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Accumulation of coumarins in *Arabidopsis thaliana*

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Abstract

The biosynthesis of coumarins in plants is not well understood, although these metabolic pathways are often found in the plant kingdom. We report here the occurrence of coumarins in *Arabidopsis thaliana* ecotype Columbia. Considerably high levels of scopoletin and its β-D-glucopyranoside, scopolin, were found in the wild-type roots. The scopolin level in the roots was ~1200 nmol/gFW, which was ~180-fold of that in the aerial parts. Calli accumulated scopolin at a level of 70 nmol/gFW. Scopoletin and scopolin formation were induced in shoots after treatment with either 2,4-dichlorophenoxyacetic acid (at 100 μM) or a bud-cell suspension of *Fusarium oxysporum*.

In order to gain insight into the biosynthetic pathway of coumarins in *A. thaliana*, we analyzed coumarins in the mutants obtained from the SALK Institute collection that carried a T-DNA insertion within the gene encoding the cytochrome P450, CYP98A3, which catalyzes 3′-hydroxylation of *p*-coumarate units in the phenylpropanoid pathway. The content of scopoletin and scopolin in the mutant roots greatly decreased to ~3% of that in the wild-type roots. This observation suggests that scopoletin and scopolin biosynthesis in *A. thaliana* are strongly dependent on the 3′-hydroxylation of *p*-coumarate units catalyzed by CYP98A3. We also found that the level of skimmin, a β-D-glucopyranoside of umbelliferone, was slightly increased in the mutant roots.
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Keywords: *Arabidopsis thaliana*; Cruciferae; Scopoletin; Scopolin; Skimmin; Biosynthesis of coumarins; Cytochrome P450; CYP98A3; T-DNA insertion tag lines

1. Introduction

Coumarins are often found as plant secondary metabolites in the plant kingdom (Zobel, 1997). Many have trivial names, such as umbelliferone (7-hydroxycoumarin), esculetin (6,7-dihydroxycoumarin), scopoletin (7-hydroxy-6-methoxycoumarin) and others (**1**–**9**) (Fig. 1). Their exact roles in plants are unclear. However, they are thought to play some role in plant defense due to the induction of their biosynthesis following various stress events (Garcia et al., 1995; Baillieul et al., 2003; Shimizu et al., 2005) as well as their antimicrobial and antioxidative activities (Valle et al., 1997; Chong et al., 2002; Gachon et al., 2004; Carpinella et al., 2005). So far, comparatively little information is available regarding the biosynthesis of coumarins in plants. The tracer experiments using ^{14}C-labelled ferulate or other intermediates showed that tobacco plants are able to produce scopoletin (**7**) from the phenylpropanoid pathway (Fritig et al., 1970). In the biosynthetic pathway of plant coumarins, it is thought that oxidation at the 2′-position of the ring of cinnamates, *cis–trans* geometrical isomerization of the side chain and lactonization occur successively (Keating and O'Kennedy, 1997; Matern et al., 1999). Several branch pathways from phenylpropanoid compounds to coumarins are probable (Fig. 1).

Arabidopsis CYP98A3 (At2g40890) encodes the cytochrome P450 called *p*-coumaroylshikimate/quinate 3′-hydrolxylase (C3′H) (Schoch et al., 2001). The 3′-hydroxylation step was reported to be important role(s) to control carbon allocation to downstream processes in the phenylpropanoid pathway (Anterola et al., 1999, 2002). Franke et al. (2002a,b) also reported that the C3′H activity is important in the lignin synthesis because the deficient

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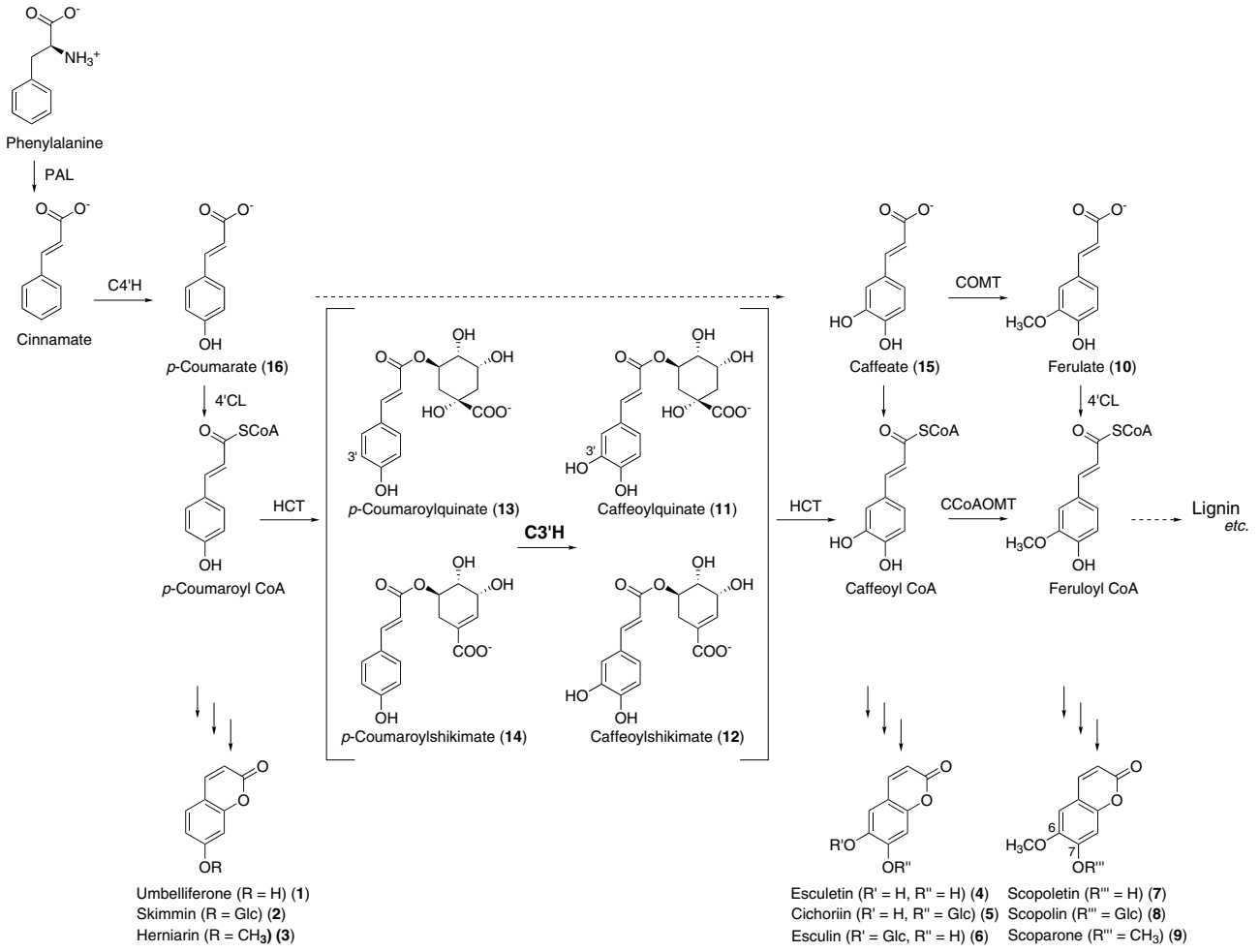

Fig. 1. Proposed biosynthesis pathways of coumarins in plants. Coumarins, such as umbelliferone (**1**) and scopoletin (**7**), are biosynthesized via the phenylpropanoid pathway. The enzymes and their abbreviations are phenylalanine ammonia-lyase (PAL); cinnamate 4′-hydroxylase (C4′H); 4′-hydroxycinnamoyl CoA ligase (4′CL); hydroxycinnamoyl CoA:shikimate/quinate hydroxycinnamoyltransferase (HCT); *p*-coumaroylshikimate/quinate 3′-hydroxylase (C3′H); caffeic/5′-hydroxyferulic acid *O*-methyltransferase (COMT); caffeoyl CoA *O*-methyltransferase (CCoAOMT).

mutant of C3′H, *ref8*, exhibits a dwarf phenotype and decreases in guaiacyl and syringyl lignin. Thus deficiency in the function of C3′H causes significant effects on the profile of the phenylpropanoid metabolism and therefore, the *c3′h* mutant is an exploitable source to investigate the biosynthesis pathways of phenylpropanoids.

We report here the occurrence of coumarins in *A. thaliana* ecotype Columbia. Levels of **7** and its β-D-glucopyranoside, scopolin (**8**), in *A. thaliana* were quantified both before and after various treatments. Furthermore, we analyzed coumarins in the *c3′h* mutants of *A. thaliana* obtained from the SALK Institute collection in order to collect information on the biosynthesis of **7** and **8**.

2. Results and discussion

2.1. Analysis of coumarins in Arabidopsis thaliana

Recently, it was reported that *A. thaliana* contains scopoletin (**7**) and its β-D-glucopyranoside, scopolin (**8**) (Rohde et al., 2004; Bednarek et al., 2005). We extensively analyzed coumarins in *A. thaliana*. Quantified levels of coumarins in *A. thaliana* are shown in Table 1. Compounds **7** and **8**, which were identified by LC–MS/MS, accumulated to considerably high levels. Trace amounts of skimmin (**2**), esculetin (**4**) and the β-glucoside of **4** were also detected. We could not identify whether the β-glucoside of **4** is cichoriin (**5**) or esculin (**6**), since the standard of **5** was unavailable. Additional coumarins were not detected in *A. thaliana*.

The roots accumulated higher amounts of the coumarins than the shoots. HPLC analysis with a fluorescence detector showed that levels of **7** and **8** in roots were ~15 and 1200 nmol/gFW, respectively, which were ~180-fold of those in the shoots (Table 1). The level of **8** was ~85-fold of that of **7** both in the shoots and roots, indicating that the majority of **7** is stored as β-glucoside in *A. thaliana*, although the glucosylation step of the 7-position has not been elucidated in *A. thaliana*. The calli also contained **7** and **8** at ~1 and 70 nmol/gFW, respectively, ~10-fold of those in the shoots.

Table 1
The occurrence of the coumarins in *A. thaliana*

Coumarins	Shoot (nmol/gFW)	Root (nmol/gFW)	Callus (nmol/gFW)
Umbelliferone (**1**)	n.d.	n.d.	n.d.
Skimmin (**2**)	n.d.	trace	n.d.
Herniarin (**3**)	n.d.	n.d.	n.d.
Esculetin (**4**)	trace	trace	n.d.
Cichoriin (**5**) or esculin (**6**)	trace	trace	trace
Scopoletin (**7**)	0.0720 ± 0.0329	14.7 ± 0.494	3.02 ± 0.686
Scopolin (**8**)	6.87 ± 0.337	1250 ± 171	69.1 ± 11.3
Scoparone (**9**)	n.d.	n.d.	n.d.

Accumulated coumarins in higher levels, **7** and **8**, were quantified with HPLC analysis using a fluorescence detector (ex 340 nm, em 420 nm). Values show the averages of **7** and **8** contents with standard errors ($n = 3$). n.d. (not detected), trace: by LC–MS/MS.

The accumulation level of **7** and **8** in the roots of *A. thaliana* was comparable to that observed in tobacco (Dieterman et al., 1964). Compound **7** has antimicrobial and antioxidative activities (Valle et al., 1997; Chong et al., 2002; Gachon et al., 2004; Carpinella et al., 2005). Levels of **7** may increase after hydrolysis of **8** by β-glucosidases when the plants are subjected to wounding or other damage. The roots are thought to always be exposed to challenges by microorganisms and physical wounding. The higher protection mechanisms seem to be needed in roots rather than in aerial parts of the plants, for example accumulating protective compounds such as coumarins. The release of coumarins in *A. thaliana* by the action of enzymes such as glucosidase(s) is also of interest to our group.

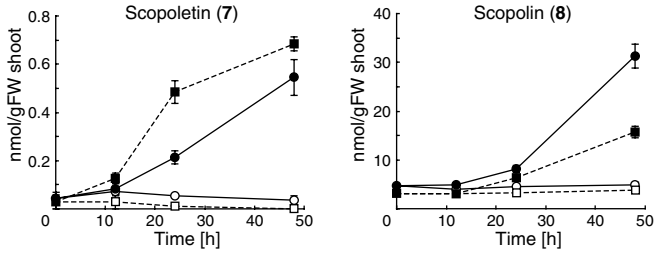

Fig. 2. Scopoletin (**7**) and scopolin (**8**) level in shoots after treatments with 2,4-D and *F. oxysporum*. 23–25-day-old plants were treated with the 2,4-D (100 μM: solid circle, control: open circle) or bud-cell suspension of *F. oxysporum* f. sp. *batatas* O-17 (5×10^7 bud-cells/ml: solid square, control: open square). Shoots were extracted with MeOH containing 4-methylumbelliferone as an internal standard. The MeOH extracts were subjected to HPLC analysis using a fluorescence detector (ex 340 nm, em 420 nm). Values show the averages of scopoletin (**7**) and scopolin (**8**) contents. The horizontal axis shows time after the treatments. Bars show standard errors ($n = 3$).

2.2. Induction of scopoletin (7) and scopolin (8) after various treatments

Coumarins were induced by various stresses or chemical treatments, such as wounding, phytohormones and interaction with microorganisms (Dieterman et al., 1964; Hino et al., 1982; Cabello-Hurtado et al., 1998; Shimizu et al., 2005). To investigate the effect of phytohormones on the content of scopoletin (**7**) and scopolin (**8**) in *A. thaliana*, we treated the shoots with salicylic acid, methyl jasmonate, 2,4-dichlorophenoxyacetic acid (2,4-D), and kinetin and incubated for 48 h. The levels of **7** and **8** in the shoots increased after treatment with 2,4-D, whereas all other plant hormones had no effect on the level (data not shown). The levels of **7** and **8** significantly increased 12 h after the treatment and reached 0.55 and 31 nmol/gFW, respectively, at 48 h (Fig. 2). Considering that the calli derived from leaves with 2,4-D and kinetin contains a higher level of **7** and **8** than the shoots (Table 1), auxin may regulate the levels of **7** and **8**.

Treatment with a bud-cell suspension of *Fusarium oxysporum* f. sp. *batatas* O-17 (5×10^7 bud-cells/ml), pathogenic against sweet potato and morning glory (Ogawa, 1988; Shimizu et al., 2000), induced **7** and **8** in the shoots of *A. thaliana*. The levels of **7** and **8** treated with a bud-cell suspension at 48 h were 0.68 and 16 nmol/gFW, respectively. Neither wounding nor treatment with laminarin and chitosan had any effect on the levels of these coumarins. Compounds **7** and **8** can be induced by the fungus stimulant *F. oxysporum* f. sp. *batatas* O-17, whose mechanism of interaction with *A. thaliana* is unknown. It is known that coumarins including furanocoumarins relate to plant defense with their antimicrobial activity (Johnson et al., 1973; Desjardins et al., 1989; Baillieul et al., 2003). Compound **7** is reported to play an important role in protecting against pathogen infection (Valle et al., 1997; Chong et al., 2002; Gachon et al., 2004; Carpinella et al., 2005) and scavenging reactive oxygen intermediate accompanied by infection (Chong et al., 2002). In *A. thaliana*, Compounds **7** and **8** may also have protective role(s) following infection by microorganisms.

2.3. T-DNA insertion mutant lines of C3′H

In the first step to investigate the biosynthesis of scopoletin (**7**) and scopolin (**8**) in *A. thaliana*, we isolated the functional deficient mutants of C3′H encoded by the cytochrome P450 gene, *CYP98A3* (At2g40890) (Schoch et al., 2001). C3′H is an important enzyme in the pathway. Therefore, it is interesting to investigate the effect of the C3′H mutation on coumarin biosynthesis. In the Salk Institute Genomic Analysis Laboratory T-DNA insertion collection, there were two candidate mutant lines in which T-

DNA was inserted in the exon of the *CYP98A3* gene. The two T-DNA insertion mutant lines (SALK_112823 and SALK_125686) were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus). After selection of the homozygous T-DNA insertion mutants, the exact position of T-DNA insertion was determined by sequencing of the PCR products amplified with the gene-specific and T-DNA specific primers (Fig. 3A). In both mutant lines T-DNA was found to be inserted in an exon of the *CYP98A3* gene. The functional deficiency of CYP98A3 was confirmed by RT-PCR (Fig. 3B). These *c3′h* mutants exhibited the dwarf phenotype (Franke et al., 2002a), which was most likely the result of low lignin levels (Franke et al., 2002b).

2.4. Reduction of scopoletin (7) and scopolin (8) level by the functional deficiency of C3′H

The two *c3′h* mutant lines (SALK_112823 and SALK_125686) were used to determine the levels of scopoletin (**7**) and scopolin (**8**). Quantification by HPLC analysis showed that the levels of **7** and **8** in the *c3′h* roots were very low compared to those in the wild-type roots (Fig. 4). The *c3′h* roots contained **7** and **8** at about 0.6 and 30 nmol/gFW, 9% and 3% of the wild-type roots, respectively. The severe reduction of the coumarins in the *c3′h* mutants indicates that **7** and **8** are biosynthesized via the phenylpropanoid pathway in *A. thaliana*. These results are consistent with previously reported research (Fritig et al., 1970), which showed that **7** is biosynthesized from ferulate (**10**) rather than via the umbelliferone–esculetin–scopoletin pathway in tobacco plants. The ring modification steps are likely to proceed at the cinnamic acid stage in *A. thaliana*. The biosynthesis of **7** and **8** is strongly dependent on the 3′-hydroxylation of *p*-coumarate units, indicating that

Fig. 3. Characterization of the T-DNA insertion mutants of CYP98A3. Two homozygous lines of the mutant were obtained. (A) The positions of the T-DNA insertion in SALK_112823 and SALK_125686 lines. The exact positions of the respective T-DNA insertion were determined by sequencing of the PCR products amplified with the gene-specific and the left border specific primers. L means the left border of T-DNA. (B) RT-PCR of *CYP98A3* transcripts. Total RNA was extracted from the homozygous mutant lines and wild-type (WT). RT-PCR was performed with the *CYP98A3* specific primers and the *ACTIN* specific primers (control).

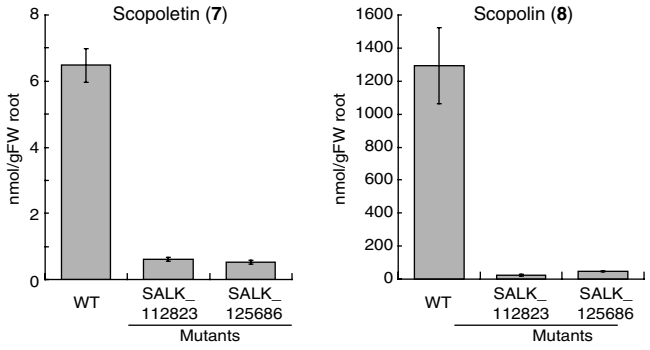

Fig. 4. The levels of scopoletin (**7**) and scopolin (**8**) in the roots of the *c3′h* mutants and wild-type. The roots of 25-day-old plants were extracted with MeOH containing 4-methylumbelliferone as an internal standard. The MeOH extracts were subjected to HPLC analysis using a fluorescence detector (ex 340 nm, em 420 nm). Values show the averages of scopoletin (**7**) and scopolin (**8**) contents. Bars show standard errors ($n = 3$).

the 3′-hydroxy group of caffeate units leads to the oxygen atom in the 6-methoxy group of **7**. Franke et al. (2002b) reported that the mutant of CYP98A3, *ref8*, exhibited a reduction in guaiacyl and syringyl lignin and soluble units. The supply of caffeoylquinate (**11**) and/or caffeoylshikimate (**12**) to the downstream biosynthesis including **7** and **8** presumably fell due to the deficiency of CYP98A3.

The presence of small amounts of **7** and **8** detected in the *c3′h* mutants indicates a slight conversion from *p*-coumarate units to caffeate units in the phenylpropanoid pathway by another enzyme(s). While CYP98A3 has been reported to be the main C3′H enzyme in *A. thaliana* (Schoch et al., 2001; Franke et al., 2002a,b; Raes et al., 2003), another enzyme(s) might exhibit C3′H activity in *A. thaliana*. The CYP98A family consists of three genes, *CYP98A3*, *CYP98A8* and *CYP98A9*, in *A. thaliana*. Although CYP98A8 and CYP98A9 do not hydroxylate *p*-coumaroylquinate (**13**) and *p*-coumaroylshikimate (**14**) (Schoch et al., 2001), they might catalyze the 3′-hydroxylation of *p*-coumarate units of other phenylpropanoid derivatives.

2.5. Increase of skimmin (2) level in the c3′h mutants

Besides the substantial reduction in the levels of scopoletin (**7**) and scopolin (**8**), we explored changes in the levels of other coumarins in the *c3′h* mutants. The level of skimmin (**2**), the β-glucoside of umbelliferone (**1**), identified by LC–MS/MS was slightly but significantly increased in the *c3′h* mutants. HPLC analysis with a fluorescence detector showed that the level of **2** in the *c3′h* roots was found to be ~2 nmol/gFW (Fig. 5). A trace amount of **1**, the aglycon of **2**, was also detected in the *c3′h* roots by LC–MS/MS (data not shown). The major form of **1** in the *c3′h* mutants was the β-glucoside **2** as well as that of **7** in the wild-type, indicating that the glucosylation in the 7-position of **1** is likely active in the *c3′h* mutants. Compound **1** and its β-glucoside **2** do not have a methoxy group at the C-6 position, and therefore, they seem to be derived from precursors

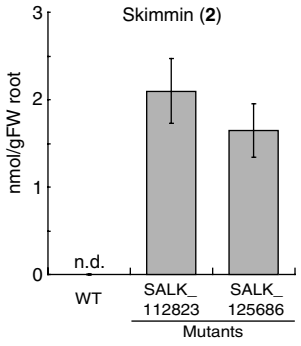

Fig. 5. The level of skimmin (**2**) in the roots of the *c3′h* mutants and wild-type. The roots of 25-day-old plants were extracted with MeOH containing 4-methylumbelliferone as an internal standard. The MeOH extracts were subjected to HPLC analysis using a fluorescence detector (ex 320 nm, em 380 nm for the detection of skimmin (**2**), ex 340 nm, em 420 nm for the detection of 4-methylumbelliferone). Values show the averages of contents of **2**. Bars show standard errors ($n = 3$). n.d.: not detected (less than 87 pmol/gFW).

before the 3′-hydroxylation reaction by C3′H. *p*-Coumarate units were accumulated in the *ref8* mutant (Franke et al., 2002b), indicating that the pathway producing *p*-coumarate units is still active in the *ref8* mutant. In this pathway, an unusual *p*-hydroxyphenyl lignin is formed from *p*-coumaryl alcohol, which accumulated instead of the guaiacyl and syringyl lignin formed from coniferyl and sinapyl alcohol, respectively. The accumulated *p*-coumarate units may be supplied as the source of **1** and **2** in the *c3′h* mutants. Furthermore, our results indicate that the *c3′h* mutants retain, to some degree, the biosynthetic activities for constructing the coumarin structure from *p*-coumarates and ferulates, in which the 2′-hydroxylation could be the key step of the coumarin biosynthesis because of its irreversibility. It was reported that phenoloxidase in *Saxifraga stolonifera* has 2′-oxidation activity for caffeate (**15**) toward esculetin (**4**) production (Sato, 1967). However, phenoloxidase cannot catalyze 2′-oxidation of *p*-coumarate (**16**) and ferulate (**10**), which lack the hydroxy groups necessary to form the quinone structure allowing the reaction. The 2′-oxidase(s) of cinnamates in plants still remains to be unclear. Our results indicate that these biosynthetic activities in *A. thaliana* constructing the coumarin structure could produce **1** from *p*-coumarate units. The level of **2** in the *c3′h* mutants was very low, ~0.16% compared to that of **8** in the wild-type roots. Two reasons could be proposed for the low level of **2** in the *c3′h* mutants. First, *p*-coumarate derivatives, which do not have the 3′-hydroxy group, may be poor substrates of the 2′-oxidase(s) in *A. thaliana*. Second, no appropriate substrates may accumulate in sufficient amounts to allow the reaction to proceed efficiently to form **1**. Biochemical and genetic characterization of such enzyme(s) would provide us further information on the biosynthetic pathway of coumarins in *A. thaliana*.

A. thaliana is an excellent system for analyzing biosynthetic pathways in plants due to the large number of available libraries of tag inserted lines and ease of culture.

3. Experimental

3.1. Instrumentation

HPLC analysis was performed using LC-10ADvp Solvent Delivery Unit (Shimadzu, Kyoto, Japan) and a Waters 470 Scanning Fluorescence Detector (Waters, Milford, MA), whereas LC–MS/MS analysis utilized an API3000 LC–MS/MS System equipped with an electrospray ion source (Applied Biosystems Japan, Tokyo, Japan).

3.2. Chemicals

Scopolin (**8**) and skimmin (**2**) were synthesized, according to the reported method by Gee et al. (1999). Scopoletin (**7**) and 4-methylumbelliferone were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Umbelliferone (**1**) and esculin (**6**) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Esculetin (**4**) was purchased from Avocado Research Chemicals Ltd. (Lancashire, UK). Herniarin (**3**) was purchased from Acros Organics (Geel, Belgium). Scoparone (**9**) was purchased from Sigma–Aldrich Co. (St. Louis, MO).

3.3. Plant material

Seeds of *A. thaliana* (ecotype Columbia and mutants) were surface sterilized with 5% (v/v) NaOCl and sown on 0.8% (w/v) agar-solidified medium supplemented with Murashige and Skoog salt, 1% (w/v) sucrose, 0.5 g/l 2-morpholinoethanesulfonic acid (pH 5.9), 100 mg/l *myo*-inositol, 1 mg/l thiamine hydrochloride, 0.5 mg/l pyridoxine hydrochloride, and 0.5 mg/l nicotinic acid. Seeds on the medium were incubated at 4 °C in darkness for 2–3 days and then placed at 22 °C under continuous light for ~4 weeks. The plates for collection of the roots were placed vertically, in which the roots grew down along the surface of the medium. Callus cultures were initiated from rosette leaves grown on 0.8% (w/v) agar of Gamborg's B5 basal medium, pH 5.7, supplemented with 2% (w/v) glucose, 0.5 mg/l 2,4-dichlorophenoxyacetic acid, and 0.05 mg/l kinetin. The plates were incubated at 22 °C under continuous light for 10 weeks.

3.4. Chemical and stress treatments

Leaves of 21-day-old plants were wounded by making two small cutting traces on each leaf with scissors. Chemical or fungal treatments were performed on 20–27-day-old plants. Treatment solutions were as follows: 2,4-dichlorophenoxyacetic acid [2,4-D, 100 μM in 0.1% (v/v) MeOH], salicylic acid [100 μM in 0.1% (v/v) MeOH], methyl jasmonate [100 μM in 0.1% (v/v) MeOH], kinetin (100 μM in 1 mM KOH), chitosan (1 mg/ml in H_2O), laminarin (1 mg/ml in H_2O), and bud-cell suspension of *F. oxysporum* f. sp. *batatas* O-17 (5×10^7 bud-cells/ml). Preparation of

bud-cell suspension was described previously (Shimizu et al., 2000). All solutions contained 0.05% (v/v) polyoxyethylene(20) sorbitan monolaurate, except for the bud-cell suspension. The solutions without hormones, polysaccharides, or bud-cells were used as the controls. The treatment solution was poured into the culture disks of the plants. The disks were gently agitated for 30 s to treat the shoots thoroughly before discard of the solution. After wounding or treatments, plants were incubated at 22 °C under continuous light, and the leaves or shoots were collected at different times.

3.5. Mutant verification

The seeds of the mutant lines (SALK_112823 and SALK_125686) in the Salk Institute Genomic Analysis Laboratory T-DNA insertion collection were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus). Genomic DNA was extracted from the leaves, according to the method reported by Liu et al. (1995). T-DNA insertions were confirmed by DNA amplification with the left T-DNA border-specific primer LBb1 (5′-GCGTGGACCGCTTGCTGCAACT-3′) and the gene-specific primers as follows: C3′H-R (5′-TTACATATCGTAAGGCACGCGTTT-3′) for the SALK_112823 line and C3′H-F (5′-ATGTCGTGGTTTCTAATAGCGGTG-3′) for the SALK_125686 line. PCR was performed using a KOD Dash kit (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's instructions. The PCR was carried out in a 10 μl reaction mixture containing genomic DNA, 0.25 U of KOD Dash polymerase, 2 nmol of dNTP, 1 pmol of each primer, and 1 × KOD Dash buffer. The reaction mixture was first denatured at 94 °C for 5 min, and the PCR amplification was performed in the following 30 cycles of 94 °C for 30 s, 54 °C for 2 s, and 74 °C for 30 s. The absence of the wild-type amplification product with gene-specific primers, C3′H-F and C3′H-R, confirmed the homozygous nature of the mutant lines. The conditions of PCR amplification were the same as above. The position of the T-DNA insertion was confirmed by the sequencing of PCR products amplified with the T-DNA specific primer, LBb1, and the gene-specific primer C3′H-R (for SALK_112823) or C3′H-F (for SALK_125686). Sequencing reactions were carried out using a BigDye terminator cycle sequencing kit (Applied Biosystems Japan). C3′H-R or LBb1 was used for the SALK_112823 line as the primer, and C3′H-F or LBb1 was used for the SALK_125686 line.

To confirm the absence of functional transcripts, total RNA was extracted from the roots, according to the method reported by Shirzadegan et al. (1991). RT-PCR was performed using a ReverTra Dash RT-PCR kit (Toyobo, Osaka, Japan), according to the manufacturer's instructions. First-strand DNA was synthesized in a 20 μl reaction mixture containing 0.7 μg of total RNA, 100 U of ReverTra Ace reverse transcriptase, 10 pmol of oligo(dT) primer, 20 nmol of dNTP, and 1 × RT buffer. The RT reactions were carried out at 42 °C for 60 min and 99 °C for 5 min. The reaction mixture was chilled to 4 °C and diluted 2 times, and 0.5 μl of aliquot was used as a template for each of the PCR amplifications with the gene-specific primers. C3′H-F and C3′H-R were used as the primers of *C3′H* amplification. Nucleotide sequences of the primers of *Actin2* amplification were as follows: act2-F, 5′-GTGAAGGCTGGATTTGCAGGA-3′ and act2-R, 5′-AACCTCCGATCCAGACACTGT-3′. The conditions of *C3′H* amplification by PCR were the same as the above except for 25 cycles. *Actin2* amplification was performed by denaturing at 94 °C for 5 min following 23 cycles of 94 °C for 30 s, 50 °C for 2 s, and 74 °C for 30 s. The mutant lines were kept as heterozygotes, because of the low viability and low fertility of homozygotes.

PCR was performed by a GeneAmp PCR system 9700 (Applied Biosystems Japan) or TaKaRa PCR Thermal Cycler Dice (Takara Bio Inc., Otsu, Japan). The PCR products were analyzed with a 1% (w/v) agarose gel containing 0.2 μg/ml of ethidium bromide, visualized with a UV transilluminator. DNA sequencing was performed with a DNA sequencer model 377 (Applied Biosystems Japan).

3.6. Identification of the coumarins by LC–MS/MS

The plant materials were soaked in MeOH overnight. The extracts were centrifuged for 10 min at 15,000*g* and the supernatants were subjected to LC–MS/MS analysis. Separation of eight coumarins was performed according to the following conditions: a COSMOSIL 5C18-AR-II (4.6 × 150 mm; Nacalai Tesque), with H_2O containing 0.1% (v/v) HCO_2H as solvent A and MeOH containing 0.1% (v/v) HCO_2H as solvent B, at a flow rate of 1.0 ml/min at 40 °C. Elution was started with isocratic conditions of 15% solvent B for 2 min, following a linear gradient flow up to 55% in 18 min. The ionspray voltage was set at 4.5 kV [for skimmin (**2**), herniarin (**3**), esculetin (**4**), esculin (**6**) and scopolin (**8**)], 4.6 kV [for scopoletin (**7**) and scoparone (**9**)], and 4.7 kV [for umbelliferone (**1**)]. The orifice potential was set at 30 V (for **8**), 35 V (for **1**, **2**, **4** and **6**), 40 V (for **7**), and 45 V (for **3** and **9**). The ring potential was set at 190 V (for **4**), 200 V (for **1**, **2** and **8**), 220 V (for **6**), 240 V (for **7** and **9**), and 250 V (for **3**). The collision energy was set at 15 V (for **8**), 20 V (for **2**), 22.5 V (for **6**), 30 V (for **1**, **3**, **7** and **9**), and 32.5 V (for **4**). The detection mode was multiple reaction monitoring (MRM) positive to detect the low-level coumarins. The MRM series for the detection of the coumarins were set at *m/z* 163.3/107.3 (for **1**), *m/z* 325.2/163.0 (for **2**), *m/z* 177.3/121.3 (for **3**), *m/z* 179.3/123.3 (for **4**), *m/z* 341.3/179.2 (for **6**), *m/z* 193.3/133.2 (for **7**), *m/z* 355.1/193.0 (for **8**), and *m/z* 207.3/151.2 (for **9**). The dwell time was 500 ms.

The coumarins that accumulated to a higher level were subjected to further LC–MS/MS analysis for identification with the product ion scan mode. Conditions were as follows: a COSMOSIL 5C18-AR-II (4.6 × 150 mm; Nacalai

Tesque), with H_2O containing 0.1% (v/v) HCO_2H as solvent A and MeOH containing 0.1% (v/v) HCO_2H as solvent B, at a flow rate of 1.0 ml/min at 40 °C. Elution was started with isocratic conditions of 15% solvent B for 2 min, following a linear gradient flow up to 35% in 14 min. For **7**, the ionspray voltage was set at 4.6 kV, the orifice potential was 30 V, the ring potential was 220 V, and the collision energy was 30 V with N_2 gas. Q1 was locked on *m*/*z* 193.0 and Q3 was scanned from 100 to 200 with a step size of 0.1 and with a dwell time of 1 ms/step. *m*/*z* (rel. int.): 178 (45), 165 (4), 150 (9), 137 (33), 133 (100), 122 (16), 105 (6). For **8**, the ionspray voltage was set at 4.5 kV, the orifice potential was 30 V, the ring potential was 200 V, and the collision energy was 50 V with N_2 gas. Q1 was locked on *m*/*z* 355.1 and Q3 was scanned from 100 to 360 with a step size of 0.1 and with a dwell time of 1 ms/step. *m*/*z* (rel. int.): 193 (100), 178 (46), 165 (7), 150 (4), 137 (25), 133 (95), 122 (7), 105 (3). For **2**, the ionspray voltage was set at 4.5 kV, the orifice potential was 35 V, the ring potential was 200 V, and the collision energy was 50 V with N_2 gas. Q1 was locked on *m*/*z* 325.2 and Q3 was scanned from 100 to 330 with a step size of 0.1 and with a dwell time of 0.5 ms/step. *m*/*z* (rel. int.): 163 (100), 135 (9), 119 (42), 107 (61).

3.7. Quantification of the coumarins

The plant materials were soaked for 22 h in MeOH containing 4-methylumbelliferone as an internal standard. The extracts were centrifuged for 10 min at 15,000*g*, after concentration or dilution, when needed. The supernatants were subjected to HPLC analysis on a COSMOSIL 5C18-AR-II (4.6 × 150 mm; Nacalai Tesque), with H_2O containing 0.1% (v/v) HCO_2H as solvent A and MeOH containing 0.1% (v/v) HCO_2H as solvent B, at a flow rate of 1.0 ml/min at 40 °C. Elution was started with isocratic conditions of 20% solvent B for 2 min, following a linear gradient flow up to 56% in 16 min. Detection was performed using a fluorescence detector with an excitation wavelength at 340 nm and an emission wavelength at 420 nm. Since skimmin (**2**) was less than 0.2% of scopolin (**8**) in the wild-type, other conditions were introduced to quantify the level of **2** in the mutant plants as follows: on a YMC-Pack Pro C18 AS-307-3 (4.6 × 75 mm; YMC Co., Ltd., Kyoto, Japan), with H_2O containing 0.1% (v/v) HCO_2H as solvent A and MeOH containing 0.1% (v/v) HCO_2H as solvent B, at a flow rate of 0.75 ml/min at 40 °C. Elution was started with isocratic conditions of 8% solvent B for 18 min, following a linear gradient flow up to 60% in 10 min followed by an isocratic flow of 60% solvent B for 5 min. The detection of **2** was performed with a fluorescence detector with an excitation wavelength at 320 nm and an emission wavelength at 380 nm (0–20 min), and the detection of 4-methylumbelliferone was performed with an excitation wavelength at 340 nm and an emission wavelength at 420 nm (20–33 min).

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