Planta (2010) 232:209–218
DOI 10.1007/s00425-010-1166-1

ORIGINAL ARTICLE

Expression of bacterial tyrosine ammonia-lyase creates a novel *p*-coumaric acid pathway in the biosynthesis of phenylpropanoids in Arabidopsis

Yasutaka Nishiyama · Choong-Soo Yun · Fumio Matsuda · Tadamasa Sasaki · Kazuki Saito · Yuzuru Tozawa

Received: 14 January 2010 / Accepted: 31 March 2010 / Published online: 16 April 2010

Abstract Some flavonoids are considered as beneficial compounds because they exhibit anticancer or antioxidant activity. In higher plants, flavonoids are secondary metabolites that are derived from phenylpropanoid biosynthetic pathway. A large number of phenylpropanoids are generated from *p*-coumaric acid, which is a derivative of the primary metabolite, phenylalanine. The first two steps in the phenylpropanoid biosynthetic pathway are catalyzed by phenylalanine ammonia-lyase and cinnamate 4-hydroxylase, and the coupling of these two enzymes forms a rate-limiting step in the pathway. For the generation of *p*-coumaric acid, the conversion from phenylalanine to *p*-coumaric acid that is catalyzed by two enzymes can be theoretically performed by a single enzyme, tyrosine ammonia-lyase (TAL) that catalyzes the conversion of tyrosine to *p*-coumaric acid in certain bacteria. To modify the *p*-coumaric acid pathway in plants, we isolated a gene encoding TAL from a photosynthetic bacterium, *Rhodobacter sphaeroides*, and introduced the gene (*RsTAL*) in *Arabidopsis thaliana*. Analysis of metabolites revealed that the ectopic over-expression of *RsTAL* leads to higher accumulation of anthocyanins in transgenic 5-day-old seedlings. On the other hand, 21-day-old seedlings of plants expressing *RsTAL* showed accumulation of higher amount of quercetin glycosides, sinapoyl and *p*-coumaroyl derivatives than control. These results indicate that ectopic expression of the *RsTAL* gene in *Arabidopsis* enhanced the metabolic flux into the phenylpropanoid pathway and resulted in increased accumulation of flavonoids and phenylpropanoids.

Y. Nishiyama and C. S. Yun contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00425-010-1166-1) contains supplementary material, which is available to authorized users.

Y. Nishiyama · C.-S. Yun · T. Sasaki · Y. Tozawa (✉)
Cell-Free Science and Technology Research Center,
and The Venture Business Laboratory, Ehime University,
3 Bunkyo-cho, Matsuyama, Ehime 890-8577, Japan
e-mail: tozaway@ccr.ehime-u.ac.jp

F. Matsuda · K. Saito
RIKEN Plant Science Center, 1-7-22 Suehiro-cho,
Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan

K. Saito
Graduate School of Pharmaceutical Sciences, Chiba University,
Yayoi-cho 1-33, Inage-ku, Chiba, Chiba 263-8522, Japan

Present Address:
C.-S. Yun
Biotechnology Research Center, The University of Tokyo,
1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

Present Address:
T. Sasaki
Department of Biological Science and Technology,
Tokyo University of Science, Yamazaki 2641,
Noda, Chiba 278-8510, Japan

Present Address:
F. Matsuda
Organization of Advanced Sciences and Technology,
Kobe University, 1-1 Rokkodaicho, Nada-ku,
Kobe 657-8501, Japan

Keywords Flavonoid · Metabolic engineering · Phenylpropanoid pathway · Tyrosine ammonia-lyase

Abbreviations

C4H	Cinnamate 4-hydroxylase
4CL	4-Coumarate:CoA ligase
ORF	Open reading frame
PAL	Phenylalanine ammonia-lyase
PCR	Polymerase chain reaction
RsTAL	*Rhodobacter sphaeroides TAL*
RT-PCR	Reverse transcription polymerase chain reaction
TAL	Tyrosine ammonia-lyase

Introduction

Flavonoids, a class of polyphenolic compounds produced via the phenylpropanoid biosynthetic pathway in higher plants, consist of more than 9,000 structures (Williams and Grayer 2004). Flavonoids have many functions in plants including protection from UV light, defense against pathogenic microorganisms, or coloration of flowers (Dixon and Paiva 1995; Dixon 2004; Tanaka et al. 2008). Over the past decade flavonoids have been attracting the attention of many researchers for their pharmacological and nutritional properties. Among the flavonoids, the anticancer and antioxidant activities of kaempferol and quercetin make them good candidates for inclusion in the human diet (Pietta 2000; Ren et al. 2003; Williams et al. 2004).

Lignins, polymers of 4-hydroxyphenylpropanoids, are also derived from the phenylpropanoid pathway (Boerjan et al. 2003). In plants, lignins play important roles in the development and resistance to biotic and abiotic stresses because the polymers confer strength and rigidity to the cell wall. On the other hand, lignins hinder the use of plant biomass in chemical pulping and processing to biofuels, besides decreasing the digestibility of forage.

The initial step in the phenylpropanoid biosynthetic pathway in higher plants is catalyzed by phenylalanine ammonia-lyase (PAL), which converts phenylalanine to *trans*-cinnamic acid by the elimination of ammonia. Cinnamate 4-hydroxylase (C4H), a membrane-anchored cytochrome P450 monooxygenase enzyme, subsequently hydroxylates *trans*-cinnamic acid to *p*-coumaric acid. *p*-Coumaric acid is a precursor of 4-coumaroyl-CoA, which serves as a substrate to form the basic skeleton of all flavonoid derivatives (Ehlting et al. 1999).

PAL is a metabolically important enzyme because its reaction links primary metabolism to secondary metabolism. Furthermore, PAL has been known as a rate-limiting enzyme in the phenylpropanoid pathway (Hahlbrock et al. 1976). The genomic DNA of the model plant *Arabidopsis* encodes four PAL isoforms, and Cochrane et al. (2004) reported that all four recombinant PAL proteins recognize phenylalanine, but not tyrosine as a substrate. It has been reported that multiple mechanisms, such as feedback inhibition and transcriptional regulation, regulate PAL activity in plants (Bolwell et al. 1986, 1988; Jorrin and Dixon 1990; Mavandad et al. 1990; Appert et al. 1994). In fact, increased expression of PAL enhances the accumulation of phenylpropanoids such as chlorogenic acid while major flavonoids such as rutin were not altered in the tobacco plants overexpressing PAL (Bate et al. 1994; Howles et al. 1996; Shadle et al. 2003). It has also been postulated that the initial metabolic flux into the phenylpropanoid biosynthetic pathway is regulated by an enzyme complex consisting of PAL and C4H, which are co-localized on the ER membrane (Blount et al. 2000; Achnine et al. 2004). Thus, the regulatory mechanisms governing the initial steps of phenylalanine metabolism are complicated.

On the other hand, it has been reported that some photosynthetic bacteria possess a unique soluble enzyme, tyrosine ammonia-lyase (TAL), that catalyzes the direct conversion of tyrosine to *p*-coumaric acid in order to produce a chromophore of a photosensory protein (Kyndt et al. 2002; Xue et al. 2007). We therefore expected that the introduction of the *TAL* gene into plants would open an extra *p*-coumaric acid pathway and increase the initial flux into phenylpropanoid biosynthesis pathway (Fig. 1). In the present study, we describe the characterization of *Arabidopsis* transformants expressing *TAL* gene that originates from *Rhodobacter sphaeroides*. We analyzed metabolites in the transformed plants, and found that the expression of TAL efficiently enhanced the metabolic flux into the phenylpropanoid pathway, resulting in the accumulation of phenylpropanoid derivatives such as quercetin glycosides.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia (RIKEN Plant Science Center, Yokohama, Japan) was used in this study. Seeds were sown on soil medium and stratified at 4°C for 3 days before transfer to a growth chamber maintained under long-day (16-h light, 8-h dark) conditions. Temperature and humidity were maintained at 23°C and 50%, respectively.

For non-targeted metabolic profiling, transformed and wild-type *Arabidopsis* were cultured on solid MS medium [Murashige and Skoog plant salt mixture, Gamborg's B5 medium vitamin mixture, 1% sucrose, 0.05% Mes buffer (pH 5.7), 0.8% agar] for 3 weeks under long-day (16-h

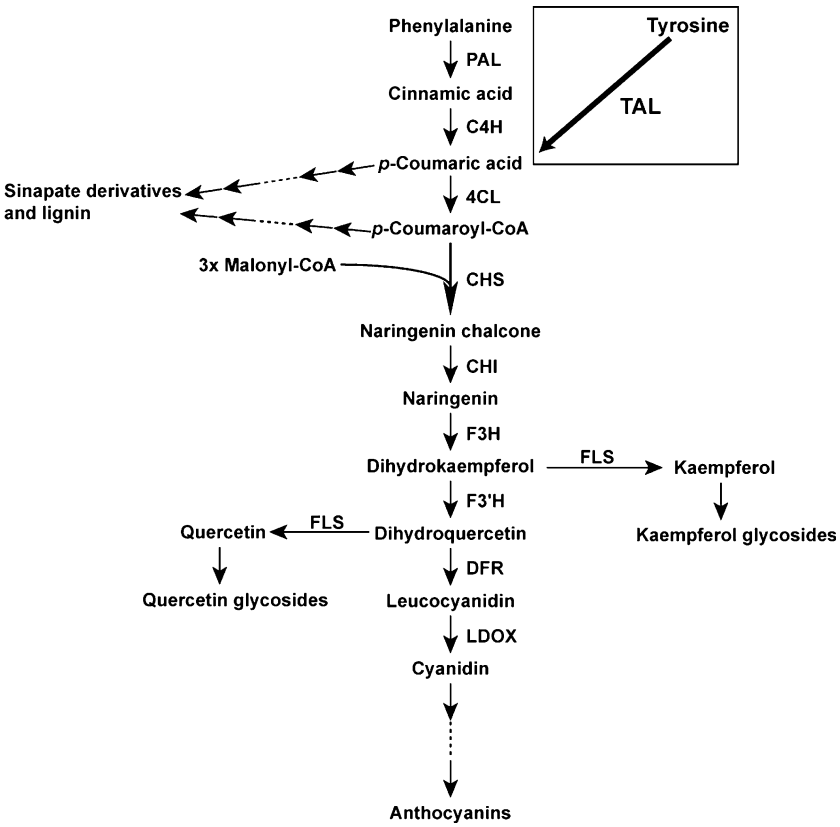

Fig. 1 Summary of phenylpropanoid and flavonoid biosynthetic pathways in plants. *TAL* tyrosine ammonia-lyase, *PAL* phenylalanine ammonia-lyase, *C4H* cinnamate 4-hydroxylase, *4CL* 4-coumarate:CoA ligase, *CHS* chalcone synthase, *CHI* chalcone isomerase, *F3H* flavanone 3-hydroxylase, *F3′H* flavonoid 3′-hydroxylase, *FLS* flavonol synthase, *DFR* dihydroflavonol 4-reductase, *LDOX* leucoanthocyanidin dioxygenase. The *boxed part* indicates the introduced conversion step catalyzed by TAL in this study

light, 8-h dark) condition. Whole aerial part of seedlings were collected and frozen in liquid nitrogen and stored at −85°C before use.

For flavonoid-targeted analysis, plants were cultured on solid 1/2 MS medium with 1% sucrose under long-day (16-h light, 8-h dark) condition. Plates were set up in vertical orientation. Five-day old seedlings were collected, frozen in liquid nitrogen and stored at −85°C until analysis.

Cloning of *TAL* from *Rhodobacter*

Rhodobacter sphaeroides (NBRC no. 12203) was obtained from NBRC [National Institute of Technology and Evaluation (NITE) Biological Resource Center, Kisarazu, Japan]. Genomic DNA of *R. sphaeroides* was extracted and used as a template to amplify a 1.6-kb DNA fragment containing the open reading frame (ORF) of the *RsTAL* gene. Primers used are as follows: RsTAL1 (5′-AAAACTAGTATGCTCGCCATGAGC-3′, *Spe*I site underlined) and RsTAL2 (5′-AAAGTCGACTCAGACGGGAGATTGC-3′, *Sal*I site underlined). The amplified fragment was cloned into the *Spe*I and *Sal*I sites of pEU3S (Kanno et al. 2005) to yield pEU3S-RsTAL. The DNA sequence of the inserted fragment was identical to that of the *R. sphaeroides* TAL gene as reported previously (GenBank accession no. YP_355075).

Construction of expression vector and plant transformation

The *Sse*8387I fragment containing *RsTAL* was excised from the pEU3S-RsTAL and cloned into the *Sse*8387I site of the binary vector pYT105 (Yun et al. 2008) to obtain pYT105-RsTAL. The binary vector pYT105-RsTAL and empty vector pYT105 were introduced into *Agrobacterium tumefaciens* C58C1 by the freeze–thaw method (Höfgen and Willmitzer 1988), respectively. Transformation of *A. thaliana* (Columbia) was performed by in planta infiltration (Clough and Bent 1998). Transformed seedlings (T_1) were identified by selection on solid MS medium containing 50 mg/l kanamycin and 200 mg/l claforan, then transferred to soil. Homozygous T_3 plants: *RsTAL*-expressing plants, TA3, TB7, TE3, TM1 and TL6; and plants transformed with empty vector, YT1, YT4 and YT5, were obtained and used in subsequent analyses.

Detection of *RsTAL* transcript

For RNA analysis, total RNA was extracted from whole seedlings of wild-type and transgenic *Arabidopsis* plants with the use of RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), and 5 μg of the RNA were subjected to RNA gel-blot hybridization. A digoxigenin-labeled antisense

RNA probe for the detection of *RsTAL* transcripts was prepared with a DIG RNA labeling kit (Roche, Mannheim, Germany). A 375-bp fragment of the *RsTAL* ORF (nucleotides 91–485) was amplified by PCR with the primers RsTAL3 (5′-AAAGAATTCCGGATTGTCCTTG-3′) and RsTAL4 (5′-CCCAAGCTTCAGAGCACCATAT-3′), then cloned into pSPT19 (Roche). The antisense RNA probe was synthesized from the obtained vector by in vitro run-off transcription with T7 RNA polymerase in the presence of digoxigenin-labeled UTP. RNA gel-blot hybridization was performed as described previously (Tozawa et al. 1998).

Non-targeted metabolic profiling analysis using LC-PDA-MS

The frozen tissues were mixed with five volumes of 80% aqueous methanol containing 0.5 mg/l of lidocaine and *d*-camphorsufonic acid and homogenized in a mixer mill (MM 300, Retsch, Haan, Germany) with a zirconia bead for 10 min at 20 Hz. Following centrifugation at 15,000*g* for 10 min, the supernatants were passed through a filter (Ultrafree-MC, 0.2 μm, Millipore, Bedford, MA, USA) to remove the insoluble materials. The sample extracts (3 μl) were analyzed using an LC-MS system equipped with an electrospray ionization interface (HPLC: Waters Acquity UPLC system; MS: Waters Q-TOF Premier, Waters, Milford, MA, USA). Data acquisition and processing were performed as previously described (Matsuda et al. 2009). Flavonoid-targeted analyses using PDA chromatograms were performed by use of reported profiles of flavonoids accumulated in *A. thaliana* (Tohge et al. 2005).

Flavonoid-targeted analysis of 5-day-old seedlings

Flavonoid-targeted analyses were performed in triplicate. Frozen seedlings were homogenized in 5 μl of extraction solvent (methanol:CH_3COOH:H_2O = 9:1:10, 0.02 mM naringenin-7-*O*-glucoside) per milligram of fresh weight of tissue in the mixer mill (MM 300, Retsch) for 5 min at 30 Hz. After centrifugation at 12,000*g*, the supernatants were immediately used for flavonoid analysis. The UPLC-MS system mentioned above was used. Data acquisition and processing were performed as previously described (Yonekura-Sakakibara et al. 2009).

Results

Transgenic *Arabidopsis* expressing *RsTAL*

To enhance the carbon flow into the phenylpropanoid pathway in plants, we cloned the gene encoding TAL from *R. sphaeroides,* which has been reported to manifest specific and efficient catalytic activity in the conversion of tyrosine to *p*-coumaric acid when expressed in *Escherichia coli* (Watts et al. 2004). To express RsTAL protein, the *RsTAL* gene under the control of CaMV 35S promoter was introduced into *Arabidopsis* (Fig. 2a). Kanamycin-resistant T_1 plants were selected and, eventually, five independent lines (TA3, TB7, TE3, TM1 and TL6) were obtained as homozygous T_3 plants. The accumulation of *RsTAL* transcripts in seedlings of these lines was confirmed by RNA gel-blot hybridization (Fig. 2b).

We detected no significant differences in morphology or growth rate among the five *RsTAL* homozygous lines and wild-type plants (data not shown), indicating that *RsTAL* expression, even at a high level, does not affect normal development of *Arabidopsis*. Four independent lines (TA3, TB7, TM1 and TL6) exhibiting comparatively higher accumulation of transcripts were selected for further investigation.

LC-PDA-MS analysis of phenylpropanoid compounds in transgenic plants

To analyze the effect of TAL expression on phenylpropanoid metabolism, we conducted LC-PDA-MS analysis of 21-day-old seedlings expressing *RsTAL.*

The PDA chromatogram showed that *RsTAL*-expressing plants accumulated more quercetin glycosides and sinapoylmalate than control plants. Sinapoylmalate and total

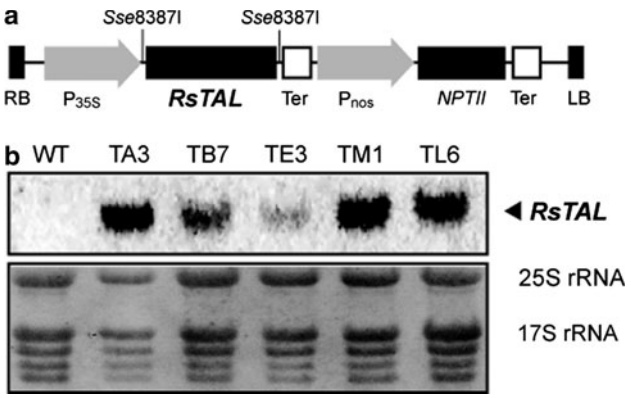

Fig. 2 Expression of *RsTAL* in transgenic *Arabidopsis*. **a** Structure of the T-DNA region of the binary vector pYT105-RsTAL. *RB and LB* right and left borders, respectively, P_{35S} 35S promoter of cauliflower mosaic virus, *Pnos and Ter* promoter and terminator of the nopaline synthase gene, respectively, *NPTII* neomycin phosphotransferase gene. **b** Detection of *RsTAL* mRNA in transgenic *Arabidopsis* lines. Total RNA (5 μg) from wild-type and five independent lines of T_3 transgenic seedlings (TA3, TB7, TE3, TM1, TL6) were subjected to the analysis. The 2.1-kb *RsTAL* transcript is indicated by an *arrowhead*. The membrane was also stained with methylene blue for visualization of 25S and 17S rRNA

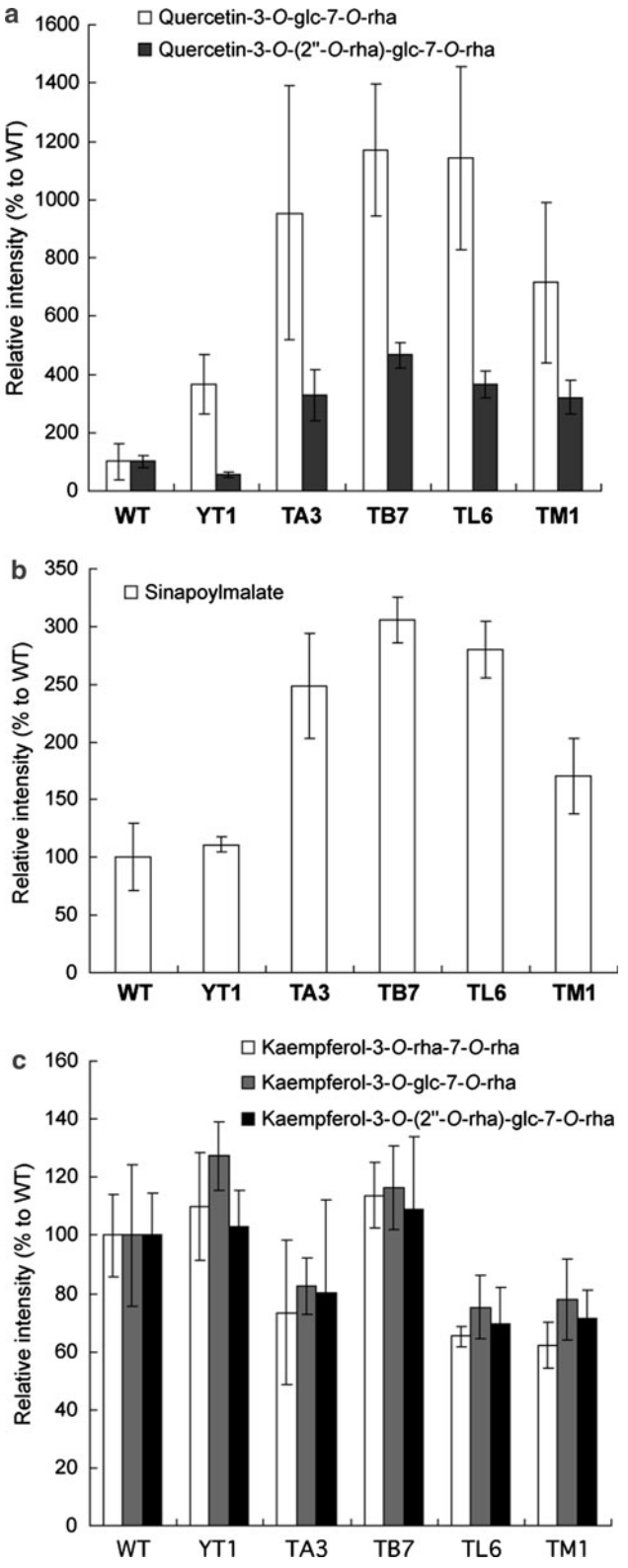

Fig. 3 Phenylpropanoid compounds accumulated in the transgenic lines expressing *RsTAL*. Extracts from 21-day-old seedlings were analyzed by LC-PDA-MS. Flavonol glycosides and sinapoylmalate were identified in the PDA chromatogram and their amounts were compared. Analyses were performed in quintuplicate for *RsTAL*-expressing lines and wild type, and in triplicate for empty vector control (YT1). Mean values with SD are shown. **a** Relative quantification of quercetin glycosides. **b** Relative quantification of sinapoylmalate. **c** Relative quantification of kaempferol glycosides

quercetin glycosides in the *RsTAL*-expressing plants are 1.7–2.5 and 5–10 times higher than those in wild type, respectively (Fig. 3a, b). The content of quercetin 3-*O*-(2″-*O*-rhamnosyl)-glucoside 7-*O*-rhamnoside, which is a more modified version of a quercetin-derivative than quercetin 3-*O*-glucoside-7-*O*-rhamnoside, is constantly higher in plants expressing *RsTAL* than in the wild type and vector control. On the other hand, kaempferol glycosides, such as kaempferol-3-*O*-rhamnoside 7-*O*-rhamnoside and kaempferol-3-*O*-(2″-*O*-rhamnosyl)-glucoside 7-*O*-rhamnoside, were not increased in three *RsTAL*-expressing lines, TA3, TL6 and TM1 (Fig. 3c). Anthocyanins were not detected in the 21-day-old seedlings.

Non-targeted metabolite profiling was also conducted on the same samples. From the 2,250 peaks detected in positive-ion mode, peaks that displayed more than threefold higher or lower average ion intensity in *RsTAL*-expressing plants when compared with those in wild-type controls were selected. There were 16 peaks with increased ion intensity (UK1–16) while no peak with decreased intensity was detected (Table 1). UK14 was identified as sinapoylmalate by comparison of the chromatographic behavior with that of authentic compound. We could not detect ions derived from quercetin glycosides in MS analysis because quercetin glycosides co-migrated with other unknown compounds during LC separation.

Among 16 peaks with increased ion intensity, there were four pairs of peaks with the same *m/z* value (UK1, UK2 (*m/z* 277); UK3, UK5 (*m/z* 369); UK4, UK6 (*m/z* 353); UK10, UK12 (*m/z* 354)). Each pair was assumed to be a set of isomers because two peaks showed similar MS/MS spectrum. MS/MS analysis of peaks UK4, UK6 (*m/z* 353), UK10, UK12 (*m/z* 354), UK3 and UK5 (*m/z* 369) gave fragments with *m/z* 207, 175, 147, 119, 91. The fragment pattern was considered to be derived from a sinapoyl moiety. Based on an accurate estimation of their molecular masses, UK4 and UK6 (*m/z* 353.1318) and peaks UK10 and UK12 (*m/z* 354.1119) were deduced to be sinapoylglutamine ($C_{16}H_{20}N_2O_7$, theoretical molecular weight of protonated molecule 353.1349) and sinapoylglutamate ($C_{16}H_{19}NO_8$, theoretical molecular weight of protonated molecule 354.1189), respectively. Each pair was supposed to consist of *cis*/*trans* isomers. However, the pertinent compounds were not found in CAS database. MS/MS analysis of peaks UK1 and UK2 (*m/z* 277.1623) gave fragments derived from cleavage of *p*-coumaroyl residue,

Table 1 Compounds increased in the *RsTAL* expressing *Arabidopsis* detected by LC-MS analyses

Peak number	Retention time (min)	*m/z*	Annotation (putative)	Fold of increase				Average peak height (cps)				
				TA3/WT	TB7/WT	TL6/WT	TM1/WT	WT	TA3	TB7	TL6	TM1
UK1	3.13	277	*p*-Coumaroylagmatine isomer	7.0	3.2	4.4	5.3	92	651	295	403	492
UK2	3.38	277	*p*-Coumaroylagmatine isomer	16.1	5.6	6.8	10.4	70	1,133	397	480	732
UK3	3.49	369	Sinapoyl-containing metabolite	14.0	5.7	10.7	21.7	12	167	68	128	260
UK4	3.56	353	Sinapoylglutamine isomer	8.1	10.7	13.0	21.9	79	639	846	1,025	1,722
UK5	3.64	369	Sinapoyl-containing metabolite isomer	8.9	4.6	6.8	14.3	22	200	103	153	319
UK6	3.68	353	Sinapoylglutamine isomer	8.3	10.1	9.5	18.5	80	660	810	761	1,473
UK7	3.77	312	n.d.	5.2	5.8	4.4	9.7	15	76	84	65	141
UK8	3.82	538	n.d.	12.4	17.6	9.7	23.1	14	169	239	132	315
UK9	3.93	404	n.d.	14.6	6.9	11.5	16.9	24	351	167	277	406
UK10	3.99	354	Sinapoylglutamate isomer	5.1	4.7	5.1	7.4	130	658	614	659	961
UK11	4.01	410	n.d.	4.1	6.4	7.0	9.3	21	85	133	148	194
UK12	4.14	354	Sinapoylglutamate isomer	5.0	4.5	3.7	5.9	187	937	836	694	1,108
UK13	4.80	225	n.d.	34.5	19.4	25.0	44.2	13	442	249	320	566
UK14	4.89	1,076	Sinapoylmalate (adduct ion)	8.1	5.2	3.0	6.7	30	243	157	91	202
UK15	4.96	737	n.d.	27.7	15.6	10.9	29.2	47	1,294	731	509	1,367
UK16	6.80	338	n.d.	22.8	9.9	17.7	29.9	18	401	174	311	526

n.d. not determined

i.e. fragments with *m/z* 147, 119 and 91. We annotated these peaks as *cis*/*trans* isomers of *p*-coumaroylagmatine ($C_{14}H_{21}N_4O_2$, theoretical molecular weight of protonated molecule 277.1665) based on a search of the CAS database. We could not deduce structures of peaks UK3 and UK5.

Increase of the compound corresponding to UK16 was also confirmed in the comparison of PDA chromatograms. MS/MS analysis of the peak gave fragments with *m/z* 207, 175, 147 and 119 though the intensities of fragment ions were relatively low. The compound was deduced to be a sinapoyl derivative whose molecular formula is $C_{17}H_{25}NO_6$ (theoretical molecular weight of protonated molecule 338.1603). Mass spectrometry data and deduced structures of UK1, UK2, UK4, UK6, UK10 and UK12 are shown in supplementary material (Suppl. data 1).

In brief, we identified one peak and putatively annotated 6 peaks out of 16 increased peaks based on their UV–Visible absorption spectra and comprehensive analysis of mass fragmentation patterns obtained by tandem MS spectroscopy (Table 1). Five peaks were deduced to have sinapoyl residues while two peaks were apparently *p*-coumaroyl derivatives among annotated peaks. Three unannotated peaks were also deduced to be sinapoyl derivatives.

Taken together, LC-PDA-MS analysis showed that plants expressing *RsTAL* had higher levels of quercetin glycosides and phenylpropanoid metabolites, which have sinapoyl or *p*-coumaroyl residues, as compared to wild-type plants.

Flavonoid-targeted analysis of 5-day-old seedlings

In order to analyze phenylpropanoid metabolism in other growth stages, we carried out metabolic profiling of flavonoids in 5-day-old seedlings of *Arabidopsis* expressing *RsTAL*. Flavonoid-targeted analysis of transgenic plants revealed that the expression of RsTAL resulted in slightly higher accumulation of quercetin 3-*O*-rhamnoside 7-*O*-rhamnoside in two lines, TA3 and TM1, when compared with wild type (1.6 and 1.9 times, respectively) (Fig. 4a). There was no apparent change in other quercetin glycosides. Increase of sinapoylmalate was less obvious than that of quercetin-derivatives, and we only observed a slight increase in TM1 line (Fig. 4b). In TM1, kaempferol glycosides, kaempferol 3-*O*-rhamnoside 7-*O*-rhamnoside and kaempferol 3-*O*-glucoside 7-*O*-rhamnoside, also increased slightly compared to wild type (1.4 and 1.5 times, respectively) (Fig. 4c). Changes in amount of these compounds in 5-day-old seedlings were not prominent as those in 21-day-old seedlings.

In contrast, *RsTAL* expression led to significantly higher accumulation of major anthocyanins when compared with the wild-type controls (Fig. 4d). The *RsTAL* transgenic lines accumulated 2.2–4.0 times higher amount of cyanidin 3-*O*-[2″-*O*-(2‴-*O*-(sinapoyl)-xylosyl)-6″-*O*-(*p*-*O*-(glucosyl)-*p*-coumaroyl)-glucoside] 5-*O*-(6⁗-*O*-malonyl) glucoside than the wild-type controls (Fig. 4d). Increase of another anthocyanin, cyanidin 3-*O*-[2″-*O*-(2‴-*O*-(sinapoyl)-xylosyl)-6″-*O*-(*p*-coumaroyl)-glucoside] 5-*O*-(6⁗-*O*-malonyl)

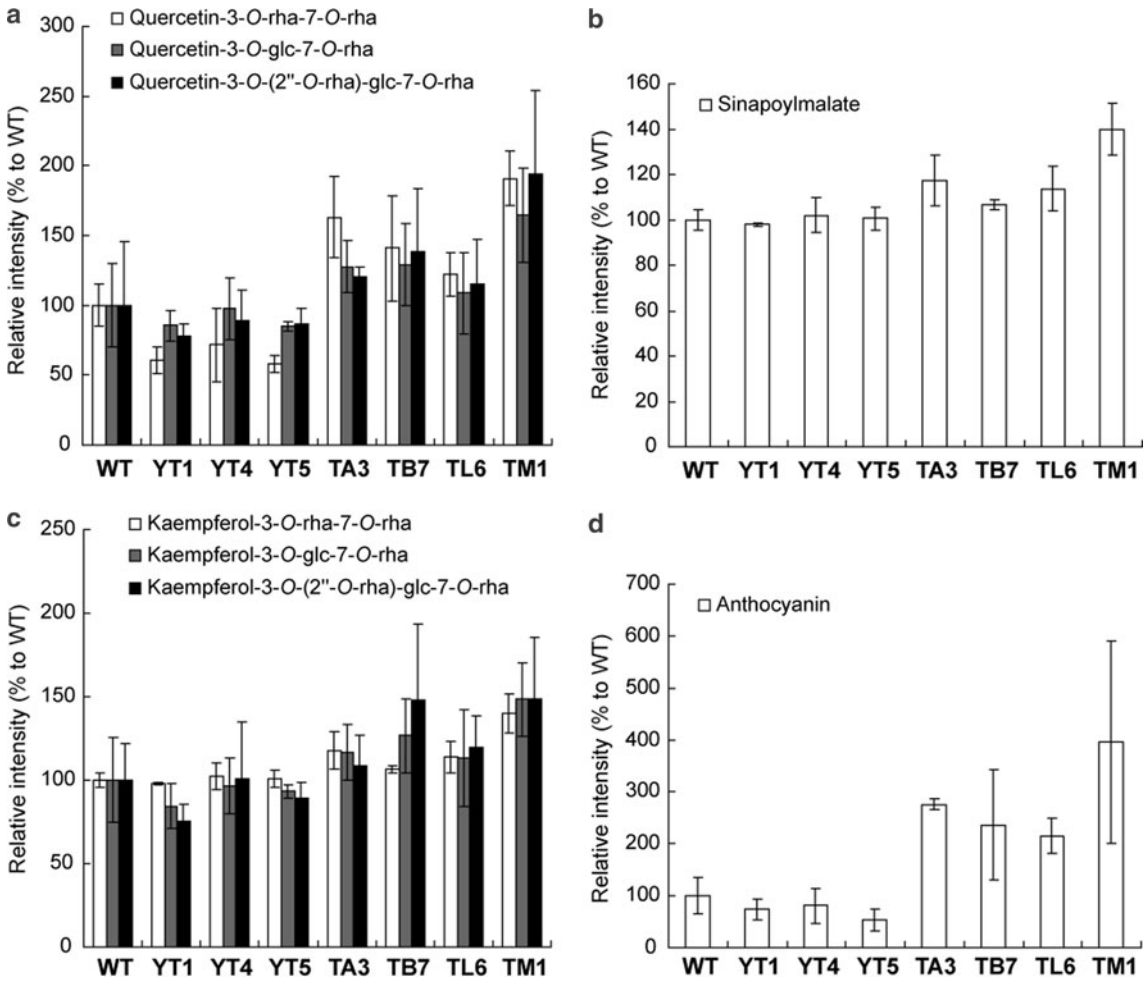

Fig. 4 Analyses of flavonoid accumulation in 5-day-old seedlings of transgenic plants expressing *RsTAL*. Extracts from 5-day-old seedlings were analyzed by LC-PDA-MS. Relative MS peak intensities of ions derived from flavonol glycosides, sinapoylmalate and anthocyanins were compared with that of wild type. YT1, YT4 and YT5 are control plants transformed with an empty vector. Mean values and SD of three biologically independent experiments are shown. **a** Relative quantification of quercetin glycosides. **b** Relative quantification of sinapoylmalate. **c** Relative quantification of kaempferol glycosides. **d** Relative quantification of anthocyanin (cyanidin 3-*O*-[2″-*O*-(2‴-*O*-(sinapoyl)-xylosyl)-6″-*O*-(*p*-*O*-(glucosyl)-*p*-coumaroyl)-glucoside] 5-*O*-(6⁗-*O*-malonyl) glucoside)

glucoside, was also dependent on the expression of *RsTAL* (data not shown).

Discussion

To date, there have been a number of reports of metabolic engineering approaches aimed at increased accumulation of flavonoids. Some reports have described modification of enzyme activity that limited the carbon flow into the phenylpropanoid pathway. Transgenic tobacco plants with higher PAL activity were selected from transgenic plants expressing a heterologous *PAL* gene (Elkind et al. 1990; Howles et al. 1996). Chlorogenic acid was increased in plants with higher PAL activity while the flavonoid rutin was not increased. Over-expression of C4H in tomato gave rise to some plants with increased rutin and naringenin, though increased level of those compounds was not directly correlated to increased level of C4H (Millar et al. 2007). Some reports revealed an increase in flavonoids by activating transcription factors. For example, anthocyanins and quercetins were specifically increased in PAP1-overexpressing *Arabidopsis* (Borevitz et al. 2000; Tohge et al. 2005) while quercetins were specifically increased in MYB12-overexpressing *Arabidopsis* (Mehrtens et al. 2005). These results indicate that over-expression of transcription factor is a useful tool in metabolic engineering for increased production of specific metabolites. Over-production of proanthocyanidins was reported by over-expression of PAP1 in combination with MYB transcription factor TT2 only in a subset of cells (Sharma and Dixon 2005). Co-expression of PAP1 and anthocyanidin reductase resulted in ectopic production of proanthocyanidins in transgenic plants (Xie et al. 2006). These results suggest

that co-expression of multiple genes may help to produce a specific class of metabolites.

In this report, we increased the metabolic flux to the phenylpropanoid biosynthetic pathway by expressing TAL, which catalyzes the reaction from tyrosine to *p*-coumaric acid. TAL has been found to be involved in the development of the photosensory system in photosynthetic bacteria, *R. sphaeroides*, by producing *p*-coumaric acid as the chromophore of the photosensory yellow protein (Kort et al. 1998; Kyndt et al. 2002). As *trans*-cinnamic acid is not detected in the bacteria, tyrosine has been considered to be the natural substrate of RsTAL in vivo. In fact, the K_m values of RsTAL are 60 and 560 μM for tyrosine and phenylalanine, respectively, while k_{cat} values for both substrates are similar (0.02 and 0.01 s^{-1} for tyrosine and phenylalanine, respectively) (Xue et al. 2007). These parameters indicate that RsTAL can catalyze the deamination of tyrosine 19 times more efficiently than that of phenylalanine.

Maize PAL was confirmed to catalyze tyrosine deamination besides phenylalanine deamination using recombinant protein (Rösler et al. 1997). k_{cat}/K_m values of recombinant ZmPAL1, measured at pH 7.7, are 0.018 and 0.023 μM^{-1} s^{-1} for phenylalanine and tyrosine, respectively. ZmPAL1 catalyzes the deamination of phenylalanine and tyrosine at similar efficiencies. On the other hand, all four PAL isoforms were supposed to accept only phenylalanine in *Arabidopsis* (Cochrane et al. 2004). For AtPAL1, 2 and 4, enzyme efficiencies for the deamination of tyrosine are 340-, 1,200- and 970-times lower, respectively, than those for phenylalanine. AtPAL3 is essentially inactive with tyrosine. Based on these catalytic properties of AtPALs and RsTAL, we expected that the expression of *RsTAL* in *Arabidopsis* will open a novel phenylpropanoid pathway by utilizing tyrosine as an extra primary substrate.

Metabolite profiling using LC-PDA-MS revealed that *RsTAL* expression in *Arabidopsis* leads to higher accumulation (5–10-fold) of quercetin glycosides. On the other hand, we did not observe increased accumulation of kaempferol glycosides in the transgenic lines. It seems that the downstream effect of the enhanced influx into flavonoid pathway is limited to an increase of quercetin. Similarly, a previous report has shown that *PAP1*-overexpressing plants only increased the level of quercetin glycosides while kaempferol glycosides decreased or remained unchanged (Tohge et al. 2005).

Besides quercetin glycosides, we expected that phenylpropanoids with sinapoyl or coumaroyl residues will be highly accumulated in seedlings expressing *RsTAL*. MS/MS analyses revealed that eight peaks with sinapoyl residue and two peaks with coumaroyl residue were increased in transgenic plants (Table 1). In addition, flavonoid-targeted analysis of 5-day-old seedlings revealed that anthocyanin is increased in *RsTAL*-expressing seedlings (Fig. 4). Taken together, metabolite analyses revealed that 21- and 5-day-old seedlings have different metabolic profiles, and confirmed that *RsTAL* expression results in the accumulation of various end products instead of just a specific class of metabolites, most likely in a growth stage-specific manner. We also conducted semi-quantitative RT-PCR analysis. However, no obvious change was observed in the expression of genes related to flavonoid biosynthesis, such as *PAL*, *C4H*, *4CL*, *CHS*, *CHI*, *F3H*, *F3′H*, *FLS*, *MYB12*, and *MYB111*, in 21-day-old seedlings of transgenic lines (Suppl. data 2). Similarly, the expression of genes related to anthocyanin biosynthesis, such as *DFR*, *LDOX*, and *PAP1*, was not changed in 5-day-old seedlings (Suppl. data 2). The expression of *RsTAL* therefore does not seem to affect gene regulation of related enzymes in the flavonoid biosynthetic pathway.

Increased amount of various end products derived from phenylpropanoid pathway suggested that TAL expression enhanced the metabolic flux into the phenylpropanoid pathway. By expressing TAL protein, plants can utilize tyrosine as an alternative initial substrate of the phenylpropanoid pathway. Increased supply of *p*-coumaric acid was assumed to enhance the downstream metabolic flux in the phenylpropanoid pathway, though we could not detect *p*-coumaric acid in either *RsTAL* expressing nor wild-type seedlings. It is possible that the conversion of *p*-coumaric acid into 4-coumaroyl-CoA by 4-coumarate:CoA ligase may be efficient enough to prevent accumulation of *p*-coumaric acid and its consequent detection by LC-PDA-MS.

Plants with high levels of *RsTAL* expression had normal wild-type morphotypes. This suggests that overexpression of *RsTAL* has no visibly adverse effect on plants. In some cases, altered accumulation levels of secondary metabolites cause adverse effects on growth, morphology, and fertility (Ylstra et al. 1994; Fischer et al. 1997; Sharma and Dixon 2005; Millar et al. 2007). Normal growth and fertility are advantageous characteristics in metabolic engineering. *TAL* is expected to be co-expressed with other gene(s) for accumulation of specific class of metabolites. We reported functional expression of flavone synthase type I in *Arabidopsis* (Yun et al. 2008). *RsTAL* expression can increase the supply of the substrate naringenin to flavone synthase.

In summary, the phenylpropanoid pathway in *Arabidopsis* was activated by expressing a *TAL* gene cloned from a photosynthetic bacterium. This approach will facilitate metabolic engineering of the phenylpropanoid pathway in many kinds of plants.

References

Achnine L, Blancaflor EB, Rasmussen S, Dixon RA (2004) Colocalization of L-phenylalanine ammonia-lyase and cinnamate 4-hydroxylase for metabolic channeling in phenylpropanoid biosynthesis. Plant Cell 16:3098–3109

Appert C, Logemann E, Hahlbrock K, Schmid J, Amrhein N (1994) Structural and catalytic properties of the four phenylalanine ammonia-lyase isoenzymes from parsley (*Petroselinum crispum* Nym.). Eur J Biochem 225:491–499

Bate NJ, Orr J, Ni W, Meromi A, Nadler-Hassar T, Doerner PW, Dixon RA, Lamb CJ, Elkind Y (1994) Quantitative relationship between phenylalanine ammonia-lyase levels and phenylpropanoid accumulation in transgenic tobacco identifies a rate-determining step in natural product synthesis. Proc Natl Acad Sci USA 91:7608–7612

Blount JW, Korth KL, Masoud SA, Rasmussen S, Lamb C, Dixon RA (2000) Altering expression of cinnamic acid 4-hydroxylase in transgenic plants provides evidence for a feedback loop at the entry point into the phenylpropanoid pathway. Plant Physiol 122:107–116

Boerjan W, Ralph J, Baucher M (2003) Lignin biosynthesis. Annu Rev Plant Biol 54:519–546

Bolwell GP, Cramer CL, Lamb CJ, Schuch W, Dixon RA (1986) L-Phenylalanine ammonia-lyase from *Phaseolus vulgaris*: modulation of the levels of active enzyme by *trans*-cinnamic acid. Planta 169:97–107

Bolwell GP, Mavandad M, Millar DJ, Edwards KJ, Schuch W, Dixon RA (1988) Inhibition of mRNA levels and activities by *trans*-cinnamic acid in elicitor-induced bean cells. Phytochemistry 27:2109–2117

Borevitz JO, Xia Y, Blount J, Dixon RA, Lamb C (2000) Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. Plant Cell 12:2383–2393

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743

Cochrane FC, Davin LB, Lewis NG (2004) The *Arabidopsis* phenylalanine ammonia lyase gene family: kinetic characterization of the four PAL isoforms. Phytochemistry 65:1557–1564

Dixon RA (2004) Phytoestrogens. Annu Rev Plant Biol 55:225–261

Dixon RA, Paiva NL (1995) Stress-induced phenylpropanoid metabolism. Plant Cell 7:1085–1097

Ehlting J, Büttner D, Wang Q, Douglas CJ, Somssich IE, Kombrink E (1999) Three 4-coumarate:coenzyme *A* ligases in *Arabidopsis thaliana* represent two evolutionarily divergent classes in angiosperms. Plant J 19:9–20

Elkind Y, Edwards R, Mavandad M, Hedrick SA, Ribak O, Dixon RA, Lamb CJ (1990) Abnormal plant development and down-regulation of phenylpropanoid biosynthesis in transgenic tobacco containing a heterologous phenylalanine ammonia-lyase gene. Proc Natl Acad Sci USA 87:9057–9061

Fischer R, Budde I, Hain R (1997) Stilbene synthase gene expression causes changes in flower colour and male sterility in tobacco. Plant J 11:489–498

Hahlbrock K, Knobloch KH, Kreuzaler F (1976) Coordinated induction and subsequent activity changes of two groups of metabolically interrelated enzymes: Light induced synthesis of flavonoid glycosides in cell suspension cultures of *Petroselinum hortense*. Eur J Biochem 61:199–206

Höfgen R, Willmitzer L (1988) Storage of competent cells for *Agrobacterium* transformation. Nucleic Acids Res 16:9877

Howles PA, Sewalt VJH, Paiva NL, Elkind Y, Bate NJ, Lamb C, Dixon RA (1996) Overexpression of L-phenylalanine ammonia-lyase in transgenic tobacco plants reveals control points for flux into phenylpropanoid biosynthesis. Plant Physiol 112:1617–1624

Jorrin J, Dixon RA (1990) Stress responses in alfalfa (*Medicago sativa* L.): II. Purification, characterization, and induction of phenylalanine ammonia-lyase isoforms from elicitor-treated cell suspension cultures. Plant Physiol 92:447–455

Kanno T, Komatsu A, Kasai K, Dubouzet JG, Sakurai M, Ikejiri-Kanno Y, Wakasa K, Tozawa Y (2005) Structure-based in vitro engineering of the anthranilate synthase, a metabolic key enzyme in the plant tryptophan pathway. Plant Physiol 138:2260–2268

Kort R, Phillips-Jones MK, van Aalten DMF, Haker A, Hoffer SM, Hellingwerf KJ, Crielaard W (1998) Sequence, chromophore extraction and 3-D model of the photoactive yellow protein from *Rhodobacter sphaeroides*. Biochim Biophys Acta 1385:1–6

Kyndt JA, Meyer TE, Cusanovich MA, Van Beeumen JJ (2002) Characterization of a bacterial tyrosine ammonia lyase, a biosynthetic enzyme for the photoactive yellow protein. FEBS Lett 512:240–244

Matsuda F, Yonekura-Sakakibara K, Niida R, Kuromori T, Shinozaki K, Saito K (2009) MS/MS spectral tag (MS2T)-based annotation of non-targeted profile of plant secondary metabolites. Plant J 57:555–577

Mavandad M, Edwards R, Liang X, Lamb CJ, Dixon RA (1990) Effects of *trans*-cinnamic acid on expression of the bean phenylalanine ammonia-lyase gene family. Plant Physiol 94:671–680

Mehrtens F, Kranz H, Bednarek P, Weisshaar B (2005) The Arabidopsis transcription factor MYB12 is a flavonol-specific regulator of phenylpropanoid biosynthesis. Plant Physiol 138:1083–1096

Millar DJ, Long M, Donovan G, Fraser PD, Boudet AM, Danoun S, Bramley PM, Bolwell GP (2007) Introduction of sense constructs of cinnamate 4-hydroxylase (CYP73A24) in transgenic tomato plants shows opposite effects on flux into stem lignin and fruit flavonoids. Phytochemistry 68:1497–1509

Pietta PG (2000) Flavonoids as antioxidants. J Nat Prod 63:1035–1042

Ren W, Qiao Z, Wang H, Zhu L, Zhang L (2003) Flavonoids: promising anticancer agents. Med Res Rev 23:519–534

Rösler J, Krekel F, Amrhein N, Schmid J (1997) Maize phenylalanine ammonia-lyase has tyrosine ammonia-lyase activity. Plant Physiol 113:175–179

Shadle GL, Wesley SV, Korth KL, Chen F, Lamb C, Dixon RA (2003) Phenylpropanoid compounds and disease resistance in transgenic tobacco with altered expression of L-phenylalanine ammonia-lyase. Phytochemistry 64:153–161

Sharma SB, Dixon RA (2005) Metabolic engineering of proanthocyanidins by ectopic expression of transcription factors in *Arabidopsis thaliana*. Plant J 44:62–75

Tanaka Y, Sasaki N, Ohmiya A (2008) Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. Plant J 54:733–749

Tohge T, Nishiyama Y, Yokota-Hirai M, Yano M, Nakajima J, Awazuhara M, Inoue E, Takahashi H, Goodenowe DB, Kitayama M, Noji M, Yamazaki M, Saito K (2005) Functional genomics by integrated analysis of metabolome and transcriptome of *Arabidopsis* plants over-expressing an MYB transcription factor. Plant J 42:218–235

Tozawa Y, Tanaka K, Takahashi H, Wakasa K (1998) Nuclear encoding of a plastid σ factor in rice and its tissue- and light-dependent expression. Nucleic Acids Res 26:415–419

Watts KT, Lee PC, Schmidt-Dannert C (2004) Exploring recombinant flavonoid biosynthesis in metabolically engineered *Escherichia coli*. Chembiochem 5:500–507

Williams CA, Grayer RJ (2004) Anthocyanins and other flavonoids. Nat Prod Rep 21:539–573

Williams RJ, Spencer JP, Rice-Evans C (2004) Flavonoids: antioxidants or signaling molecules? Free Rad Biol Med 36:838–849

Xie DY, Sharma SB, Wright E, Wang ZY, Dixon RA (2006) Metabolic engineering of proanthocyanidins through co-expression of anthocyanidin reductase and the PAP1 MYB transcription factor. Plant J 45:895–907

Xue Z, McCluskey M, Cantera K, Sariaslani FS, Huang L (2007) Identification, characterization and functional expression of a tyrosine ammonia-lyase and its mutants from the photosynthetic bacterium *Rhodobacter sphaeroides*. J Ind Microbiol Biotechnol 34:599–604

Ylstra B, Busscher J, Franken J, Hollman PCH, Mol JNM, van Tunen AJ (1994) Flavonols and fertilization in *Petunia hybrida*: localization and mode of action during pollen tube growth. Plant J 6:201–212

Yonekura-Sakakibara K, Tohge T, Matsuda F, Nakabayashi R, Takayama H, Niida R, Watanabe-Takahashi A, Inoue E, Saito K (2009) Comprehensive flavonol profiling and transcriptome coexpression analysis leading to decoding gene–metabolite correlations in *Arabidopsis*. Plant Cell 20:2160–2176

Yun CS, Yamamoto T, Nozawa A, Tozawa Y (2008) Expression of parsley flavone synthase I establishes the flavone biosynthetic pathway in *Arabidopsis thaliana*. Biosci Biotechnol Biochem 72:968–973