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Original Paper

Metabolic profiling analysis of genetically modified rice seedlings that overproduce tryptophan reveals the occurrence of its inter-tissue translocation

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Abstract A metabolic profiling approach using high performance liquid chromatography-mass spectrometry (LC-MS) was applied to seedlings of transgenic rice plants (*Oryza sativa*) that over-express the *OASA1D* gene encoding a feedback-insensitive α-subunit of anthranilate synthase (AS, EC 4.1.3.27). Analysis revealed that the seedlings accumulated tryptophan (Trp) at a high concentration without marked effects on the amounts of other major metabolites. Some minor indole metabolites showed a certain degree of increase in the amounts in Trp-accumulating tissues, while no active catabolic conversion of Trp was indicated. Analysis also revealed that the distribution of Trp in the plant was uneven, with the highest level being observed in young developing tissues, despite tissue-independent expression of the *OASA1D* gene under the control of ubiquitin promoter. Differences in AS activity and anthranilate content among organs were small. A feeding experiment with radiolabeled Trp clearly demonstrated one-way Trp movement from old to young leaves; thus, the uneven distribution of Trp in *OASA1D* plants is the manifestation of Trp translocation. The negligible effects of Trp accumulation in other metabolic pathways, low metabolic activity of Trp, and efficient translocation of Trp in rice plants expressing *OASA1D* transgene are favorable characters from the aspect of metabolic engineering of Trp production.

Key words: Amino acid translocation, anthranilate synthase, indole alkaroid, tryptophan, metabolic engineering, metabolic profiling.

Tryptophan (Trp), an essential amino acid for mammals, has been one of the important targets of plant metabolic engineering. One of the committed steps for Trp synthesis is the conversion of chorismate to anthranilate, which is catalyzed by anthranilate synthase (AS) (Niyogi et al. 1992; Niyogi et al. 1993; Poulsen et al. 1993). This enzyme regulates biosynthetic flux for Trp by feedback inhibition from Trp (Bohlmann et al. 1996; Kreps et al. 1996; Bohlmann, 1996; Li et al. 1996; Song et al. 1998). Thus, the Trp content in plants is controlled at a low level, which somehow reduces the nutritional value of many crops as food or feed. Accordingly, the elevation of Trp levels has long been one of the important goals in plant breeding, and recent molecular-based approaches have demonstrated that transgenic plants over-expressing the gene of feedback-insensitive AS α (ASA) subunit show a significant increase in free Trp contents (Cho et al. 2000; Cho et al. 2004; Inaba et al. 2007; Tsai et al. 2004; Zhang et al. 2001). In particular, the effectiveness of a modified rice ASA gene (*OASA1D*), in which 323rd aspartate was substituted with asparagine, was outstanding not only in calli, vegetative tissues, and seeds of rice, (Tozawa et al. 2001; Wakasa et al. 2006), but also in plants of different species such as Arabidopsis (Ishihara et al. 2006), potato (Matsuda et al. 2005; Yamada et al. 2004), azuki bean (Hanafy et al. 2006) and soybean (Ishimoto et al. 2010) raising hopes for its practical use to generate crop plants with elevated Trp contents.

As a step toward practical application, metabolic analysis is required to evaluate the effects of modification of Trp biosynthesis pathway on other

Abbreviations: AS, anthranilate synthase; ICA, independent component analysis; LC-ESI-Q-MS, liquid chromatography-quadruple mass spectrometry equipped with electrospray ionization probe; NB, Nipponbare; Trp, tryptophan

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metabolic pathways. Techniques of comprehensive analysis of phytochemical components, or metabolic profiling have been developed to date for this purpose, and are applied for the safety assessment of genetically modified (GM) crops, in combination with other toxicity tests (Baker et al. 2006; Beckmann et al. 2007; Catchpole et al. 2005; Dixon et al. 2006; Domingo 2007; Goodman et al. 2008; Kuiper et al. 2001; Kuiper et al. 2003; Shepherd et al. 2006). In the case of *OASA1D* plants, profiling analyses of transformed rice calli, potato tubers and Arabidopsis tissues have been performed using liquid chromatography connected with a photodiode array detector (LC-PDA) to demonstrate that the marked accumulation of Trp only had a limited effect on the profiles of other UV-active metabolites except for the accumulation of a few minor indole alkaloids in these tissues (Ishihara et al. 2006; Matsuda et al. 2005; Morino et al. 2005). Microarray and targeted metabolite analyses of young rice seedlings also showed that the effect of the transgene was very limited (Dubouzet et al. 2007). Thus, the activity of tryptophan-utilizing pathways was considered to be generally low in these tissues, although these results were somewhat surprising, taking into account that Trp bridges primary and secondary metabolism, and a number of biologically active substances of Trp origin have been described in many plants (Salmoun et al. 2002; van Der Heijden et al. 2004).

The previous studies, however, are yet inadequate in that they have only covered the effects on UV-active metabolites, and those on other UV-inactive as well as minor metabolites still need to be considered. In addition, the activity of converting Trp into secondary metabolites can depend on the age and the tissues of developed mature plants, as has been observed for the production of alkaloids (Grubb et al. 2006; Suttipanta et al. 2007). Furthermore, the accumulation of the target compounds can be influenced by the tissue-specific degradation capability as well as by translocation among tissues.

Taken these into consideration, the metabolic phenotype of *OASA1D*-transformed rice plants was intensively examined in this study for full assessment of the effects of manipulating Trp biosynthetic activity at the whole plant level. Non-targeted metabolic profiling analyses using liquid chromatography-mass spectrometry (LC-MS) were employed to detect a wider range of metabolites (Bottcher et al. 2008; Dunn 2008; Fiehn 2002; Huhman et al. 2002; Sumner et al. 2003; Villas-Boas et al. 2005). The results indicate that accumulated Trp is not actively utilized or metabolized even in aged plants, while Trp itself is unevenly distributed in the transformant. The occurrence of inter-tissue Trp translocation will be proposed as a possible mechanism causing the uneven distribution of Trp, based on the results of AS activity assay, quantification of anthranilate, and tracer experiments of exogenous radio-labeled Trp.

Materials and methods

Plant material

HW1 and HW5 lines were established from the original Agrobacterium-mediated transformation of rice calli (*Oryza sativa L.* cv. Nipponbare) with *OASA1D* (Wakasa et al. 2006). Two lines, HW1 and HW5, were grown in a non-containment greenhouse (T5). Seeds of T5 were then grown in an isolated field (T6). Seeds of both HW1 T5 generation and HW5 T6 generation were used in this study. After soaking in 5% sodium hypochlorite solution in water for 30 min, the seeds of transformed and untransformed rice were incubated at 28°C with a 16-h photoperiod per day. Rice seedlings were then grown in a pot containing Bonsol soil® (Sumitomo Chemical, Osaka) in a growth chamber under the same conditions for four weeks.

Quantification of Trp and anthranilate

Tissue samples were cut into small pieces and extracted with ten volumes (w/v) of 2% acetic acid in water at 100°C for 10 min. The extracts were centrifuged at 15,000 g for 10 min, and filtered through a Cosmonice Filter S (pore size 0.5 μm, filter diameter 13 mm, Nacalai Tesque, Kyoto). The resulting filtrates were subjected to analyses. For the quantification of Trp, the filtrate (10 μl) was analyzed with an HPLC system (Hitachi L7000 series) coupled with a UV detector (Hitachi L7400). Chromatography was performed with a COSMOSIL 5C18AR-II column (150×4.6 mm; particle size, five μm, Nacalai Tesque) and a solvent mixture of methanol and 0.1% phosphoric acid in water at a flow rate of 0.8 ml min^{-1} at 35°C. The ratio of methanol to 0.1% phosphate in water was programmed as: 10:90, v/v, for 5 min, and then from 10:90 to 70:30 over 30 min. The detection wavelength was set at UV280 nm. The levels of anthranilic acid were determined by a previously reported method (Dubouzet et al. 2007).

Non-targeted metabolic profiling analysis using LC-MS

Tissue samples were cut into small pieces and extracted with 40 volumes (w/v) of extraction solvent (methanol : water (50 : 50, v/v) containing 0.2% acetic acid) at room temperature for one hour. Extracts (500 μl) were added to 2.0 ml dilution solvent (methanol : water (90 : 10, v/v) containing 0.2% acetic acid) and applied to a Sep-Pak Plus C18 Cartridge (Waters, Milford, USA) equilibrated with dilution solvent. The cartridge was washed with 2.0 ml dilution solvent and all solvent in the cartridge was eluted by aeration. The eluate was evaporated under a vacuum and the residue was dissolved in 200 μl of 20% methanol in water. Following centrifugation at 15,000 g for 10 min, supernatants were subjected to LC-ESI-Q-MS analyses. Sample extracts (5 μl) were analyzed using a LC-MS system equipped with an electrospray ionization (ESI) interface (HPLC: Shimadzu LC-10VP system, MS; Shimadzu LCMS-2010). The analytical conditions were as follows: HPLC

column: Cadenza CD-C18, Imtact Co., Kyoto, Japan, 2.0×75 mm; solvent system: acetonitrile (0.1% formic acid) : water (0.1% formic acid), gradient program: 5:95, v/v, at 0 min; 60:40 at 11 min; 98:2 at 12 min; 98:2 at 13 min; 5:95 at 13.1 min; 5:95 at 20 min, flow rate: 0.25 ml min^{-1}, temperature: 35°C, MS detection; CDL temperature: 250°C, block heater temperature: 200°C, probe voltage: +4.5 kV, Q-array voltage: scanning mode, nebulizing gas flow: 1.5 l min^{-1}, detection mode: scan mode (*m*/*z* 100–700), scan time: 2 sec. The scans were repeated for 15 min (750 times) in a single run. Data were recorded with the aid of LCMS-Solution version 2.0 software (Shimadzu, Kyoto).

Data processing

Raw data files of LC-MS apparatus are converted to a peak table using COWtool (Nielsen et al. 1998) and self-made Perl scripts. The data of ions generated from Trp other than protonated molecule (*m*/*z* 205 $[M+H]^+$), e.g. sodium adduct, isotope and fragment ions, were excluded from the peak intensity table before statistical analyses. Blank cells in the table were filled with a value of '1000' as the ion intensity of noise level. Student's *t*-test and calculation of the correlation coefficient for data analysis were performed using Microsoft Excel 2000. Independent component analysis (ICA) was performed with a web-based tool, MetaGeneAlyse (http://metagenealyse.mpimp-golm.mpg.de/) (Scholz et al. 2004). All peak intensity data in the peak table were logarithmically transformed for ICA. Hierarchical clustering analysis was performed using TMeV version 4.0 (Saeed et al. 2006).

Assay of AS activity

The activity of AS in rice plant tissues was assayed by a previously described method (Matsuda et al. 2005; Tozawa et al. 2001).

Tracer labeling experiment

Dehulled rice seeds (NB and HW1) were incubated on MS medium for seven days at 28°C with a 16-h photoperiod per day. The seedlings were moved to Kimura's B solution medium and incubated for three days under the same conditions. The back of the third expanded leaf (leaf III) was scratched with Carborundum and dipped for one day in 10 mL Trp aqueous solution (1.0 mM) containing [3-^{14}C]Trp (1.85×10^4 Bq, American Radiolabeled Chemicals Inc., St. Louis, MO). At two, six, and thirteen days after treatment, Trp in leaves II, IV, and V was separately extracted with 20 volumes (w/v) of 80% methanol in water and the levels of radioactivity were determined using a liquid scintillation counter. Trp fractions were recovered from crude extracts using HPLC and radioactivity was also determined.

Results

Time-dependent and tissue-specific accumulation of Trp in OASA1D*-transformed rice plants*

To analyze the distribution of Trp in a plant, rice seedlings were separated into three parts, respectively referred to as root, leaf sheath, and leaves. Leaves were numbered I to V in the order of expansion. Leaf sheath represented the rest of seedlings after root and leaf blades were removed, in which stem, developing small leaves and shoot apical meristem were included. The levels of Trp in each part of transformed (HW1 and HW5) and untransformed (NB) rice plants are shown in Figure 1.

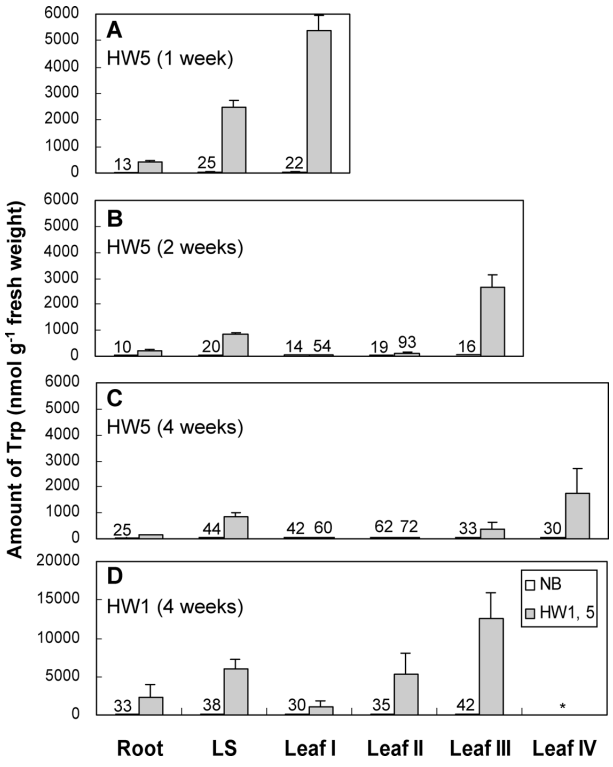

Figure 1. Tryptophan contents in leaves, leaf sheath (LS), and roots of untransformed rice (Nipponbare, NB) and of rice transformants expressing the *OASA1D* transgene (HW1, HW5) at one (A), two (B) and four (C, D) weeks after germination. Data are the means ±SD of values from three plants. The asterisk in (D) means that leaf IV was not developed in this experiment.

Over-expression of the *OASA1D* transgene caused marked accumulation of Trp in HW5 rice plants, as has been observed (Tozawa et al. 2001; Wakasa et al. 2006); however, while Trp was evenly distributed in the whole plant of NB, levels of Trp in transformants depended on the tissues as well as the time after germination. The highest level was found in leaf I of HW5, reaching 5,300 nmol g^{-1} fresh weight one week after germination. The leaf sheath and root contained about 2,800 and 380 nmol g^{-1} fresh weight of Trp, respectively. When the second and third leaf blades (leaf II and III) were expanded in the HW5 plant two weeks after germination, the highest amount of Trp (about 2,700 nmol g^{-1} fresh weight) was accumulated in the youngest leaf (leaf III). The levels of Trp in leaves I and II decreased to the level of NB. Four weeks after germination, the highest level of Trp (about 1,800 nmol g^{-1} fresh weight) was again found in the youngest leaf (leaf IV), when the amount in leaf III

decreased to 450 nmol g^{-1} fresh weight. A similar distribution pattern of Trp was also observed in the HW1 line four weeks after germination, although the accumulated amount was much higher than in HW5. The level of Trp reached 14,800 nmol g^{-1} fresh weight in the youngest leaf (leaf III). The growth of shoots and roots of both HW1 and HW5 was not significantly different from that of untransformed plants with respect to their length and weight (data not shown).

Effects of OASA1D *expression on the metabolic profile*

Given the time-dependent and tissue-specific change in the levels of Trp in the transformed rice plants, their metabolic profiles were very likely changed depending on time and tissues. To study this, non-targeted metabolic profiling analysis using LC-MS (Bottcher et al. 2007; De Vos et al. 2007) was conducted for the rice seedlings of HW5 and Nipponbare at one or two weeks after germination. A total of 1673 peaks were detected in the extracts prepared from the leaf, leaf sheath, and root tissues, and they were serially numbered from #1 to #1673 to formulate a peak intensity table consisting of data from 80 samples. Intensity data are graphically shown as a heat map in Supplemental Figure 1 (see online version).

When the peaks whose intensities were increased by more than three-fold in the transformants with the significance of $\alpha<0.05$ by Student's *t*-test were deemed as those significantly affected by the expression of *OASA1D*, the intensities of 46 peaks were judged to be increased in leaf I of the one-week-old rice plant (W1L1). By contrast, the corresponding number for the root tissues (W1R) was only three, as shown in the Venn diagram (Figure 2A). There was no specific peak whose intensity was increased only in roots. The change in the metabolic profile of the leaf sheath (W1LS) was fairly similar to that of leaf tissue (W1L1), where intensities of about 50% of peaks (22 peaks) were commonly increased in leaves and leaf sheath. These results indicated that major effects of *OASA1D* expression on the metabolic profile were exerted in the aerial part of the rice plant. No peaks with significantly decreased intensity were found.

The number of peaks showing that increased intensity in leaf I was markedly decreased from 46 to eight in a week (Figure 2B), accompanied with a marked decrease in the level of Trp. On the other hand, the number in leaf III of the two-week-old plant, in which the highest level of Trp was accumulated, was comparable to that in leaf I of the one-week-old plant (29 and 46 peaks, respectively). Thus, the effect of *OASA1D* expression on the metabolic profiles in rice plant tissues was closely related to the magnitude of Trp accumulation, and the disappearance of Trp in old leaves was unlikely to be due to its conversion to other components.

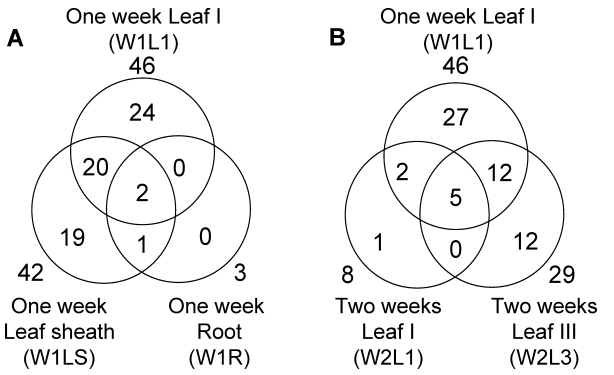

Figure 2. Venn diagrams of peaks with significantly increased intensities by *OASA1D* over-expression in each plant tissues at one week after germination (A) and in leaves at one and two weeks after germination (B).

Characterization of peaks whose intensities were increased by OASA1D *expression*

Metabolic profile data obtained by LC-ESI-Q-MS were analyzed in more detail using three different statistical methods, i.e. (1) independent component analysis (ICA) (Scholz et al. 2004), (2) correlation analysis (Morgenthal et al. 2006; Roessner et al. 2001) and (3) Student's *t*-test, in order to focus the peaks of particular significance among those whose intensities were increased by transgene expression.

ICA is an improved method of principal component analysis (PCA) (Scholz et al. 2004) that is often used to extract the factors characterizing the difference of metabolic profiles between transgenic plants and untransformed plants (Baker et al. 2006; Catchpole et al. 2005). The factors, which are referred to as principal components, are derived by linear combinations of original variables (i.e. the amounts of metabolites) in order to account for the variance in the dataset. The coefficient of each term (i.e. loading factors) in the extracted principal components provides information about the contribution of each metabolite to the characterization of the metabolic profile. In this study, peak intensity value of all the detected metabolites in one-week-old rice plants were logarithmically transformed to be analyzed by ICA. The plots representing the profiles of root, leaf sheath, and leaf I of NB, and those of HW5 were successfully separated to form six clusters in a space defined by the axes of two components (Figure 3). Of these two axes, the clusters of NB and HW5 appeared to be well separated in the direction of the horizontal one (factor 1 in Figure 3), and therefore, this component was considered to explain the effect of *OASA1D* expression on the metabolic profiles. Thus, 18 peaks with high loading factors (higher than 0.067) in formulating this component were selected as the metabolites that importantly characterize the profile

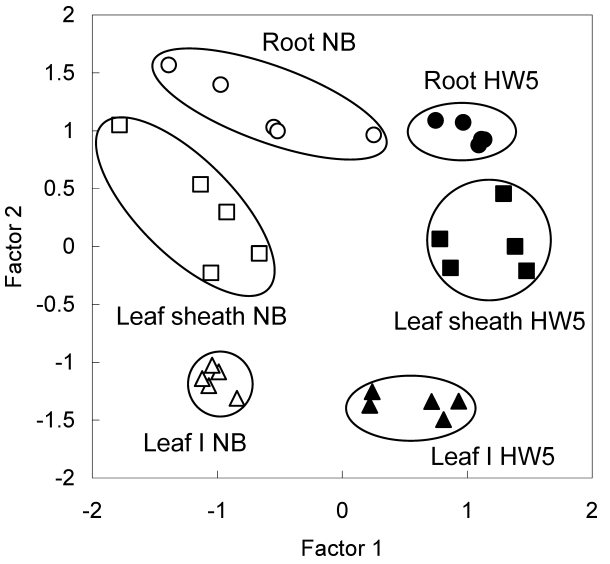

Figure 3. Independent component analysis (ICA) of non-targeted metabolic profile of rice plant tissues. Square, triangle, and circle symbols represent samples obtained from leaf I, leaf sheath and root tissues of NB (open symbol) and HW5 (closed symbol) rice plants, respectively.

difference between NB and HW5 (Table 1).

Secondly, Pearson's correlation coefficients were calculated between the intensities of respective peaks and the intensity of Trp in all samples. The correlation coefficients ranged from -0.35 to 0.96, and 16 peaks with large coefficients ($r>0.7$) were chosen as metabolite peaks whose levels were closely associated with that of Trp (Table 1). Thirdly, the significance of the difference of peak intensities was tested using Student's *t*-test by setting $\alpha=0.01$ for peaks whose mean intensities were increased or decreased more than ten-fold by *OASA1D* expression. The number of peaks selected by at least one of these three analyses amounted to 27, including Trp (Table 1). The intensities of the 26 peaks other than that of Trp were weak compared to the markedly accumulated level of Trp (peak #220).

The chemical components of these 26 peaks were structurally characterized by LC-MS/MS, and eight (#198, 243, 321, 366, 501, 1025, 1098 and 1144) were assigned to five metabolites (**1**–**5**) depicted in Figure 4. The structures of metabolites **1**–**3** were deduced by MS/MS, and confirmed to be indole-3-propionic acid, 1,2,3,4-tetrahydro-β-carbolin-3-carboxylic acid and γ-glutamyltryptophan, respectively, by comparing with the authentic samples. Metabolite **4** (#1025 and 1098) was identical with the compound in peak #46 of the extract from rice calli overexpressing the *OASA1D* transgene, the structure of which was presumably determined as 6-(1,2-dihydroxyethyl)-3a,6a-dihydroxy-3-(1*H*-indol-3-yl)-2-(β-D-glucopyranosyloxymethyl)tetrahydrofuro[3,4-*b*]furan-4-one on the basis of spectroscopic analyses, including MS, ^{1}H- and ^{13}C-NMR (Morino et al. 2005). Metabolite **5** was also found in *OASA1D*-overexpressing rice calli. Molecular formula of **5** was deduced as $C_{28}H_{33}N_3O_9$ by MS and NMR spectroscopic analyses, following the chromatographic isolation from the extract of the calli. UV and NMR spectra indicated the presence of a hexose, two indole moieties, and a 2,3-dihydroxypropyl group. Further intensive analyses after the chemical derivatization showed that one of the indole moieties constituted a tryptophan substructure in the molecule, and the deduced partial structures were assembled based on the HMBC and NOESY experiments to conclude the structure of **5** as 2-[2-hydroxy-3-β-D-glucopyranosyloxy-1-(1*H*-indol-3-yl)propyl] tryptophan. The detailed process of structure analysis is described in the Appendix (see online version). These metabolites commonly contain indole rings in their structures, which is relevant to the activation of AS-catalyzed reaction in the *OASA1D* transformant. The accumulation of these metabolites could not be detected in the previous analysis of rice seedlings using LC-PDA (Dubouzet et al. 2007 in online version). MS/MS analysis (see Appendix in online version) suggested that the components of the other five peaks (#252, 253, 254 1218 and 1602) were also likely to be indole-containing metabolites, but no structural information about other 13 peaks was obtained.

AS activity and anthranilate levels

In order to examine whether the distribution of Trp observed in *OASA1D* transformants is dependent on the activity of AS in tissues, AS activity in each part of four-week-old NB and HW1 plants was determined (Table 2). In NB, AS activities in the leaf sheath and root were about 0.7 and 2.2 pkat mg^{-1} protein, respectively, while that in leaves I, II, and III was about 0.2 to 0.5 pkat mg^{-1} protein. The relatively high level of activity in the root is probably because of the much lower concentration of protein in the root compared to other parts. The expression of *OASA1D* affected AS activity in each part in a different manner. AS activity in leaf I, II, and III of HW1 rice was about half of that of NB, whereas activity in the leaf sheath was not changed. In the root, activity increased to 3.9 pkat mg^{-1} protein, which was about 1.8 times as much as that of NB.

Figure 5 shows the relationship between the concentration of Trp in the reaction mixture and the relative activity of AS. The activities of AS in any part of NB rice were reduced to 10 to 30% of the control in the presence of Trp at 10 to 20 μM, and completely inhibited by 50 μM Trp. On the other hand, the activity of AS in HW1 leaves retained 40% of the activity in the control even in the presence of 50 μM of Trp. The inhibition profile by Trp was similar in leaves I, II, and III, indicating that the *OASA1D* gene was expressed and translated in these leaves to the same extent. AS in the HW1 root appeared to have lower sensitivity than in

Table 1. Metabolites whose levels were significantly increased by *OASA1D* expression in a transformed rice plant (HW5) compared to an untransformed plant (NB) one week after germination

Peak number #	Mass number *m/z*	Retention time min	Identity[a]	Correlation coefficient value with Trp (>0.7)	Factor loadings of independent component 1[b] (>0.067)	Peak intensity ratio (>10) and *P* values of Student's *t*-test (<0.01)						Peak intensity (cps)					
						Leaf I		Leaf sheath		Root		Leaf I		Leaf sheath		Root	
						HW5/NB	*P*	HW5/NB	*P*	HW5/NB	*P*	NB	HW5	NB	HW5	NB	HW5
23	112	16.37	n.d.		0.079							1000	2002	1286	2258	1271	2857
61	128	12.67	n.d.					12.6	0.001			3420	8636	1000	12627	10192	5039
100	141	0.73	n.d.		0.071							4946	10317	1904	9434	9551	10554
198	190	6.03	indole-3- propionic acid, **1**		0.069							1706	3160	1583	9244	1000	1709
220	205	4.73	tryptophan	1.00	0.092	162.9	<0.001	67.5	<0.001	19.4	<0.001	6365	1036770	10733	724074	7927	153913
243	217	5.60	TCCA, **2**	0.96		39.1	<0.001	22.9	0.003			2810	109841	3579	81787	5698	13759
252	221	1.30	hydroxy-tryptophan?	0.76	0.080	11.5	<0.001					1750	20075	1086	5776	1217	1697
253	221	1.93	hydroxy-tryptophan?	0.77		18.9	<0.001					1000	18885	1401	4465	1473	1159
254	221	3.20	hydroxy-tryptophan?	0.81	0.067	27.2	0.002					1000	27207	1247	9335	1064	1593
295	243	4.40	n.d.	0.80								1225	4307	1185	2680	1089	1279
321	258	5.27	acetonitirile adduct of **2**	0.92		28.1	0.003	15.0	0.002			1860	52298	2360	35457	2828	5485
366	334	5.90	γ-glutamyl-tryptophan, **3**	0.96	0.071			10.8	0.000			1534	15069	937	10118	915	1764
496	392	4.77	n.d.	0.78	0.093			11.5	0.001			1872	7299	1917	22080	1000	4004
501	394	7.00	fragment of **5**					13.7	0.006			1622	6682	1116	15268	1882	1226
540	406	6.27	n.d.					17.2	0.002			6674	6584	1435	24616	1447	5629
546	408	4.63	n.d.	0.71	0.086			12.5	0.004			1555	6589	1426	17897	1000	2378
593	422	5.93	n.d.		0.075							1611	2111	1090	7820	1165	3298
726	456	4.80	n.d.	0.73	0.073							1212	9428	1441	7319	1016	1820
789	472	4.43	n.d.	0.76	0.073							3555	9909	1000	4420	1000	1681
794	473	4.63	n.d.	0.78	0.078							1652	5546	1000	5521	1000	1142
1025	528	5.03	indole alkaloid, **4**		0.082							1334	1502	1815	1576	2183	2756
1098	545	5.00	ammonium adduct of **4**			28.7	0.002					1070	30673	1493	10754	1144	1014
1144	556	6.97	indole alkaloid, **5**	0.81	0.115	50.3	0.000	173.1	<0.001			2114	106322	1000	173072	1212	2243
1184	566	5.40	n.d.	0.72	0.084							1000	9741	1000	6215	1000	1000
1218	574	4.53	putative indole metabolite	0.70				12.3	0.001			2077	5140	1051	12890	1047	1236
1299	594	6.10	n.d.		0.074							1216	2412	1000	2273	1000	1171
1602	690	6.63	hexoside of **4**?		0.071	12.9	0.002	14.4	<0.001			1281	16514	973	13978	1000	1000

[a] n.d., not determined: intensity of fragment ions of MS/MS spectrum was too weak to obtain structural information. The numbers indicated by boldface correspond to those used for referring to the compounds in Figure 4.

[b] See Figure 3.

leaves and leaf sheath, where 35% of activity in the absence of Trp was maintained even in the presence of 100 μM Trp. These results suggest that the localized accumulation of Trp in the transformant could not be explained by the distribution of AS activity in the rice plant.

The level of anthranilate, a product of the reaction catalyzed by AS, in NB two weeks after germination was low (<0.7 nmol g^{-1} fresh weight, Table 2). The anthranilate produced by the AS-catalyzed reaction was considered to be rapidly converted by phosphoribosylanthranilate transferase that catalyzes the next step of the Trp biosynthetic pathway. Expression of *OASA1D* caused an increase in the amount of anthranilate in all parts of the transformant, although the levels (1.1 to 5.9 nmol g^{-1} fresh weight) were much lower than those of Trp (30 to 2500 nmol g^{-1} fresh weight, Figure 1). Anthranilate was also unevenly distributed in the transformants; however, the distribution pattern of anthranilate among the tissues was different from that observed for Trp, with the highest level found in roots (Figure 1).

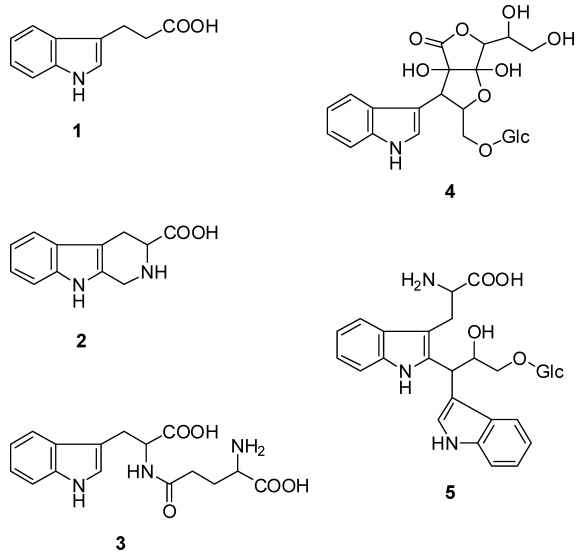

Figure 4. Structures of indole-3-propionic acid (**1**), 1,2,3,4-tetrahydro-β-carbolin-3-carboxylic acid (**2**), γ-glutamyltryptophan (**3**) and indole alkaloids (**4** and **5**) identified as the metabolites increased in *OASA1D*-transformed rice plants. The numbers of the compouds are referred to in the text and in Table 1. Glc represents D-glucose.

Translocation of isotopically labeled Trp

Given that no correlation was found between the Trp levels in the tissues of *OASA1D* transformants and AS activity, nor between the Trp levels and the anthranilate levels, the inter-tissue translocation of Trp was likely involved for its uneven distribtution in the plant. In order to investigate this, radioisotope-labeled Trp ([3-^{14}C]Trp)

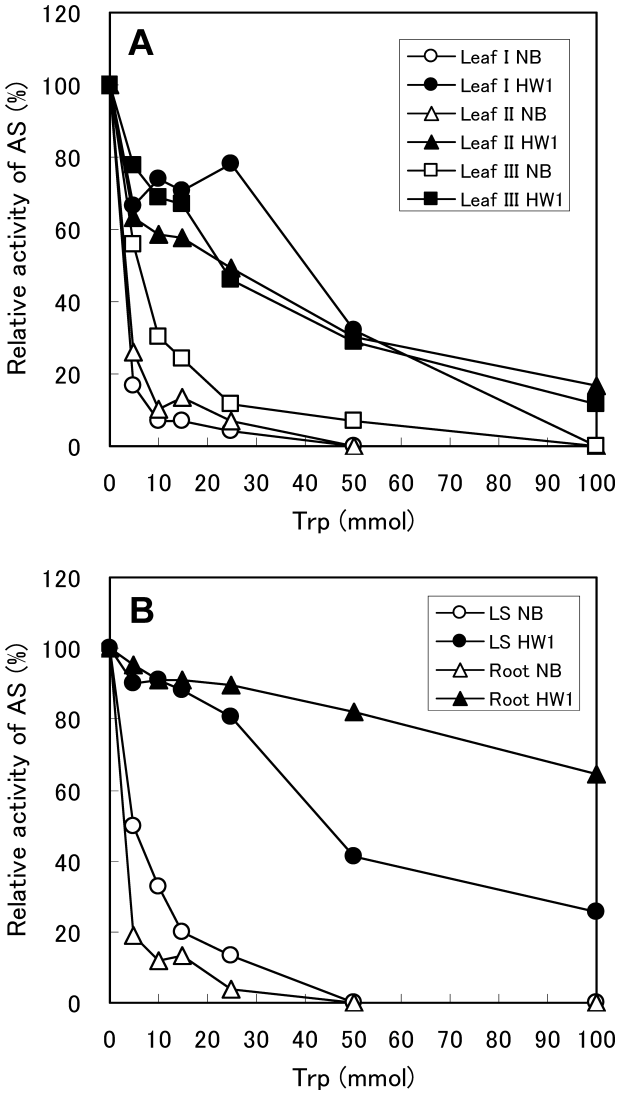

Figure 5. Sensitivity of AS activity in leaves (A), leaf sheath and roots (B) to feedback inhibition by Trp. All data are expressed as the means ±SD of values from three separate experiments.

Table 2. Levels of anthranilate and activity of anthranilate synthase (AS) in an untransformed rice plant (NB) and transformed rice plant expressing OASA1D (HW1)

Tissues	Anthranilate level (nmol g^{-1} fresh weight)		AS activity (pkatal mg^{-1} protein)	
	NB	HW1	NB	HW1
Root	0.17 ± 0.02	5.91 ± 1.26	2.14 ± 0.23	3.80 ± 0.10
Leaf sheath	0.34 ± 0.09	4.64 ± 1.60	0.70 ± 0.05	0.78 ± 0.10
Leaf I	0.23 ± 0.05	1.10 ± 0.20	0.30 ± 0.04	0.07 ± 0.01
Leaf II	0.28 ± 0.02	1.81 ± 0.13	0.25 ± 0.03	0.10 ± 0.01
Leaf III	0.69 ± 0.05	3.19 ± 0.57	0.44 ± 0.10	0.20 ± 0.04

The data are expressed as the mean ±SD (n=3).

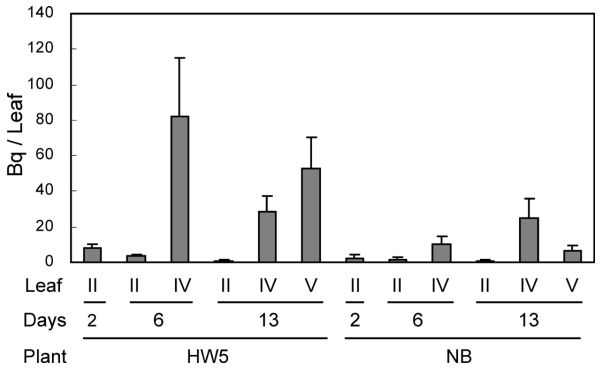

Figure 6. Inter-tissue translocation of exogenously applied radioisotope labeled Trp. All data are expressed as the means ±SD of values from three separate experiments.

was applied to leaf III of 10-day-old seedlings of NB and HW5, and the change in the radioactivity in leaves II, IV, and V was monitored . ^{14}C levels in the applied leaf (leaf III) decreased with time for both NB and HW5, where radioactivity of 625 (HW5)/724 (NB), 401/417, and 318/348 Bq $(leaf)^{-1}$ was detected at two, six and thirteen days after treatment, respectively. Correspondingly, radioactivity in younger leaves (leaf IV and V) increased with time in both HW5 and NB (Figure 6), while activity in the older leaf (leaf II) remained low throughout the experiment. The level of radioactivity in younger leaves was higher in HW5 than in NB. Free Trp accounted for 65 and 46% of the total radioactivity in leaf V of HW5 and NB, respectively, thirteen days after treatment. These results suggest that a considerable portion of exogenously applied Trp was transported to young leaves without conversion to other metabolites.

Discussion

Effects of OASA1D *transgene on the metabolic profiles of rice plant tissues*

In the present study, non-targeted metabolic profiling analysis was conducted using LC-ESI-Q-MS to evaluate the effects of *OASA1D* transgene on the metabolite composition of rice plant tissues. Of several profiling techniques currently available, the LC-MS-based method is the primary choice in plant metabolomics, in light of its capacity to cover a wide range of metabolites with relatively high sensitivity (Bottcher et al. 2007; De Vos et al. 2007). The present results demonstrated that metabolic profiles were changed in a tissue-dependent manner, and the uneven accumulation of Trp was most noteworthy, in spite of the uniform expression of *OASA1D* transgene in the whole plant tissues under the control of ubiquitin promoter. Inter-tissue comparison of the metabolic profile revealed that the effect of *OASA1D* expression on the metabolic profiles in rice plant tissues was closely related to the magnitude of Trp accumulation. While a certain number of metabolite peaks increased in size, no decrease in the levels of other major constituents, such as flavonoids and phenylpropanoids that share the biosynthetic pathway (shikimate pathway) with Trp was observed (data not shown), indicating no remarkable competition among these pathways in the *OASA1D* transformant.

A combination of three types of statistics revealed that the levels of 26 peaks were most closely related to the expression of *OASA1D* (Table 1). Mass spectroscopic analysis of the chemical components in these increased 26 peaks suggested that 13 were assigned to metabolites containing indole moieties in their structures, of which five compounds were structurally characterized, as shown in Figure 4. Compounds **1**, **2**, and **3** were derivatives of Trp, and their increase is well accountable as a consequence of Trp overproduction. While no biological activity has been reported for **2** and **3**, except toward mammals (Herraiz and Galisteo 2003; Smith et al. 2003), compound **1** has been detected in the seedlings of squash and pea as an naturally-occurring auxin (Segal and Wightman 1982; Schneider et al. 1982); however, no significant changes associated with auxin were observed in the seedlings of HW1 or HW5, and the increase of **1** possibly had a negligible effect. In this relation, the levels of indole-3-acetic acid (IAA) should be of greater concern, although the profiling analysis in this study failed to detect the changes by the expression of *OASA1D*. The targeted analysis to quantify IAA in the seedlings was somehow hampered in the presence of high levels of Trp, and an improved analytical method is currently being sought for to determine the precise IAA levels in the transformants. An increase in the IAA levels was observed in calli as well as in seeds of *OASA1D*-transformed rice, by which no significant change in growth rate or other morphological traits was observed (Morino et al. 2005; Wakasa et al. 2006).

Compound **4** was also detected as a metabolite with increased content in rice calli overexpressing *OASA1D* (Morino et al. 2005). Aglycone of compound **5**, 2-[2,3-dihydroxy-1-(indol-3-yl)propyl]-L-tryptophan, has been reported as a by-product in a commercial Trp preparation (Simat et al. 2003; Williamson et al. 1997). It is likely that compounds **4** and **5** are innate minor secondary metabolites that originate from indole-3-glycerol phosphate in rice plants. The functions of these compounds in plants are unknown, and will be the subjects of future studies. The intensities of the peaks in Table 1, including 13 indole compounds, are generally weak, and hence, their contributions to the mass balance in the whole metabolism of rice plants are thought to be minor. Thus, the excess amount of Trp accumulated in a rice plant expressing *OASA1D* is relatively stable and 'reluctant' to undergo metabolic and/or catabolic conversion, which is favorable for utilizing the *OASA1D* gene in crop breeding. This strict control might be

associated with the fact that Trp is one of the most biosynthetically expensive amino acids, and plants may have developed a mechanism to prevent such precious metabolites from active utilization. It is also likely that the capability of crop plants to convert Trp into indole alkaloids has been weakened in the evolution of breeding, because indole alkaloids often taste bad and are considerably toxic to humans.

Uneven distribution of Trp in OASA1D*-transformed rice plant as a consequence of inter-tissue translocation*

The present study has shown that the concentration of Trp in each tissue of *OASA1D* rice plant markedly changed in a time-dependent manner to establish an uneven, tissue-dependent profile of accumulation. Marked accumulation was observed in young developing tissues, whereas the levels of Trp in old expanded leaves were similar to those in untransformed plants (Figure 1). Such uneven distribution has never been noted in *OASA1D* transformants with elevated Trp levels, including young rice seedlings (Tozawa et al. 2001; Wakasa et al. 2006), Arabidopsis (Ishihara et al. 2006), potato (Matsuda et al. 2007; Yamada et al. 2004), and azuki bean (Hanafy et al. 2006): in these studies, it was implicitly assumed that the levels of Trp in tissues uniformly increased, since the expression of the *OASA1D* transgene is under the control of the ubiquitin promoter. A tendency toward uneven Trp distribution among tissues has been observed in five-week-old seedlings of Arabidopsis expressing *OASA1D*, (Ishihara et al. 2006), although unevenness in rice plants is far more evident.

The enzyme assay of AS showed that activity was certainly increased, and its sensitivity to feedback inhibition was decreased in all parts of four-week-old rice seedlings expressing *OASA1D* (Figure 4), indicating that the observed uneven accumulation of Trp in the transformant was unlikely to be attributed to uneven expression of the *OASA1D* transgene. Likewise, a significant increase in the amount of anthranilate, the reaction product catalyzed by AS (Table 2) in each tissue confirmed that in vivo AS activity was certainly up-regulated in a tissue-independent manner. In addition, Trp-associated metabolites and/or catabolites were not detected to a significant level by metabolic profiling analysis (Supplemental Figure S1 in online version and Table 1) in leaves I two weeks after germination, and therefore, the marked decrease in the level of Trp in old leaves is unlikely to be a result of the conversion of Trp into other metabolites. Thus, other mechanisms were considered to be responsible for the uneven distribution of Trp.

The tracer experiment using ^{14}C-Trp in this study revealed that inter-tissue translocation may account for the disappearance of an excess amount of Trp in old leaves, in which exogenous ^{14}C-Trp was shown to definitely move upward into younger tissues (Figure 6). This observation manifests the potential of Trp translocaton in wild-type rice plants, while only the translocation of glutamine and asparagine has been noted to date (Hayashi and Chino 1990). The capacity of Trp translocation could be fairly large, considering the amount of Trp that disappeared from young leaves in *OASA1D* plants (Figure 1), and such an efficient translocation system is likely to exist to salvage amino acids from leaves undergoing senescence during the developmental process, or under stressed conditions. It has been observed that senescence causes remobilization of amino acids, including Trp, in oat leaves by hydrolysis of constitutive proteins (Soudry et al. 2005). Translocation of remobilized amino acids was suggested by the difference in the amounts of free amino acids between detached senescent leaves and in those remaining attached to the plant: a marked increase in amino acid contents was observed in the detached leaves, while no increase occurred in the attached leaves, probably due to the translocation to other tissues.

Implication for metabolic engineering

Trp is a biosynthetic precursor of many pharmaceutically active indole secondary metabolites in plants. The successful accumulation of Trp in an excess amount by transformation with *OASA1D* may represent a step on the way to producing such valuable compounds in plants by introducing additional genes for further conversion. The relatively low activity of metabolizing Trp in rice seems to be favorable for this purpose. In this regard, we have already developed rice calli lines that accumulate a larger amount of tryptamine instead of Trp by co-expressing the rice tryptophan decarboxylase gene with *OASA1D* (Dubouzet et al., in preparation). Tryptamine is a key intermediate for the biosynthesis of indole alkaloids. Metabolic engineering using *OASA1D* combined with other genes would produce crops possessing a novel ability to synthesize valuable metabolites.

In this study, it was demonstrated that an excess amount of Trp was unevenly accumulated among the tissues of transgenic rice plants by an as yet unknown inter-tissue transportation mechanism. From the viewpoint of plant metabolic engineering, these results provide important insight into designing metabolite overproduction in a plant. For example, although the marked accumulation of Trp in *OASA1D* transgenic rice seeds has been established (Wakasa et al. 2006), it is very likely that Trp originates from photosynthesizing tissues and is translocated into seeds, and therefore, introduction of the *OASA1D* gene under control of the leaf-specific promoter (for example, rice *rbcS* promoter (Suzuki et al. 2007)) is considered to be sufficient to bring about high Trp in seeds: this strategy is preferable

in that the transgene is not expressed in edible seeds. Clarification of the molecular mechanisms responsible for the inter-tissue translocation of amino acids should provide good insight not only for further understanding of plant functions, but for more elaborate engineering of plant metabolism.

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