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Metabolic changes in *Arabidopsis thaliana* expressing the feedback-resistant anthranilate synthase α subunit gene *OASA1D*

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Abstract

Anthranilate synthase (AS) is a key enzyme in tryptophan (Trp) biosynthesis. Metabolic changes in transgenic *Arabidopsis* plants expressing the feedback-resistant anthranilate synthase α subunit gene *OASA1D* were investigated with respect to Trp synthesis and effects on secondary metabolism. The Trp content varied depending on the transgenic line, with some lines showing an approximately 200-fold increase. The levels of AS activity in crude extracts from the transgenic lines were comparable to those in the wild type. On the other hand, the enzyme prepared from the lines accumulating high levels of Trp showed a relaxed feedback sensitivity. The AS activity, determined in the presence of 50 μM L-Trp, correlated well with the amount of free Trp in the transgenic lines, indicating the important role of feedback inhibition in control of Trp pool size. In *Arabidopsis*, Trp is a precursor of multiple secondary metabolites, including indole glucosinolates and camalexin. The amount of indol-3-ylmethyl glucosinolate (I3M) in rosette leaves of the high-Trp accumulating lines was 1.5- to 2.1-fold greater than that in wild type. The treatment of the leaves with jasmonic acid resulted in a more pronounced accumulation of I3M in the high-Trp accumulating lines than in wild type. The induction of camalexin formation after the inoculation of *Alternaria brassicicola* was not affected by the accumulation of a large amount of Trp. The accumulation of constitutive phenylpropanoids and flavonoids was suppressed in high-Trp accumulating lines, while the amounts of Phe and Tyr increased, thereby indicating an interaction between the Trp branch and the Phe and Tyr branch in the shikimate pathway.

Keywords: *Arabidopsis*; Cruciferae; Anthranilate synthase; Feedback inhibition; Tryptophan; Secondary metabolism

1. Introduction

Tryptophan (Trp, **4**, Fig. 1) is an essential amino acid that largely functions as a building block of proteins. The biosynthesis of Trp (**4**) takes place via the shikimate pathway, and the first committed step of Trp (**4**) synthesis is catalyzed by anthranilate synthase (AS). AS transfers an amino group of glutamine to chorismate (**2**) and generates anthranilate (**3**) and pyruvate. It has been demonstrated that AS activity is regulated by Trp (**4**) through feedback inhibition (Belser et al., 1971; Widholm, 1972).

Purified AS enzymes are heterotetramers composed of distinct α and β subunits (Poulsen et al., 1993; Bohlmann et al., 1995; Niyogi and Fink, 1992; Niyogi et al., 1993). While ASβ transfers the amino group from glutamine to ASα, ASα converts chorismate (**2**) to anthranilate (**3**) with ammonium ion the amino donor, and is responsible for feedback inhibition by Trp (**4**). Feedback insensitive ASα subunits have been characterized in *Ruta graveolens* (Bohlmann et al., 1996) and cultured tobacco cells (Brotherton et al., 1986), but naturally occurring ASα subunits in other

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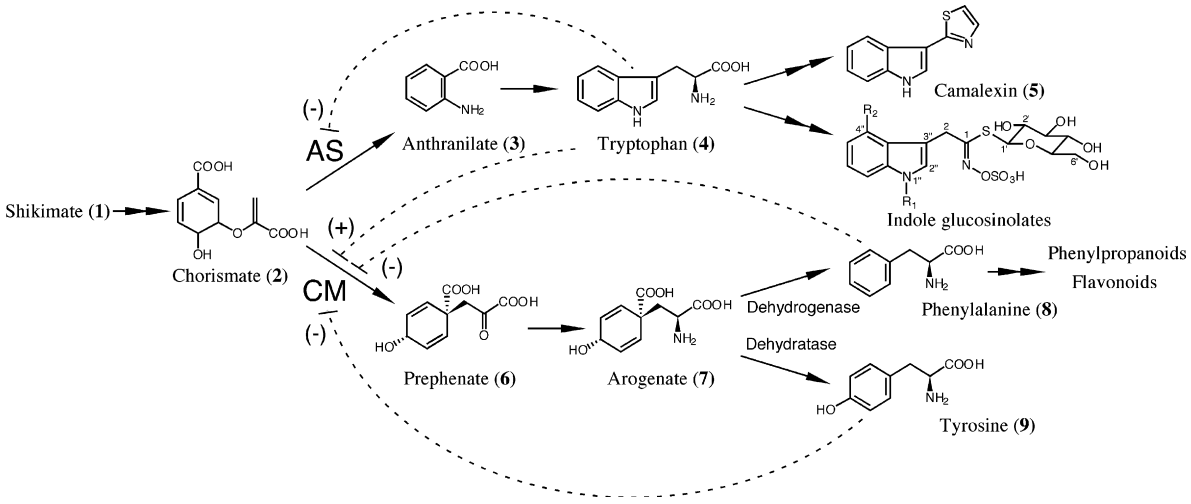

Fig. 1. Schematic representation of the metabolic pathway closely related to Trp (**4**) synthesis.

species are feedback sensitive. A mutant *trp5* that has relaxed feedback sensitivity was acquired by selection on the basis of resistance to α-methyltryptophan (Kreps et al., 1996) and 6-methylanthranilate (Li and Last, 1996) in *Arabidopsis*. α-Methyltryptophan is a false feedback inhibitor of AS, and 6-methyanthranilate is converted to another false feedback inhibitor, 4-methyltryptophan, in planta. The mutated *ASA1* was found to be responsible for the relaxed feedback sensitivity in the *trp5* plant.

A mutated cDNA of rice ASα, *OASA1D*, was designed to encode a protein with asparagine substituted for aspartate at position 323 which corresponds to position 341 of *Arabidopsis* ASA1 (Tozawa et al., 2001). The original enzyme OASA1 is similar to ASA2 in *Arabidopsis* with respect to its expression pattern, which is constitutively expressed in rice plants at a low level. An extract from calli expressing *OASA1D* under control of the maize ubiquitin promoter showed relaxed feedback sensitivity. The OASA1D enzyme was further extensively characterized using a protein prepared with an in vitro cell-free expression system (Kanno et al., 2004). The reconstituted complex of OASA1D and rice ASβ (OASB1) exhibited a 3.4-fold greater K_i value for Trp (**4**) than did the wild-type complex OASA1-OASB1.

The importance of the feedback regulation of AS in the control of metabolic flow in the Trp (**4**) pathway has been demonstrated by measuring the Trp (**4**) contents of plants expressing feedback-resistant ASα genes. The *Arabidopsis trp5* mutant accumulated approximately 3-fold more Trp (**4**) than the wild-type plant (Li and Last, 1996), and the introduction of the mutated *trp5* gene into hairy root cultures of *Catharanthus roseus* caused an increase in Trp (**4**) content from an almost undetectable level to 2.5 mg/gDW (Hughes et al., 2004). The overexpression of the naturally occurring feedback-resistant *ASA1* gene in tobacco (Song et al., 1998) also resulted in an approximately 3-fold increase in the amount of free Trp (**4**) (Tsai et al., 2005). By contrast, the utilization of *OASA1D* has been shown to be very effective in increasing Trp (**4**) content. In rice calli transformed with *OASA1D*, the Trp (**4**) content increased up to 180-fold, and the leaves and seeds of progeny plants accumulated an approximately 35-fold and 430-fold greater amount of Trp (**4**) than wild type, respectively (Tozawa et al., 2001; Wakasa et al., 2006). Potato plants expressing *OASA1D* also accumulated a 2- to 20-fold greater amount of free Trp (**4**) (Yamada et al., 2004; Matsuda et al., 2005). As shown by these examples, while the introduction of a feedback-resistant ASα gene was generally effective in enhancing Trp (**4**) accumulation, the degree of enhancement depended on the system. The feedback sensitivity of the ASα protein encoded by the mutated transgene and its expression level are two possible factors leading to the difference in Trp (**4**) content. However, a quantitative correlation between these factors and Trp (**4**) content has not clearly been demonstrated.

The Trp (**4**) pathway provides numerous secondary metabolites in plants. For instance, acridone alkaloids in the Rutaceae family (Gröger and Johne, 1968) and avenanthramide phytoalexins in oats (Ishihara et al., 1999; Matsukawa et al., 2002) are synthesized from anthranilate (**3**). Benzoxazinones, the defensive secondary metabolites in graminaceous plants, originate from indole formed from anthranilate (**3**) by three enzymatic reactions leading to Trp (**4**) (Frey et al., 1997; Nomura et al., 2002). Trp (**4**) is also converted into a diverse range of secondary metabolites including indole alkaloids and terpenoid indole alkaloids. Hence, given that the feedback regulation of AS can modulate the Trp (**4**) pathway and is important for controlling the Trp (**4**) pool size, the change in the feedback sensitivity of AS also likely affects the secondary metabolism derived from Trp (**4**). In *Arabidopsis*, indole glucosinolates (IGs) and the indolic phytoalexin, camalexin (**5**), have been found to originate from Trp (**4**) (Hull et al., 2000; Mikkelsen et al., 2000; Glawischnig et al., 2004). IGs are representative constitutive secondary metabolites, while camalexin (**5**) formation is inducible in response to invasion by pathogens. Since changes in the accumulated amounts of Trp-derived metabolites have been observed to be accompanied or preceded by changes in the expression level of AS genes

(Niyogi and Fink, 1992; Bohlmann et al., 1995, 1996), AS has been suggested to play a role in the control of substrate supply for these secondary metabolites.

Increased supply of Trp (**4**) in plants may also affect the levels of Phe (**8**) and Tyr (**9**), because Trp (**4**) shares part of its biosynthetic pathway with these aromatic amino acids. The first committed reaction in Phe (**8**) and Tyr (**9**) biosynthesis is catalyzed by chorismate mutase (CM). The sharing of a precursor by the two branches suggests putative competition between the two branches. Also, the feedback activation of CM by Trp (**4**) is another possible influence on the synthesis of Phe (**8**) and Tyr (**9**) in plants. The presence of cytosolic and plastidic CM isoforms has been shown in various plants (Romero et al., 1995). Plastidic CM has been demonstrated to be negatively regulated by Phe (**8**) and Tyr (**9**), while it is positively regulated by Trp (**4**) (Singh et al., 1986; Eberhard et al., 1996). Considering that Phe (**8**) is the precursor of various secondary metabolites such as phenylpropanoids and flavonoids, its effects on Phe (**8**) biosynthesis is of particular interest.

Recently, a series of transgenic lines expressing *OASA1D* at various levels were established in *Arabidopsis* (Kawagishi-Kobayashi et al., 2005). These transformants are useful for examining the relationship between the accumulation of Trp (**4**) and the level of AS activity in a plant. In the present study, we analyzed the levels of Trp (**4**) and AS activity in these transgenic lines in order to demonstrate that the feedback sensitivity of AS rather than total activity is the factor controlling the level of Trp (**4**) in *Arabidopsis*. In addition, we also analyzed the accumulation of representative secondary metabolites, as well as the amounts of Phe (**8**) and Tyr (**9**) in order to elucidate the influence of the overproduction of Trp (**4**) on the closely related metabolic pathways as depicted in Fig. 1.

2. Results

2.1. Effect of introduction of OASA1D on Trp (4) synthesis

The amount of Trp (**4**) in the transgenic lines bearing the *OASA1D* gene was analyzed. Two-week-old seedlings of transgenic lines were extracted and their Trp (**4**) levels were determined by HPLC analysis. In rosette leaves of W551 and W552, the amount of Trp (**4**) was 2.6 and 3.0 μmol/gFW. These values are more than 200-fold greater than the amount in wild-type leaves (Fig. 2a). Other lines, except W311 and W433, also showed 4- to 20-fold increases in Trp (**4**) content. Based on these results, we categorized the transgenic lines into three groups for convenience, i.e., high-Trp (**4**) accumulating lines (W551 and W552), moderate-Trp (**4**) accumulating lines (W133, W146, W151, W163, W521), and low-Trp (**4**) accumulating lines (W311 and W433). In the roots, a similar accumulation of Trp (**4**) was observed although the magnitude of the increase in Trp (**4**) content was smaller than that observed in the leaves (Fig. 2b).

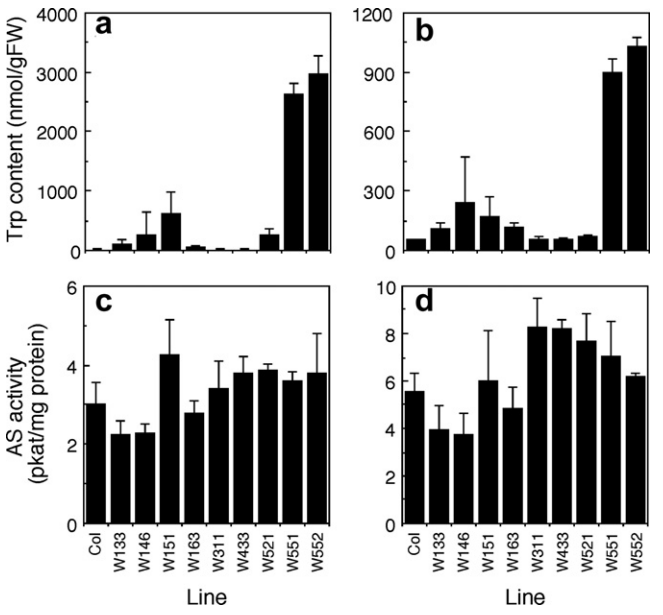

Fig. 2. Trp (**4**) accumulation (a and b) and AS activity (c and d) in transgenic *Arabidopsis* seedlings. Two-week-old seedlings were divided into rosette leaves (a and c) and roots (b and d). The error bars indicate the standard deviations of three replicates.

To analyze the distribution of Trp (**4**) in the seedlings at the reproductive stage of growth, transgenic seedlings germinated on plates containing Murashige and Skoog (MS) medium were transplanted onto vermiculite 2 weeks after sowing, and were incubated a further 3 weeks. The plants were divided into roots, rosette leaves, stems, cauline leaves, flowers, and siliques, and the Trp (**4**) content of each organ was analyzed. The transgenic and wild-type plants showed similar patterns of Trp (**4**) accumulation (Fig. 3). At this stage of growth, the silique and flower were the organs that accumulated the highest concentration of Trp (**4**), while the roots and rosette leaves contained Trp (**4**) at lower concentrations. Similar levels of Trp (**4**) were observed in lines W133 and W163 and wild-type plants, with the exception of an approximatley 2-fold increase in the silique of W133. Siliques and flowers of W521 showed a 13-fold increase in Trp (**4**) content when compared with the wild type, while the amounts of Trp (**4**) in siliques and flowers of W551 were approximately 65- and 135-fold greater, respectively, than those in the wild type. Other free protein amino acids also accumulated at higher concentrations in siliques and flowers than in rosette leaves (data not shown). Their concentrations were not significantly different between the wild type and W551 except that the concentration of Phe (**8**) in W551 (307 nmol/gFW) was 1.6-fold greater than that in the wild type (194 nmol/gFW).

To examine the correlation between Trp (**4**) content and AS activity in the transgenic lines, the crude enzyme was extracted from the leaves and roots of 2-wk-old seedlings, and the AS activity was determined with Gln and chorismate (**2**) as substrates. As shown in Fig. 2c and d, the respective activities in the rosette leaves and roots of transgenic lines ranged from 75% (W133) to 140% (W151) and from

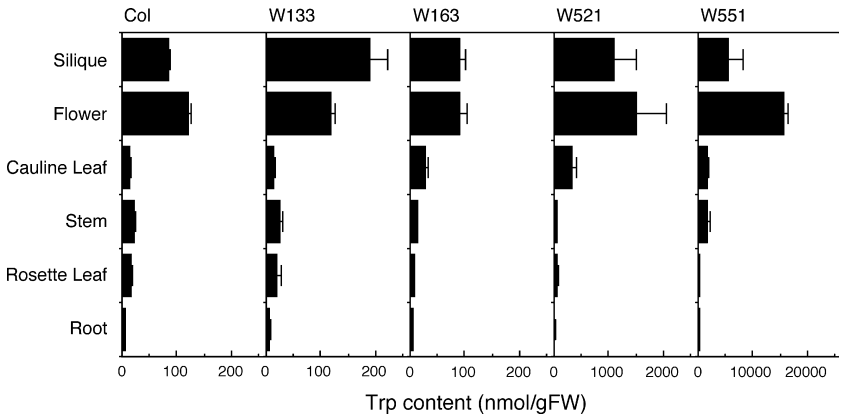

Fig. 3. Accumulation of Trp (**4**) in various parts of *Arabidopsis* seedlings. Two-week-old seedlings were transplanted to soil, and further incubated for 3 wks. The seedlings were divided into siliques, flowers, cauline leaves, stems, rosette leaves, and roots, and extracted with MeOH. The error bars indicate the standard deviations of three replicates.

68% (W146) to 139% (W521) of the activity detected in wild type seedlings. The results suggested that the Trp (**4**) content could not be correlated to total AS activity either in the rosette leaves (Fig. 2a and c) or in the roots (Fig. 2b and d).

The feedback sensitivity of AS was also determined by measuring the enzyme activity in the presence of various concentrations of L-Trp (**4**) or D,L-5-methyltryptophan (5MT). The inhibition curves obtained with L-Trp (**4**) and 5MT in the leaves of the wild type and a high-Trp accumulating line W551 are depicted in Fig. 4a and b, respectively. In the wild-type leaves, no activity was detected in the presence of either 50 μM L-Trp (**4**) or 50 μM of 5MT, while activity was detected in W551 even at 100 μM of L-Trp (**4**) or 5MT. AS activity from W551 was not completely inhibited in the presence of up to 5 mM of 5MT, with 18% of the activity detected in the absence of 5MT (data not shown). Almost the same inhibition curves were obtained for the other high-Trp (**4**) accumulating line W552 (data not shown). Moderate-Trp (**4**) accumulating lines also showed relaxed feed-back sensitivity although the degree of relaxation was less than in high-Trp (**4**) accumulating lines. In low-Trp (**4**) accumulating lines, no difference was found in the inhibition curves with those from the wild type. The Trp (**4**) content in each line was plotted as a function of the AS activity that was recorded in the presence of 50 μM L-Trp (**4**) in the reaction mixture (Fig. 4c and d). A good correlation was observed between Trp (**4**) content and AS activity measured in the presence of 50 μM Trp (**4**) in both the rosette leaves and roots.

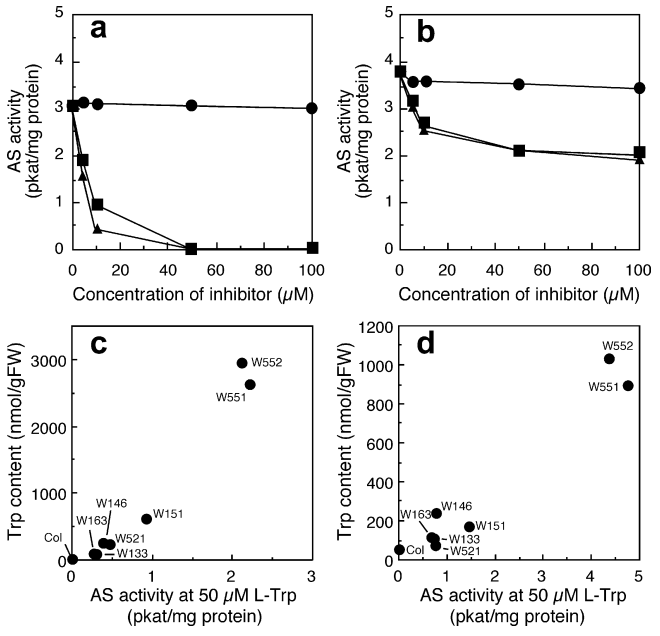

Fig. 4. Feedback inhibition of AS activity. AS activity in rosette leaves of 2-wk-old seedlings of the wild type (a) and line W551 (b) was measured in the presence of various concentrations of L-Trp (**4**) (squares), D-Trp (circles), and 5MT (triangles). AS activity in leaves (c) and roots (d) of 2-wk-old seedlings was assayed with 10 mM glutamine and 4 mM chorismate (**2**) as substrates in the presence of 50 μM Trp (**4**). Trp (**4**) content in each line, which was determined in the experiments in Fig. 2a and b, was plotted as a function of AS activity.

The accumulation of the *OASA1D* transcripts in the transgenic lines also correlated with the amount of Trp (**4**) (Fig. 5). The transcript was detected in all transgenic lines by RT-PCR analysis. On the basis of differences in the level of the *OASA1D* transcript, the transgenic lines were classified into three groups, corresponding to the high-, moderate-, and low-Trp (**4**) accumulating lines. The introduction of *OASA1D* did not affect the levels of endogenous *ASA1* and *ASA2* transcripts.

2.2. Effects of introduction of OASA1D on secondary metabolism

Arabidopsis plants constitutively accumulate IGs such as indole-3-ylmethyl glucosinolate (I3M, **10**) (Brown et al., 2003). The amount of I3M (**10**) has been shown to increase in leaves treated with jasmonic acid (JA) (Brader et al., 2001). Hence, we analyzed the amount of I3M (**10**) in rosette leaves that were treated with JA (Fig. 6a). In the untreated leaves, the amount of I3M (**10**) in the high-Trp (**4**) accumulating lines was approximately 2-fold

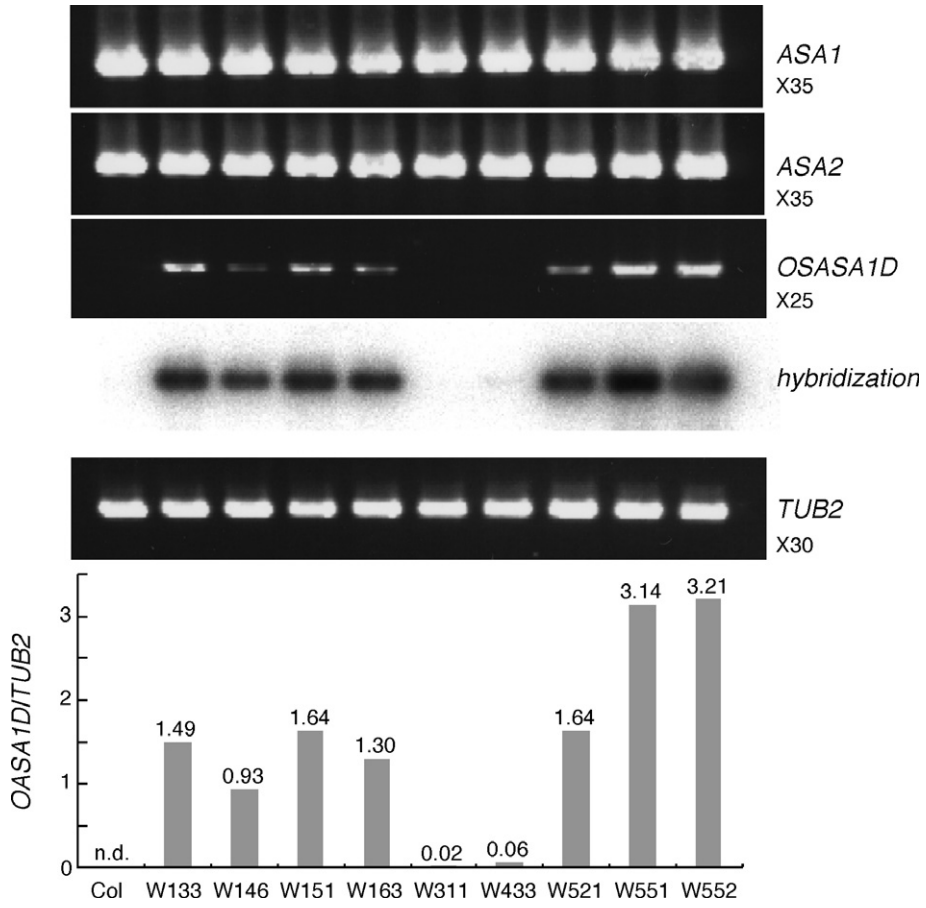

Fig. 5. Accumulation of the *OASA1D* transcript in transgenic *Arabidopsis* seedlings. Total RNA was extracted from 2-wk-old seedlings, and the accumulation of transcripts of *ASA1*, *ASA2*, and *OASA1D* was analyzed by RT-PCR. Amplified *OASA1D* cDNA was quantified by hybridization with the radio-labeled probe. Accumulation of the *OASA1D* transcript was expressed relative to that of the *TUB2* transcript.

greater than that in the wild-type leaves, but no significant difference was observed in the other transgenic lines. In the wild-type leaves, the treatment with JA resulted in a 2.8-fold increase in I3M (**10**) content, while the rate of increase was relatively greater in W551 and W552 with 4.9- and 7.7-fold increases, respectively.

Camalexin (**5**) is another Trp-derived secondary metabolite present in *Arabidopsis* (Tsuji et al., 1992; Glawischnig et al., 2004). For the induction of camalexin (**5**) formation, the conidia of the pathogenic fungus *Alternaria brassicicola* were inoculated in the rosette leaves of 2-wk-old seedlings, and the amount of accumulated camalexin (**5**) was determined 72 h after inoculation (Fig. 6b). The accumulation of camalexin (**5**) was detected in the inoculated leaves of all lines, but not in the non-inoculated control leaves. The amount of camalexin (**5**) accumulated in transgenic plants including the high-Trp (**4**) accumulating lines was approximately 200 nmol/gFW, which was similar to the concentration in the wild-type plants. No difference in the size of lesions was observed among the lines tested.

To further investigate the effects of Trp (**4**) accumulation on phenolic secondary metabolism, major peaks detected on HPLC analysis of the extract of *Arabidopsis* seedlings were characterized. After being purified by fractionation by ODS column chromatography and preparative HPLC, the compounds corresponding to each peak were identified by positive ion-spray MS and NMR spectroscopic analyses. The compounds identified in the rosette leaves were I3M (**10**), 4-methoxyindol-3-ylmethyl-glucosinolate (4MI3M, **11**), 1-sinapoylglucose (**13**), kaempferol 3-*O*-glucosylrhamnoside 7-*O*-rhamnoside (**14**), 1-methoxyindol-3-ylmethyl-glucosinolate (1MI3M, **12**), kaempferol 3-*O*-glucoside 7-*O*-rhamnoside (**15**), kaempferol 3-*O*-rhamnoside 7-*O*-rhamnoside (**16**), and sinapoyl malate (**17**). The compounds identified in the root extract were I3M (**10**), coniferin (**19**), syringin (**20**), 4MI3M (**11**), kaempferol 3-*O*-glucosylrhamnoside 7-*O*-rhamnoside (**14**), 1MI3M (**12**), quercetin 3-*O*-glucoside 7-*O*-rhamnoside (**15**), and kaempferol 3-*O*-glucoside 7-*O*-rhamnoside (**16**). The identified compounds were classified into IGs (**10**–**12**), phenylpropanoids (**13**, **18**–**20**), and flavonoinds (**14**–**17**) based on their chemical structures (Fig. 7). The changes in the levels of compounds in the transgenic lines were simultaneously analyzed by HPLC with a gradient elution system (Figs. 8a and 9a). No new peaks that were specifically detected in the transgenic lines were present under the analytical conditions employed. However, the analyses revealed differences in amounts of phenylpropanoids and flavonoids among the

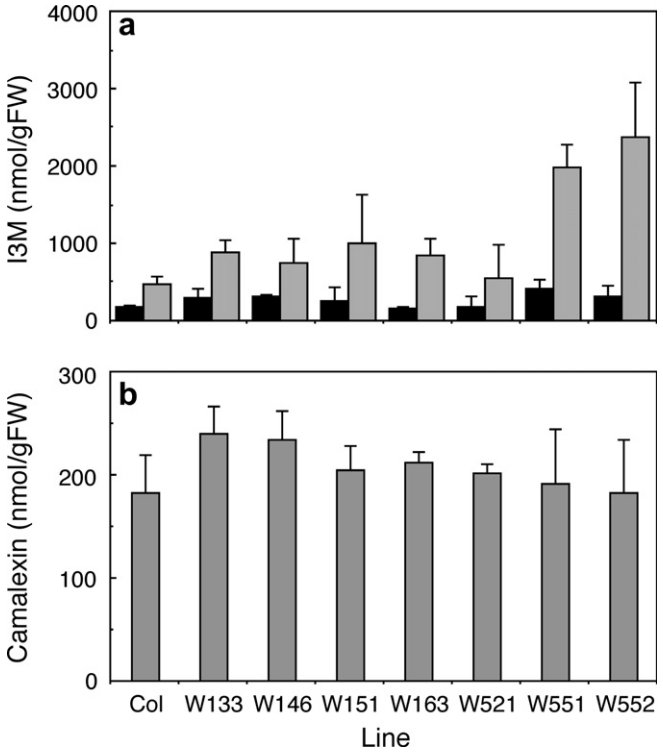

Fig. 6. Accumulation of I3M (**10**) (a) and camalexin (**5**) (b) in response to exogenous stimuli. (a) Rosette leaves were excised from 3-wk-old seedlings and floated in the 500 μM MeJA solution (gray bars) or dist. H_2O (black bars). After a 48-h incubation, the leaves were extracted by MeOH. (b) Rosette leaves of 2-wk-old seedlings were inoculated with conidia of *A. brassicicola* (5×10^5 conidia/ml). The leaves were extracted with MeOH 72 h after the inoculation. No accumulation of camalexin (**5**) was detected in non-inoculated control of all lines. The error bars indicate the standard deviations of three replicates.

lines as shown in Figs. 8b and 9b. The amounts of major flavonoids, namely, **14**–**16**, showed a 63–57% decrease in the leaves of high-Trp (**4**) accumulating lines. A decrease in the amount of major phenylpropanoids, i.e., **13** and **18**, was also detected in the leaves (49–28% decrease). Similarly, unidentified compounds (peaks F, L and M) showed a significant decrease (80–25% decrease). In roots, flavonoids (**14**, **15**, **17**; 60–9% decrease) and phenylpropanoids (**19** and **20**; 64–32% decrease) as well as unidentified compounds (peaks d and k; 71–48%) decreased in the high-Trp (**4**) accumulating lines. The amounts of flavonoids and phenylpropanoids in the other transgenic lines were not significantly different from those in the wild-type seedling.

2.3. Effects on phenylalanine (*8*) and tyrosine (*9*) contents

Flavonoids and phenylpropanoids are biosynthesized from Phe (**8**). The decrease in both flavonoids and phenylpropanoids in the high-Trp (**4**) accumulating lines suggested competition between the Trp (**4**) branch and the branch for Phe (**8**) and Tyr (**9**) of the shikimate (**1**) pathway in the transgenic lines. In order to examine this possibility, we analyzed the amounts of Phe (**8**) and Tyr (**9**) in the transgenic lines (Fig. 10). The high-Trp (**4**) accumulating lines showed about 2-fold and 1.3-fold increases in Phe (**8**) content in the leaves and roots, respectively, when compared with the wild type. Similarly, the lines showed 4.6- and 1.7-fold increases in Tyr (**9**) content in the leaves and roots, respectively. In addition, a comparison of Fig. 10 with Fig. 2 revealed that the pattern of Phe (**8**) and Tyr (**9**) accumulation was similar to that of the accumulation

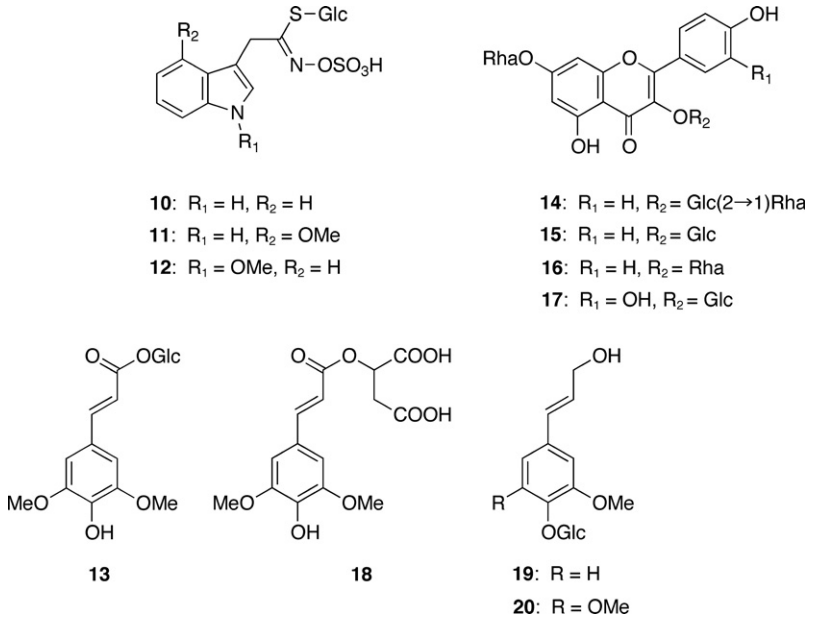

Fig. 7. Chemical structures of identified compounds. Identified compounds include indole glucosinolates [I3M (**10**, peaks A and a in Figs. 8 and 9, respectively), 4MI3M (**11**, peaks C and e), and 1MI3M (**12**, peaks H and h)], flavonoids [kaempferol 3-*O*-glucosylrhamnoside 7-*O*-rhamnoside (**14**, peaks G and g), kaempferol 3-*O*-glucoside 7-*O*-rhamnoside (**15**, peaks I and j), kaempferol 3-*O*-rhamnoside 7-*O*-rhamnoside (**16**, peak J), and quercetin 3-*O*-glucoside 7-*O*-rhamnoside (**17**, peak i)], phenylpropanoids [1-sinapoylglucose (**13**, peak E), sinapoyl malate (**18**, peak K), coniferin (**19**, peak b), and syringin (**20**, peak c)] and tryptophan (**4**, peaks D and f).

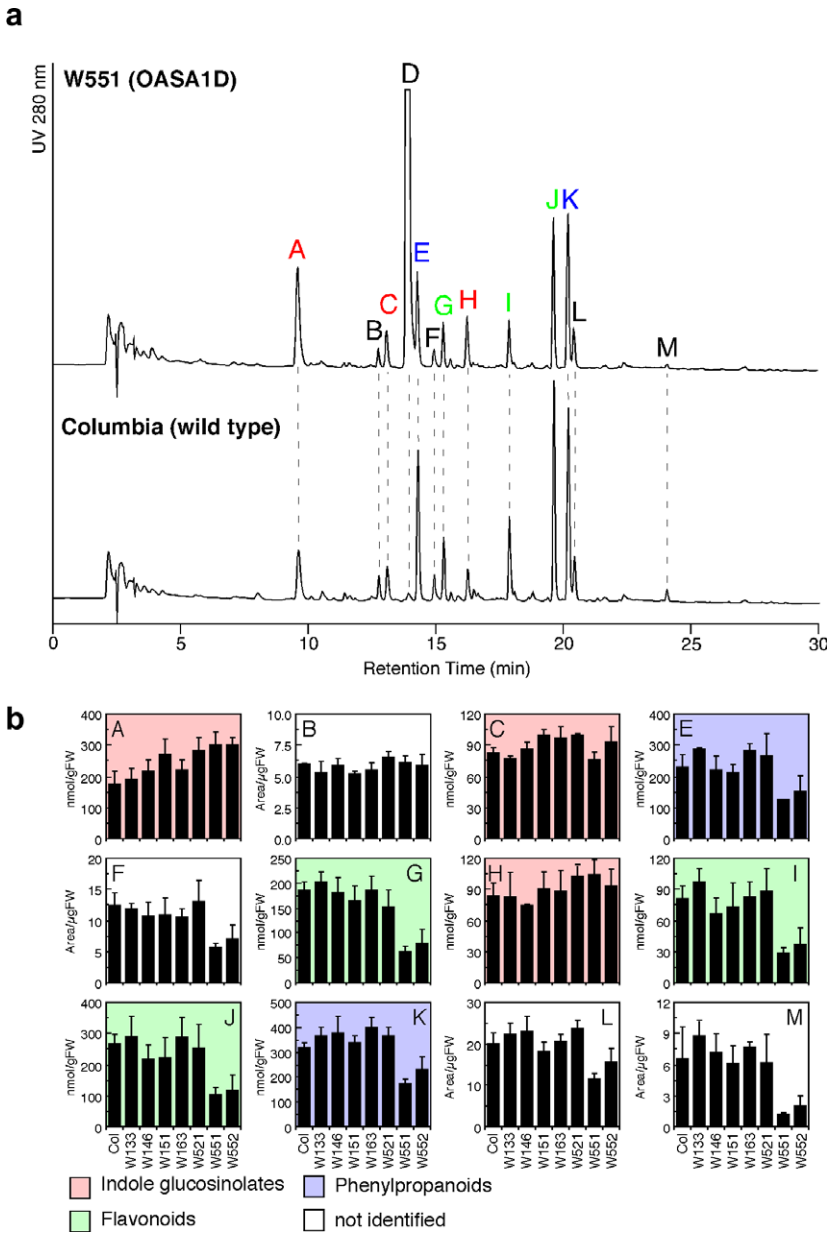

Fig. 8. HPLC analysis of secondary metabolites in aerial parts of transgenic seedlings. (a) MeOH extract of the aerial parts of 2-wk-old seedlings was subjected to reversed-phase HPLC analysis. (b) Amounts of major secondary metabolites in aerial parts of the seedlings. Identities of peaks are shown in Fig. 7. The error bars indicate the standard deviations of three replicates.

of Trp (**4**) among the lines examined; the lines accumulating high concentrations of Trp (**4**) showed a tendency to accumulate high concentrations of Phe (**8**) and Tyr (**9**). The difference in the accumulated amount of Tyr (**9**) among the lines was more pronounced than that of Phe (**8**).

3. Discussion

3.1. Effect on Trp (4) accumulation

In the present study, we analyzed the free Trp (**4**) content in transgenic lines bearing the feedback resistant ASα gene, *OASA1D*. The amount of Trp (**4**) varied with each line, and some lines showed approximately 220- and 20-fold increases in the rosette leaves and roots, respectively. In addition, the high-Trp (**4**) accumulating lines were more resistant to 5MT in the growth medium. However, the levels of AS activity in the transgenic lines were not significantly different from those in the wild type irrespective of the difference in Trp (**4**) content. Rather, the lines that accumulated a higher level of Trp (**4**) showed more relaxed feedback sensitivity, thereby indicating the importance of feedback regulation in Trp (**4**) accumulation and 5MT resistance. It is of note that no obvious increase in total AS activity was detected in the lines accumulating high levels of Trp (**4**) because the relaxed feedback sensitivity found in these lines is the consequence of a strong

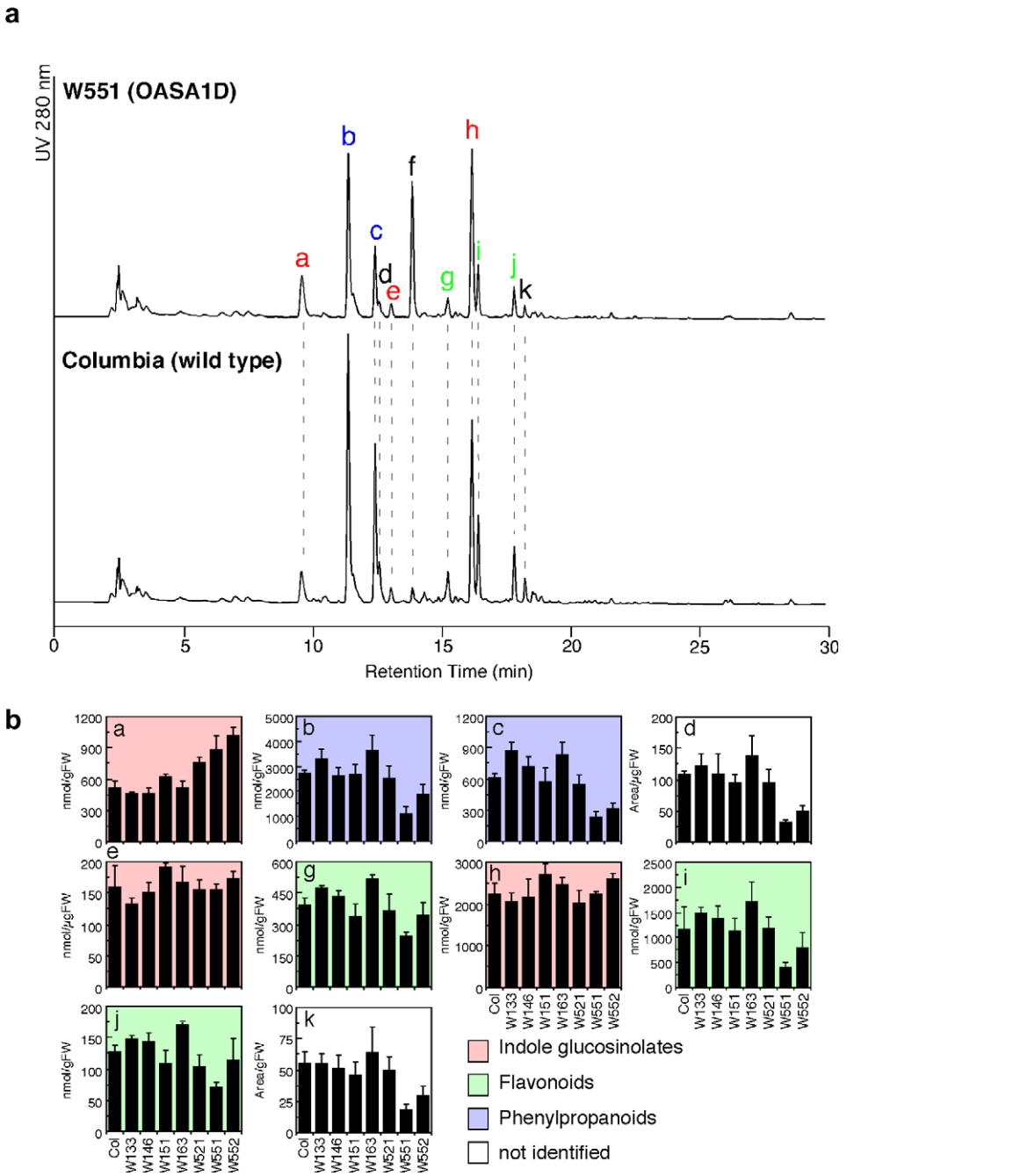

Fig. 9. HPLC analysis of secondary metabolites in roots of transgenic seedlings. (a) MeOH extract of roots of 2-wk-old seedlings was subjected to reversed-phase HPLC analysis. (b) Amounts of major secondary metabolites in roots of the seedlings. Identities of peaks are shown in Fig. 7. The error bars indicate the standard deviations of three replicates.

expression of *OASA1D*. The formation of anthranilate (**3**) from chorismate (**2**) and glutamine is catalyzed by the complex of α and β subunits of AS. Thus, that there was no obvious increase in AS activity in the transgenic lines suggests that total AS activity is determined by the amount of ASβ subunit in both the wild-type and transgenic plants. A similar unchanged level of AS activity was also reported in potato plants expressing *OASA1D* (Matsuda et al., 2005).

The Trp (**4**) content reported for the *trp5* mutant was approximately 3-fold greater than that in the wild type. The crude AS enzyme preparation from the *trp5* mutant in *Arabidopsis* showed a K_i value of 8 μM for Trp (**4**) (Li and Last, 1996). On the other hand, the K_i value of OASA1D–OASB complex for Trp (**4**) was reported to be approximately 135 μM (Kanno et al., 2004). This difference in feedback sensitivity between OASA1D and the mutated ASA1 in *trp5* well accounts for the more relaxed feedback sensitivity in the transgenic lines expressing *OASA1D* compared to the *trp5* plant, and resulted in the higher Trp (**4**) level.

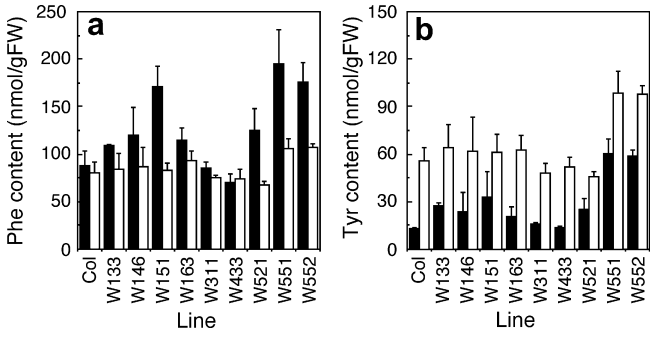

Fig. 10. Accumulation of Phe (**8**) (a) and Tyr (**9**) (b) in rosette leaves (black bars) and roots (gray bars) of 2-wk-old seedlings. The Phe (**8**) and Tyr (**9**) contents were determined by LC/MS/MS analysis in the positive MRM mode. The error bars indicate the standard deviations of three replicates.

The maximal Trp (**4**) level attained by transgenic lines expressing *OASA1D* was approximately 3,000 nmol/gFW, which is similar to the level in the leaves of transgenic rice expressing *OASA1D* (Tozawa et al., 2001). The inhibition curves obtained from the high-Trp (**4**) accumulating lines were similar to the curve reported for the OASA1D–OASB complex (Kanno et al., 2004). Given the similar inhibition curve of the complex of OASA1D and *Arabidopsis* ASB and the complex of OASA1D and OASB, it is very likely that almost all AS complexes in the transgenic lines are comprised of OASA1D and *Arabidopsis* ASB. Thus, a stronger expression of *OASA1D* would not result in a more relaxed feed-back sensitivity. The Trp (**4**) levels attained in the high-Trp (**4**) accumulating lines are around the maximal limit attained by the introduction of the *OASA1D* gene into *Arabidopsis*.

The RT-PCR analysis revealed that the level of *OASA1D* transcripts in the transgenic lines largely correlated with the relaxed feedback sensitivity and Trp (**4**) content. In addition, the levels of transcripts of endogenous *ASA1* and *ASA2* were not affected by the introduction of *OASA1D*, suggesting that the accumulation of Trp (**4**) nor free ASα subunits had no effects on the transcription of *ASA* genes. In the high-Trp (**4**) accumulating lines, the active transcription of *OASA1D* resulted in the accumulation of abundant OASA1D protein, and thus ASB had relatively higher chances to bind to OASA1D in spite of the presence of endogenous ASα subunits.

In the reproductive stage of growth, the siliques and flowers accumulated the highest concentration of Trp (**4**) among the organs analyzed, while the Trp (**4**) concentrations in rosette leaves and roots were relatively low both in the wild type and in the transgenic lines. The Trp (**4**) concentration in the siliques and flowers of the wild type was approximately 100 μM, which is beyond the concentration inhibitory to the wild-type AS. Thus, Trp (**4**) in these organs was considered not to be synthesized "on site", but to be translocated from the vegetative organs, although the possibility that the sequestration of Trp (**4**) in the vacuoles may enable the feedback-sensitive AS to generate anthranilate (**3**) in plastids was not excluded. Supporting the translocation of Trp (**4**), the presence of almost all amino acids including Trp (**4**) has been shown in the phloem sap from various plants (Tilsner et al., 2005; Caputo et al., 2001).

Phloem loading of amino acids has been assumed to be mostly unspecific on the basis of the findings that the amino acid composition in the phloem sap resembles that in the cytosol of source cells, with no individual amino acids preferentially enriched in the phloem (Riens et al., 1991; Lohaus et al., 1995). In the high-Trp (**4**) accumulating line W551, the Trp (**4**) content of siliques and flowers was around 65- and 135-fold greater than that in the wild type. This enhancement of Trp (**4**) accumulation was similar in magnitude to that found in the rosette leaves and roots of 2-wk-old seedlings. Thus, the translocation in the plant does not appear to be a major limitation to the accumulation of Trp (**4**) in siliques and flowers of the high-Trp (**4**) accumulating lines.

3.2. Effects on secondary metabolism

Trp (**4**) is a central precursor of secondary metabolites in plants. In *Arabidopsis*, IGs and camalexin (**5**) have been shown to be synthesized from Trp (**4**) (Hull et al., 2000; Mikkelsen et al., 2000; Glawischnig et al., 2004). It has been suggested that glucosinolates play a role in defense against herbivores (Lambrix et al., 2001; Nielsen et al., 2001) and pathogens (Brader et al., 2001; Tierens et al., 2001), or function as attractants of specialist pests (van Loon et al., 1992; Giamoustaris and Mithen, 1995), while camalexin (**5**) has been characterized as a phytoalexin (Tsuji et al., 1992). An investigation of the effects of enhanced substrate supply for secondary metabolism is essential as a basis for understanding the interaction between primary and secondary metabolism.

In the high-Trp (**4**) accumulating lines, the amount of constitutively accumulated I3M (**10**) increased, as shown in the control of Fig. 7. However, considering greater than 200-fold increase in Trp (**4**) content in W551 and W552, the increase in the amount of I3M (**10**) was small, which indicates that IG biosynthesis is regulated in a relatively strict manner independently of the supply of precursor. The first committed step in the biosynthesis of IGs is the formation of indole-3-acetaldoxime (IAOx) from Trp (**4**) catalyzed by CYP79B2 and CYP79B3 (Hull et al., 2000; Mikkelsen et al., 2000), and has been considered the rate-limiting step of the pathway because overexpression of *cyp79B2* resulted in an increase in IG content (Mikkelsen et al., 2000; Zhao et al., 2002). The high-Trp (**4**) accumulating lines showed a more pronounced accumulation of I3M (**10**) when the leaves were treated with JA. It has been demonstrated that JA treatment induces the expression of genes involved in the synthesis of IGs including *cyp79B2* and *cyp79B3* (Brader et al., 2001). The pronounced increase in I3M (**10**) in JA-treated leaves of high-Trp (**4**) accumulating lines suggests that induced capacity to produce IGs in the JA-treated leaves is high enough for the already accumulated Trp (**4**) to be converted to IGs, resulting in the accumulation of

IGs in larger amounts in the transformants than in the wild type.

The first step in the biosynthesis of the camalexin (**5**) is the *N*-hydroxylation catalyzed by CYP79B2 and CYP79B3, which is common to the synthesis of IG (Glawischnig et al., 2004). The amount of camalexin (**5**) produced after the inoculation of *A. brassicicola* in all transgenic lines was similar to that in the wild-type plant, which was in contrast with the results obtained in the case of IGs. Recently, raphanusamic acid was identified in a root culture of *Arabidopsis*, and was proposed to be the precursor of the thiazole ring of camalexin (Bednarek et al., 2005). Substrate availability or the efficiency of enzyme activity in downstream reactions may be rate limiting in the biosynthesis of camalexin (**5**).

The high-Trp (**4**) accumulating lines W551 and W552 showed a decrease in the amount of phenylpropanoids and flavonoids. The simultaneous decrease in the amounts of phenylpropanoids and flavonoids indicates the suppression of the biosynthetic steps shared by these secondary metabolites, i.e., Phe (**8**) biosynthesis and the phenylpropanoid pathway. Given the funneling of metabolic flow into the Trp (**4**) branch in the transgenic lines, a reduction in the synthesis of Phe (**8**) is expected to occur and give rise to a decrease in the amounts of phenylpropanoids and flavonoids. However, the actual level of Phe (**8**) in transgenic plants that accumulated high concentrations of Trp (**4**) tended to be higher than or similar to that in the wild type. These findings suggest that the decrease in the amounts of Phe-derived secondary metabolites was not a simple consequence of the competition between the two [Trp (**4**) and Phe (**8**)] branches. Thus, biosynthetic steps downstream of Phe (**8**), i.e., the reactions in the phenylpropanoid pathway, are plausible sites for the down-regulation of accumulation of secondary metabolites, which may be a mechanism to retain the Phe (**8**) concentration in response to the accumulation of Trp (**4**).

The marked resemblance between the pattern of accumulation of Trp (**4**) and that of Phe (**8**) and Tyr (**9**) among 10 tested lines indicates that the increased accumulation of Phe (**8**) and Tyr (**9**) is related to Trp (**4**) accumulation. The smaller enhancement of Phe (**8**) and Tyr (**9**) accumulation in the roots compared to the leaves was consistent with the smaller increase in the Trp (**4**) content of roots in the transgenic lines. In *Arabidopsis*, plastidic isoforms of chorismate mutase that catalyzes the first committed step toward Phe (**8**) and Tyr (**9**) were shown to be sensitive to feedback activation by Trp (**4**) (Eberhard et al., 1996; Mobley et al., 1999). Therefore, the increase in Phe (**8**) and Tyr (**9**) in the lines that accumulate high levels of Trp (**4**) may be attributable to the feedback activation of CM by Trp (**4**). An increase in Trp (**4**) content accompanied by a slight increase in Phe (**8**) and Tyr (**9**) contents was also observed in potato plants expressing *OASA1D* (Matsuda et al., 2005), suggesting the generality of the accumulation of Phe (**8**) and Tyr (**9**) in response to the overproduction of Trp (**4**).

3.3. Concluding remarks

The Trp (**4**) content in the transgenic lines was correlated with the relaxed feedback sensitivity of the crude enzyme and the accumulation of *OASA1D* transcripts. The transgenic lines that strongly expressed *OASA1D* accumulated greater amounts of Trp (**4**) than the feedback resistant *trp5* mutants (Li and Last, 1996). Thus, both the feedback property of the enzyme encoded by the transgene and its expression level are the major factors that control the Trp (**4**) accumulation. In addition to *Arabidopsis*, rice (Tozawa et al., 2001) and potato (Yamada et al., 2004; Matsuda et al., 2005) plants expressing *OASA1D* have been reported to accumulate high levels of Trp (**4**), suggesting that the introduction of *OASA1D* is an effective strategy for the modification of Trp (**4**) level in a broad range of plant species.

In contrast to the marked increase of in the content of Trp (**4**), the effects of *OASA1D* expression on the secondary metabolism downstream of Trp (**4**) were limited, indicating strict regulation of the pathways. Thus, coupling of the introduction of *OASA1D* with other approaches including modification of specific reactions to a metabolite may be effective in the manipulating secondary metabolism. We also found that there is an increase in the amount of the other aromatic amino acids, Phe (**8**) and Tyr (**9**), and a decrease in the amount of phenylpropanoids and flavonoids in the high-Trp (**4**) accumulating lines. These uncharacterized cross-talks may contribute to amino acid homeostasis by controlling the synthesis of Phe (**8**) and Tyr (**9**), and the flux of Phe (**8**) to secondary metabolism.

4. Experimental

4.1. General

Positive ion-spray mass spectra were recorded on an API-165 (ion-spray voltage: 5000 V, orifice voltage: 30 V, nebulizer gas: N_2, curtain gas: N_2, Perkin–Elmer–Sciex, Foster, CA, USA). ^1H and ^{13}C NMR spectra were recorded on a Bruker AC300 spectrometer with TMS as an internal standard. Chromatography on an ODS column was performed with Cosmosil 75 C18-OPN (Nacalai Tesque, Kyoto, Japan). Preparative HPLC was performed on an ODS column (Wakosil II 5C18HG, 250×20 mm, Wako Pure Chemical Industries, Osaka, Japan) with a column temperature of 40 °C. A two-solvent system was used to generate the mobile phase for the preparative HPLC: solvent A, 0.1% TFA; solvent B, CH_3CN.

4.2. Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia was used as the wild type. *OASA1D*-overexpressing plants were generated with the plant transformation vector pBI101-Hm containing *OASA1D* cDNA under control of CaMV35S promoter

(Yamada et al., 2004). Transformation of *Arabidopsis* with the vector and selection of the transgenic plants were carried out according to Kawagishi-Kobayashi et al. (2005). The T3 plants harboring a homozygous T-DNA insertion were used for analysis. Plants were aseptically cultured. Seeds were surface sterilized with 1.5% (v/v) NaOCl and 0.02% Tween 20 with vigorous shaking, washed three times with sterile H_2O, and sown on Murashige and Skoog basal salt mixture (Sigma), supplemented with Gamborg B5 vitamins, 2.5% sucrose, and 0.2% gelrite (Sigma). The plates were kept in darkness at 4 °C for three days to synchronize germination, and grown in a growth chamber at 21 °C with light/dark cycles of 16 h/8 h. The age of plants indicated as "-days-old" in the text means number of days after imbibition.

4.3. RNA extraction and quantitative analysis of the transcript's accumulation

Total RNA was isolated by use of an RNAwiz solution (Ambion) following the manufacturer's instruction. Semi-quantitative RT-PCR experiments were performed to analyze the level of mRNA accumulation. One microgram of total RNA was treated with RNA-secure solution (Ambion) and digested with RNase-free DNase (Promega) for use as a template. First strand cDNA synthesis was performed using SuperScriptIII (Invitrogen) followed by a polymerase chain reaction with ExTaq (Takara). *ASA1* (5′-atgtcttcctctatgaacgtagcga-3′ and 5′-attcctcagtcatggtttcattgttg-3′), *ASA2* (5′-tatcaaatgctcatatactccgtcgtt-3′ and 5′-tagaggttagagattccaaccgattc-3′), *OASA1D* (5′-gataccaaggagcattatggaacagtg-3′ and 5′-aactttgtcttctgctttcgattttcc-3′), and *TUB2* (5′-ctcaagaggttctcagcagta-3′ and 5′-tcaccttcttcatccgcagt-3′) were amplified using the indicated primers according to the following cycling conditions: 95 °C for 4 min followed by 25 cycles (*OASA1D*), 30 cycles (*TUB2*) or 35 cycles (*ASA1* and *ASA2*) of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. Amplified *OASA1D* cDNA was separated on a 1.5% agarose gel by electrophoresis and transferred to a GeneScreenPlus membrane (DuPont), hybridized with radio-labeled probes. Blots were exposed to a phosphorimaging screen and quantified using MacBAS ver. 2.5 software (Fuji Photo and Kohshin Graphic Systems, Inc.). The photostimulated luminescence (PSL) density of the signal was measured using the Quant Mode in the MacBAS software and was corrected by subtracting the image background. The accumulation of the *OASA1D* transcript was shown relative to that of the *TUB2* transcript.

4.4. Chemicals

Anthranilic acid, chorismic acid barium salt, and jasmonic acid were obtained from Wako. Camalexin (**5**) was synthesized from indole (Wako) and 2-bromothiazole (Sigma), by a Grignard reaction according to the method described by Ayer et al. (1992).

IGs were purified from broccoli according to the method of Visentin et al. (1992) with modifications to generate standard curves on HPLC. Flower buds (600 g) of broccoli were extracted with MeOH–H_2O [7:3 (v/v), 1.5 l × 2] at 70 °C for 30 min. The extract was washed with hexane, and evaporated to a small volume. The concentrated extract was applied to an ODS column equilibrated with MeOH–H_2O [5:95 (v/v)], and the column was eluted with the same solvent system. The flow-through fraction and MeOH–H_2O (5:95) fraction were combined. The mixture was evaporated and applied to a DEAE-Sepharose column (Amersham Biosciences, Picataway, NJ, USA) equilibrated with 50 mM imidazole–HCO_2H buffer (pH 4.15). The column was washed with HCO_2H–*iso*-PrOH–H_2O [3:2:5 (v/v)] and eluted with 0.5 M K_2SO_4 containing 5% *iso*-PrOH. The 0.5 M K_2SO_4 fraction was concentrated, and the precipitated $K_2SO_4$was extracted with MeOH. The MeOH solution was subjected to preparative HPLC [linear gradient: 0–40 min, 5–40% B/(A + B); flow rate: 7 ml/min: detection: 280 nm]. The peaks corresponding to IGs were collected, neutralized with solid $NaHCO_3$, and concentrated to a small volume. The concentrated fractions were applied onto a Sephadex G-10 column equilibrated with dist. H_2O. The fractions containing IGs were collected and evaporated (**10**, 60.3 mg; **11**, 6.9 mg; and **12**, 28.9 mg). Their identities were confirmed by using positive ion-spray mass spectra and by comparison of UV and ^1H NMR spectra with those reported previously (Agerbirk et al., 1998) except for **11** for which no ^1H NMR spectrum had been reported.

4MI3M (**11**): ^1H NMR (500 MHz, CD_3OD): δ 7.07 (1H, *s*, H-2″), 6.99 (1H, *t*, $J = 7.8$ Hz, H-6″), 6.94 (1H, *d*, $J = 7.8$ Hz, H-7″), 6.48 (1H, *d*, $J = 7.8$ Hz, H-5″), 4.72 (1H, d, $J = 9.9$ Hz, H-1′), 4.60 (1H, *d*, $J = 16.4$ Hz, H-2), 4.18 (1H, *dd*, $J = 16.4$, 1.2 Hz, H-2), 3.92 (3H, *s*, MeO), 3.67 (1H, *dd*, $J = 12.2$, 2.3 Hz, H-6′), 3.61 (1H, *dd*, $J = 12.2$, 4.2 Hz, H-6′), 3.31 (1H, *t-like*, $J = 9.2$ Hz, H-2′), 3.13 (1H, *t-like*, $J = 9.3$ Hz, H-4′), 3.01 (1H, *t-like*, $J = 8.9$ Hz, H-3′), 2.95 (1H, *ddd*, $J = 9.3$, 4.2, 2.3 Hz, H-5′). ^{13}C NMR (125 MHz, CD_3OD): δ 162. 5 (C-1), 155.8 (C-3″a), 139.7 (C-7″), 123.4 (C-5″), 123.2 (C-2″), 111.1 (C-3″), 106.1 (C-6″), 100.2 (C-4″), 83.8 (C-1′), 81.6 (C-5′), 79.4 (C-3′), 74.0 (C-2′), 70.5 (C-5′), 62.2 (C-6′), 55.6 (MeO), 31.6 (C-2).

Positive ion-spray MS. **10**, *m/z* (int.) 449 (5 $[M+H]^+$), 369 (69 $[M-SO_3+H]^+$), 207 (100 $[M-SO_3-162+H]^+$); **11**, *m/z* (int.) 479 (17 $[M+H]^+$), 399 (39 $[M-SO_3+H]^+$), 237 (100 $[M-SO_3-162+H]^+$); **12**, *m/z* (int.) 479 (6 $[M+H]^+$), 399 (42 $[M-SO_3+H]^+$), 237 (100 $[M-SO_3-162+H]^+$).

1-Sinapoylglucose (**13**), sinapoyl malate (**18**), and kaempferol glycosides (**14**–**16**) were purified from the rosette leaves of 2-wk-old seedlings of *Arabidopsis*. The leaves (140 g) were extracted MeOH (0.7 l × 2). After adding dist. H_2O (175 ml) to the extract, the mixture was passed through an ODS column equilibrated with MeOH–H_2O [4:1 (v/v)] with the column eluted with the

same solvent system. The flow-through fraction and MeOH–H_2O (4:1) fraction were combined and evaporated. The solution was applied to an ODS column equilibrated with MeOH–H_2O [5:95 (v/v)] and the column was eluted with MeOH–H_2O [1:4 (v/v)] and MeOH–H_2O [1:1 (v/v)]. The MeOH–H_2O (1:1) fraction was subjected to preparative HPLC [linear gradient: 0–30 min, 5–40% B/(A + B); flow rate: 6 ml/min: detection: 280 nm] to yield **13** (3.3 mg), **18** (6.6 mg), **14** (9.8 mg), **15** (2.6 mg), and **16** (10.6 mg). The identities of the compounds were confirmed by using positive ion-spray mass spectra and by comparison of UV and ^{1}H NMR spectra with those in previous reports (Mulinacci et al., 1995; Veit and Pauli, 1999; Linscheid et al., 1980).

Positive ion-spray MS. **13**, *m/z* (int.) 404 (13 $[M+NH_4]^+$), 387 (6 $[M+H]^+$), 225 (76 $[M+H-162]^+$), 207 (100 $[M+H-180]^+$); **18**, *m/z* (int.) 358 (5 $[M+NH_4]^+$), 341 (11 $[M+H]^+$), 207 (100 $[M-133]^+$); **14**, *m/z* (int.) 741 (74 $[M+H]^+$), 595 (43 $[M-146+H]^+$), 433 (100 $[M-146-162+H]^+$), 287 (72 $[M-2\times146-162+H]^+$); **15**, *m/z* (int.) 595 (96 $[M+H]^+$), 433 (82 $[M-162+H]^+$), 287 (100 $[M-146-162+H]^+$); **16**, *m/z* (int.) 579 (63 $[M+H]^+$), 433 (100 $[M-146+H]^+$), 287 (94 $[M-2\times146+H]^+$).

Similarly, coniferin (**19**) (26.7 mg), syringin (**20**) (10.6 mg), and quercetin 3-*O*-glucoside 7-*O*-rhamnoside (**17**) (9.5 mg) were purified from *Arabidopsis* roots (60 g) of 2-wk-old seedlings by fractionation on a ODS column and preparative HPLC under the same conditions. Their identities were confirmed by using positive ion-spray MS and comparison of UV and ^{1}H NMR spectra with those in previously published works (Hemm et al., 2004; Iwashina et al., 1995; Mulinacci et al., 1995).

Positive ion-spray MS. **19**, *m/z* (int.) 365 (100 $[M+Na]^+$), 360 (57 $[M+NH_4]^+$), 343 (5 $[M+H]^+$), 163 (98 $[M-180+H]^+$); **20**, *m/z* (int.) 395 (100 $[M+Na]^+$), 390 (29 $[M+NH_4]^+$), 373 (5 $[M+H]^+$), 193 (92 $[M-180+H]^+$); **17**, *m/z* (int.): 611 (71 $[M+H]^+$), 449 (57 $[M-162+H]^+$), 303 (100 $[M-162\text{-}146+H]^+$).

4.5. AS activity

All procedures were carried out at 4 °C. Plant materials were frozen in liquid N_2 and ground until they were well powdered. The powder was extracted with 5 vol. of 100 mM Tris–HCl buffer (pH 7.5) containing 1 mM EDTA, 2 mM 2-mercaptoethanol, 20 mM glutamine, 4 mM $MgCl_2$ and 10% glycerol, and centrifuged for 10 min at 12,000*g*. The supernatant was passed over a PD-10 column (Amersham Biosciences) equilibrated with the same buffer. The fraction containing proteins was used for the enzyme assay. Protein concentration was determined by the method of Bradford by using BSA as the protein standard.

The AS assay was carried out according to the method of Bücker et al. (1995) with slight modifications. A reaction mixture containing 95 μl of 4 mM chorismate (**2**), 100 μl of crude extract, and 5 μl of dist. H_2O was incubated at 30 °C for 60 min. In the experiments to investigate feedback inhibition, a solution of 0.2–4 mM L-Trp, D-Trp, and 5MT was added to the mixture instead of dist. H_2O. The reaction was terminated by inactivating the enzyme in boiling H_2O. The precipitated proteins were removed by centrifugation for 10 min at 12,000*g*, and the resulting supernatant was subjected to a reversed-phase HPLC analysis (column: HiQsil C18V, 150 mm, 4.6 mm i.d., KYA Technology, Yokohama, Japan). Anthranilate (**3**) was eluted with 15% MeOH in H_2O containing 0.1% H_3PO_4 at a flow rate of 0.8 ml/min, and was detected with a fluorescence detector (Shimadzu RF-10AXL; Ex = 340 nm, Em = 400 nm). In this system, anthranilate (**3**) was eluted at a retention time of 15.2 min.

4.6. Analysis of soluble secondary metabolites

Arabidopsis leaves and roots were extracted with 5 and 10 vol. of MeOH, respectively. The extract was analyzed by HPLC. The conditions for HPLC were as follows: column, Mightysil RP-18 GP, 150 × 4.6 mm; solvent, A = 0.1% TFA, B = MeCN; gradient, 5–40% B/(A + B) within 30 min; flow rate; 0.8 ml/min; detection, 280 nm; column temperature, 40 °C.

4.7. Analysis of IGs and camalexin (*5*)

For the analysis of IGs, rosette leaves of 3-wk-old seedlings were excised and floated in a solution of 500 μM jasmonate. After incubation for 72 h at 22 °C, the leaves were extracted with MeOH. The extract was subjected to a reversed-phase HPLC analysis under the conditions described above. For the analysis of camalexin (**5**), rosette leaves of 2-wk-old seedlings were inoculated with *A. brassicicola* (NBRC No. 31226, NITE Biological Resource Center, Department of Biotechnology, National Institute of Technology and Evaluation, Kisarazu, Japan). Droplets (5 μl) of a suspension comprising 5×10^5 conidial spores/ml were placed on the leaves of seedlings grown under sterile conditions. Four leaves of each plant were inoculated. After a 72-h incubation period, the aerial parts of the inoculated plants were collected and extracted with 10 vol. of MeOH. The camalexin (**5**) content was determined by HPLC under the conditions described above.

4.8. Analysis of Phe (*8*) and Tyr (*9*) contents

Two-week-old seedlings were extracted with 10 vol. of MeOH–H_2O [4:1 (v/v)] for 12 h, with the extract was subjected to LC/MS/MS analysis. Separation by HPLC was performed with an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a Mightysil RP-18 GP column (150 × 2.0 mm, 3 μm particle size, Kanto Chemical, Tokyo, Japan) at a flow rate of 200 μl/min. A linear gradient [0.1% HOAc (A) and MeOH (B), 5–30% B/(A + B) for 10 min] was applied.

MS detection was performed on an API-3000 triple stage quadrupole mass spectrometer equipped with a TurboIonSpray ionization source (Applied Biosystems, Foster City, CA, USA). Concentrations of Phe (**8**) and Tyr (**9**) were determined by multiple reaction monitoring (MRM). The monitored mass transitions were *m*/*z* 166 to *m*/*z* 120 for Phe (**8**) and *m*/*z* 182 to *m*/*z* 136 for Tyr (**9**). The conditions for MS were optimized for MRM using authentic Phe (**8**) and Tyr (**9**) (Wako). The optimized conditions included nebulizer gas flow (NEB), curtain gas flow (CUR), ionspray voltage (IS), TurboIonSpray temperature (TEM), collision gas pressure (CAD), declustering potential (DP), focusing potential (FP), entrance potential (EP), collision energy (CE), and cell exit potential (CEP).

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