supernatant (200 mL) was loaded onto a (10 mL of 10 mg/mL) DEAE Sephadex A25 column. The column was rinsed with HCO_2H–*i*-PrOH–H_2O (2 × 5 mL, 3:2:5, v/v/v) and deionized H_2O (4 × 5 mL), and the eluates discarded. Samples were then eluted using EtOH–H_2O (25 mL, 96:4, v/v) and 20 mM K_2SO_4/15% *i*-PrOH (25 mL). After the eluent was agitated and cooled for 10 min at 4 °C, samples were centrifuged at 1500*g* for 5 min and the supernatant was concentrated. EtOH (30 mL) was added to the concentrate, followed by concentration to near-dryness. The residue was transferred using MeOH (10 mL) and stored at −15 °C for 15 min. The solution was centrifuged at 1500*g* for 5 min and the supernatant concentrated to dryness. For removal of K_2SO_4, the steps of the sinalbin (**1**) dissolution with EtOH and MeOH were repeated until precipitation did not appear during centrifugation.

For analysis of sinalbin (**1**) purity, sinalbin (**1**) was derivatized using the same protocol as that of the Arabidopsis samples. Sinalbin (**1**) purity was measured by GC-FID and calculated by the normalization method, which indicated a percentage content of 88%.

4.7. Extraction and analysis of glucosinolates

Harvested seedlings were lyophilized to dryness and ground to a powder. Lyophilized samples (10–20 mg) were extracted in MeOH–H_2O (4 mL, 80:20, v/v) containing the internal standard (sinalbin (**1**), 0.8 μmol, 800 μL of 1 mM). The other protocols of extraction were the same as that of Reichelt et al. (2002). In the protocols used, sulfatase was employed to convert glucosinolates to their desulfated derivatives (desulfoglucosinolates). Desulfoglucosinolates were separated and identified by an HPLC/PDA/ESI-MS system (Agilent 1100 Series LC/MSD Trap). HPLC was carried out on a Venusil MP-C18, 5 μm, 4.6 × 250 mm column (Agela technologies Inc. USA) operated at a flow rate of 1 ml min^{-1} and a column temperature of 30 °C. Elution was accomplished with a gradient (solvent A: H_2O, solvent B: CH_3CN) of 1.5–8% B (14 min), 8–30% B (10 min), 30–36% B (5 min), 36–40% B (3 min). The eluent was monitored by diode array detection between 210 and 400 nm. The ESI source parameters were as follows: capillary voltage of 3.5 kV (positive mode), nebulization with N_2 at 30 psi, dry gas flow of 8 L min^{-1} at a temperature of 320 °C, fragmentation amplitude set to 1 V. Full scan mass spectra were acquired from 50 to 1000 *m/z*. Desulfoglucosinolates were identified by mass fragmentation in conjunction with diode array UV spectra and quantified by A_{229nm} relative to the internal standard.

4.8. Sulfate determination

To exclude effects of SO_4^{2-} in sinalbin (**1**) on Arabidopsis, the percentage content of SO_4^{2-} in sinalbin (**1**) was analyzed by ion chromatography. The ground plant material (ca 200 mg fresh weight) under liq. N_2 was extracted with deionized H_2O (4 mL) at 95 °C for 15 min, after which the extract was centrifuged at 23,000*g* for 5 min. The SO_4^{2-} concentration in the supernatant was determined by a Shimadzu ion chromatogram fitted with a Shimpack IC-A3 anion separator column. The percentage content of SO_4^{2-} was 0.05%, which was considered to be low enough and almost insufficient to provide SO_4^{2-} for Arabidopsis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2011.06.002.

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