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Metabolic flux analysis in plants using dynamic labeling technique: Application to tryptophan biosynthesis in cultured rice cells

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Abstract

The concept and methodology of using dynamic labeling for the MFA of plant metabolic pathways are described, based on a case study to develop a method for the MFA of the tryptophan biosynthetic pathway in cultured rice cells. Dynamic labeling traces the change in the labeling level of a metabolite in a metabolic pathway after the application of a stable isotope-labeled compound. In this study, [1-^{13}C] L-serine was fed as a labeling precursor and the labeling level of Trp was determined by using the LC–MS/MS. The value of metabolic flux is determined by fitting a model describing the labeling dynamics of the pathway to the observed labeling data. The biosynthetic flux of Trp in rice suspension cultured cell was determined to be 6.0 ± 1.1 nmol (g FW h)$^{-1}$. It is also demonstrated that an approximately sixfold increase in the biosynthetic flux of Trp in transgenic rice cells expressing the feedback-insensitive version of anthranilate synthase alpha-subunit gene (*OASA1D*) resulted in a 45-fold increase in the level of Trp. In this article, the basic workflow for the experiment is introduced and the details of the actual experimental procedures are explained. Future perspectives are also discussed by referring recent advances in the dynamic labeling approach.

Keywords: Anthranilate synthase; Dynamic labeling experiment; Metabolic flux analysis; *Oryza sativa*; Tryptophan over-production

1. Introduction

Metabolic flux analysis (MFA), by which the amount of a metabolite flowing in a pathway is determined in a quantitative manner, is a method of investigating the regulatory mechanisms of the metabolic systems of various organisms (Ratcliffe and Shachar-Hill, 2006; Schwender et al., 2006) which could be a rational basis for metabolic engineering in biotechnology (Kinney, 1998; Verpoorte and Memelink, 2002) and a component of functional genomics (Fernie et al., 2005). The modern methods for MFA have been mainly based on monitoring the labeling process of metabolites by the feeding of a stable isotope-labeled precursor. There are two methodologies for the determination of metabolic flux: "steady state" labeling and "dynamic" labeling (Ratcliffe and Shachar-Hill, 2006). Steady state labeling has long been applied in the MFA of the primary metabolic network in microorganisms (Wiechert, 2001). The objective of MFA in the metabolic engineering of microorganisms is to utilize the results to maximize the yield of a desired product from carbon sources such as glucose in a fermentation process. Various methods have been developed for the determination of metabolic flux from glucose to a specified metabolite via the primary metabolic pathway (Stephanopoulos et al., 1998). In most cases, metabolic flux is determined by the flux balancing and/or [^{13}C]-labeling methods. The flux balancing is a classical approach where

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the metabolic fluxes are estimated based on the substitution of the measurable data concerning the transformation processes (for example, substrate uptake ratio or product formation) for the variables in stoichiometry equations representing the metabolic interconversions in cells. The [^{13}C]-labeling experiment consists of an analysis of the extract obtained from microorganisms cultured in a medium containing [^{13}C]-labeled glucose (Wiechert, 2001). The labeled carbon atoms derived from [^{13}C]-labeled glucose are distributed throughout the metabolic network to form the various isotopomers of each metabolite. Then, the abundance of these isotopomers is determined using NMR or MS instruments to estimate the intracellular flux by fitting a metabolic network model to the data obtained. For example, Wittmann and Heinzle (2001) reported the MFA of lysine-producing *Corynebacterium glutamicum* in which 37 fluxes of the primary metabolic network were calculated from stoichiometric data and selected mass intensity ratios of lysine, alanine, and trehalose measured by MALDI-TOF MS in tracer experiments using either [1-^{13}C] glucose or mixtures of $^{13}C_6/^{12}C_6$ glucose. This approach is based on the assumptions that isotopomer ratios and the metabolic fluxes in the pathway are in the "steady state" during the experimental period (Wiechert and Wurzel, 2001). The assumptions are adequate enough since the conditions for the growth of microbes are usually strictly controlled by the chemostat culture. The steady state methodology has been thoroughly investigated for the last two decades and is established as a powerful tool for the metabolic engineering of microorganism. This technique has also been applied to the MFA of primary metabolism in plants (Ettenhuber et al., 2005; Roscher et al., 2000; Sriram et al., 2004).

Dynamic labeling traces the change in the labeling level of a metabolite in a metabolic pathway after the application of a stable isotope-labeled compound. The time-dependent decrease in the labeling level of the precursor (Okazaki et al., 2004; Yanagimachi et al., 2001) as well as the increase in that of a down-stream metabolite in the pathway (Matsuda et al., 2005a) is monitored as a dynamic process. The value of metabolic flux is determined by fitting a model describing the labeling dynamics of the pathway to the observed labeling data.

Although the dynamic labeling strategy is an alternative for MFA, its progress has been very slow compared to that of the steady state approach. For example, the standard textbook in this field, "Metabolic Engineering", describes the dynamic labeling approach in only one page (Stephanopoulos et al., 1998). Recently, an advantage of dynamic labeling has been demonstrated in dealing with the MFA of "unsteady" state metabolic pathways in microorganisms (Noh et al., 2006; Noh and Wiechert, 2006; Wang and Hatzimanikatis, 2006a,b). Applications to the MFA of linear or complex secondary metabolic pathways in plants have also been reported (Boatright et al., 2004; Matsuda et al., 2005a, 2003; Okazaki et al., 2004). However, practical methods for dynamic labeling inevitably require improvements on a trial and error basis. Thus, experiences obtained by the analyses of relatively simple metabolic pathways will provide the fundamentals for the future application to a large and complex metabolic network.

In this article, the concept and methodology of dynamic labeling for the MFA of plant metabolic pathways are described, based on our development of a method for the MFA of the tryptophan biosynthetic pathway in rice suspension cell cultures. Following an explanation of the basic workflow of the experiment, the actual experimental procedures are explained in a step-by-step manner. Finally, future perspectives are discussed by introducing recent advances in the dynamic labeling approach.

2. Tryptophan-overproducing rice cells transgenic for a mutant rice anthranilate synthase α-subunit gene (*OASA1D*)

The metabolic engineering of essential amino acids including Lys (Galili, 2002), Met (Nikiforova et al., 2002), and Trp (Tozawa et al., 2001) has been attained using gene manipulation techniques to improve the nutritional quality of crops (DellaPenna, 2001; Galili and Höfgen, 2002). Since the activity for this biosynthesis is under the control of feed-back inhibition by the products or intermediates of the pathway, the release of regulatory mechanisms has been considered an important approach to elevating the content of target amino acids (Galili and Höfgen, 2002). In the case of the biosynthesis of Trp, the feed-back inhibition of anthranilate synthase (AS) by Trp is mainly responsible for regulating the pathway (Bohlmann et al., 1996; Kreps et al., 1996; Li and Last, 1996; Song et al., 1998). Based on these findings, further gene modifications were undertaken for AS to produce transgenic crops accumulating larger amounts of Trp. Following the cloning of two AS alpha-subunit genes (*OASA1*, *OASA2*) from rice (*Oryza sativa* cv. Nipponbare), Tozawa et al. (2001) modified one of these genes to obtain *OASA1D* (first referred to as *OASA1(N323D)*). The transformed rice calli and rice plants had remarkably raised levels of free Trp, increased up to 180- and 35-fold, respectively (Tozawa et al., 2001). It has also been shown that the expression of *OASA1D* in potato and Arabidopsis effectively increased free Trp levels (Ishihara et al., 2006; Matsuda et al., 2005b; Yamada et al., 2004). The metabolic profiling of transgenic rice calli (Morino et al., 2005), seeds (Wakasa et al., 2006), potato tubers (Matsuda et al., 2005b), and Arabidopsis (Ishihara et al., 2006) demonstrated that the over-production of Trp did not affect levels of other metabolites in the tissues.

The marked accumulation of Trp in the transformed rice cell culture suggests that the metabolic flux of Trp biosynthesis is much increased. The metabolic flux analysis of Trp biosynthesis in these plants undoubtedly provides an interesting opportunity to investigate the relationship between the metabolic flux and the level of product of the pathway, which has not been well understood so far.

3. The workflow of the dynamic labeling experiment

A dynamic labeling experiment consists of the following six parts:

1. Construction of a hypothetical metabolic network considering the prior literature and preliminary experiment for selecting a suitable labeling precursor.
2. Construction of an analytical method for the quantification of labeled and unlabeled metabolites.
3. Establishment of a mathematical model for the labeling dynamics of the target pathway.
4. Calculation of metabolic flux by fitting the mathematical model to the time-course data on the isotope abundance of target metabolites.
5. Validation of the metabolic model based on the data obtained from the dynamic labeling experiment as well as on additional data of independent experiments and/or examinations.
6. Determination of metabolic flux.

3.1. Selection of a suitable labeling precursor

The determination of flux using the steady state labeling approach is based on the detection of re-arrangements of the carbon skeletons of target metabolites during the reaction of the primary metabolic network by using a partially labeled [^{13}C]-glucose as a labeling precursor (Wiechert and Wurzel, 2001). Thus, the approach cannot be applied to the MFA of a linear metabolic pathway without a rearrangement of carbon skeletons such as the last several steps of the biosynthetic pathway for aromatic amino acids and secondary metabolic pathways in plants. On the other hand, the dynamic approach is applicable to the MFA of a linear metabolic pathway using suitable labeling precursors. For example, MFA studies were successfully performed for the phenylpropanoid pathway in potato tubers and for the pathway of benzoic acid metabolism in peas by employing [ring-$^{2}H_5$] phenylalanine as the labeling precursor (Boatright et al., 2004; Matsuda et al., 2005a, 2003).

The feeding of a stable isotope-labeled precursor to the target metabolic pathway is the first procedure in a dynamic labeling experiment. It is desirable that the labeled compound be immediately incorporated into cells of the plant tissue. Moreover, the metabolic flux of the target pathway must not be disturbed by the application of the labeled precursor. Thus, not only the endogenous concentration of a metabolite, but also its physicochemical properties are key to the selection of a suitable labeling precursor. Furthermore, regulatory mechanisms, and rate-limiting (high flux control-coefficient) steps of the target pathway should be taken into consideration. Flux control coefficient is a quantitative representation of a concept such as ''rate-limiting'' or ''rate-controlling'' which was originally introduced by Kacser and Burns (1973) and later described in more detail by Fell (1997).

In the case of tryptophan biosynthesis, the conversion proceeds as shown in Fig. 1. A key intermediate of this pathway is chorismate which is derived from one molecule of erythrose-4-pyruvate and phosphoenolpyruvate via the shikimate pathway. Chorismate is also a precursor for the biosynthesis of two other aromatic amino acids, phenylalanine and tyrosine. For Trp biosynthesis, chorismate is converted to anthranilate by anthranilate synthase. Trp is then synthesized through a linear four-step reaction. In this process, one molecule of serine is incorporated into Trp at the final step of the pathway catalyzed by tryptophan synthase (TS).

It has been demonstrated that the activity for Trp biosynthesis is mainly regulated by the feed-back inhibition of AS by Trp as referred to above. Indeed, the activity of AS in the crude extract of an untransformed rice callus was inhibited to 15% of the control level in the presence of 50 μM Trp (Fig. 2a), and as a result, the concentration of Trp in the untransformed callus was kept at 49.4 nmol (g FW)$^{-1}$ (Table 1). By contrast, AS activity in a transgenic rice callus line (45-5) expressing a feedback-

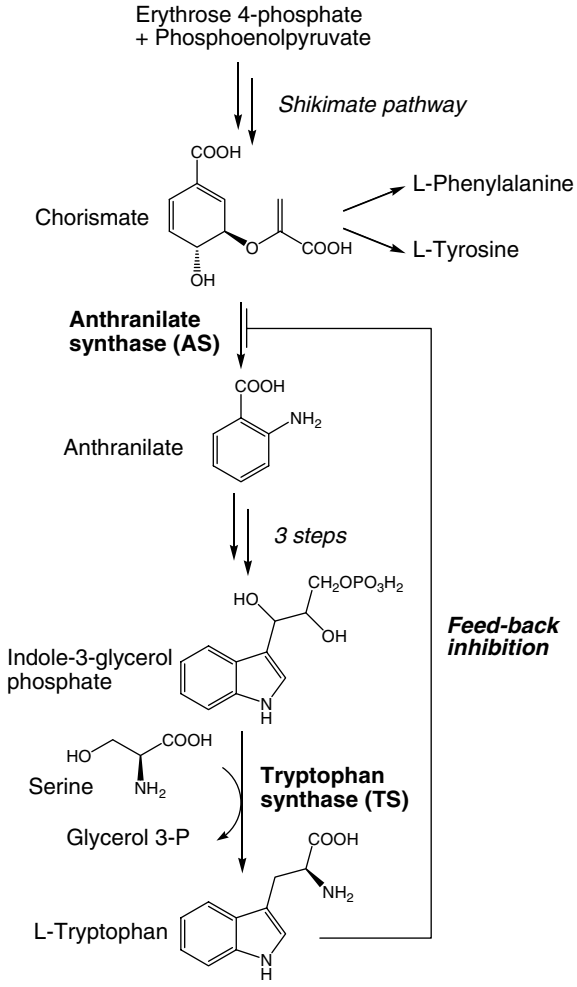

Fig. 1. Biosynthetic pathway of tryptophan (Trp). Feed-back regulation from Trp to anthranilate synthase (AS) is also shown.

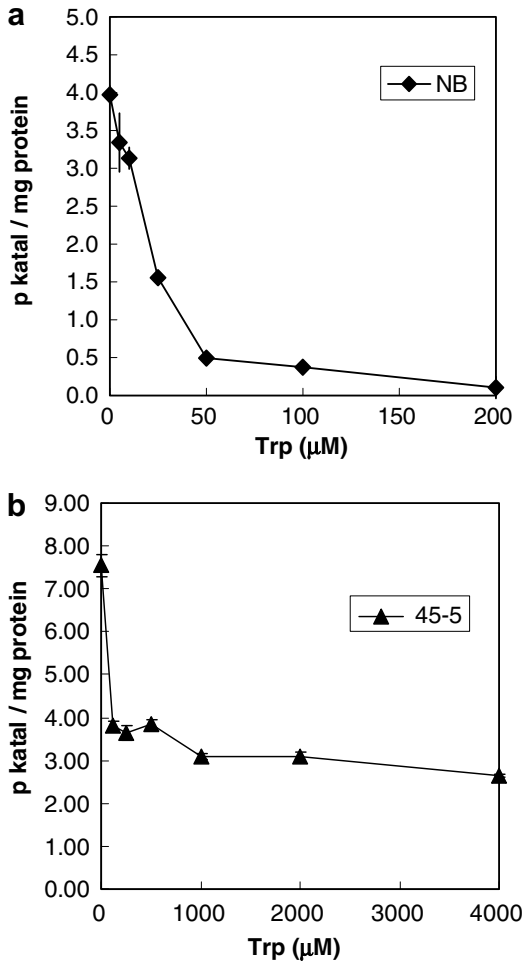

Fig. 2. Sensitivity of AS activity in the calli of NB (a) and the *OASA1D*-transformant (b) to feedback inhibition by Trp. All data are expressed as means of values from three separate experiments.

Table 1
Abundance of Trp in the untransformed rice callus (NB) and transformant (21-1 and 45-5) expressing *OASA1D*

Callus	Trp (nmol g FW^{-1})	Fold of increase
NB	49.4 ± 7.8	1.0
21-1	2385 ± 122	48.3
45-5	2288 ± 481	46.3

Data are means ± SD of values from three samples.

insensitive version of the AS α subunit gene (*OASA1D*) was increased twofold and only 50–65% of this activity was inhibited by the addition of 500 μM Trp (Fig. 2b). Such a relaxation of the feed-back control caused a drastic increase in the amount of Trp in the transgenic calli up to the 2200 nmol (g FW)$^{-1}$ level which is 46 times as much as that in untransformed calli. Metabolic profiling analysis of the transformant indicates that almost all of the anthranilate generated by the activity of feedback-insensitive AS is quickly converted to Trp without an accumulation of the intermediates or other products of the pathway. Therefore, it was suggested that the step catalyzed by AS is the rate-limiting (high flux control-coefficient) step for the flux of the Trp biosynthetic pathway (Morino et al., 2005).

In the dynamic labeling experiment, intermediates located downstream of the so-called rate-limiting (high flux control-coefficient) step cannot be selected as the labeling precursor, since the down-stream steps cannot control the metabolic flux of the pathway (Teusink and Westerhoff, 2000). For example, if the tissues were treated with a stable-isotope labeled anthranilate, the exogenously applied anthranilate would immediately be converted to Trp regardless of the actual biosynthetic flux of Trp (J). A biased J value would be obtained from the experiment. Thus, only the intermediates upstream of AS are candidates for labeling agents. However, it is difficult to obtain enough stable-isotope-labeled chorismate or shikimate. The application of a precursor in the shikimate pathway such as phosphoenolpyruvate or erythrose-4-phosphate is not realistic because the use of the precursors higher up in the biosynthetic pathway requires a more complex model to take the additional steps into consideration, and hence is ineffective.

The biosynthetic pathway shown in Fig. 1 indicates that L-serine is also a precursor for Trp biosynthesis. The pathway could be considered a one-step pathway in which one molecule of Ser is converted to Trp by the function of TS. It is unlikely that the supply of Ser to the pathway is rate-limiting (high flux control-coefficient) for Trp biosynthesis since a large amount of Ser is accumulated in rice calli (Tozawa et al., 2001). Thus, the reaction catalyzed by TS,

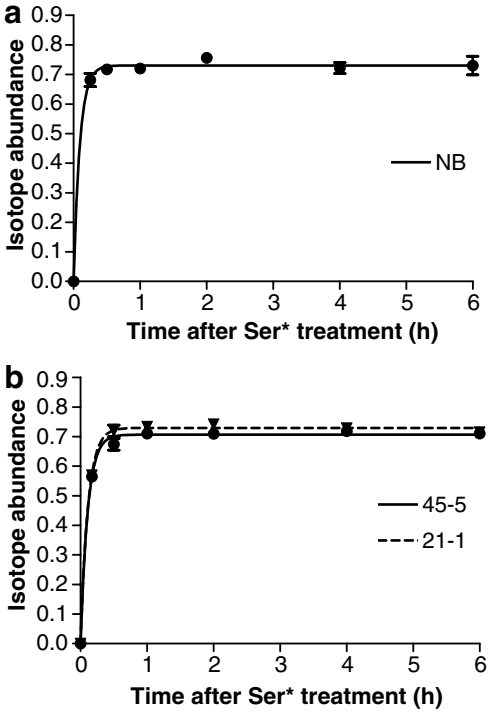

Fig. 3. Time-dependent increases of isotope abundance of Ser (F_{Ser}) in untransformed (a) and *OASA1D*-transformed (b) cultured rice cells. Five millimolar of [1-^{13}C]Ser (Ser*) was added in the medium at $t = 0$.

more accurately, the supply of indole-3-glycerol phosphate, is most likely a rate-limiting factor in this one-step pathway. In order to investigate the availability of Ser as a precursor for the dynamic labeling experiment, we treated rice callus cells suspended in the liquid medium with 5 mM of [1-^{13}C] Ser. The isotope abundance of Ser (F_{Ser}) in rice cells was rapidly increased and saturated at one hour after the application (Fig. 3). The concentration of Trp in rice cells was not disturbed by the Ser treatment (Fig. 4). These results indicated that the exogenously applied Ser was immediately incorporated into rice cells and did not affect the Trp biosynthetic flux. As discussed in the following section, a double or triple labeled stable isotope is preferable for the dynamic labeling approach. But the application of a multi-labeled Ser is unrealistic due to its poor availability.

Thus, we attempted the determination of metabolic flux for Trp biosynthesis using [1-^{13}C] Ser as a labeling precursor. The C-1 labeled preparation is favorable compared to those labeled at C-2 or C-3, since its relatively simple behavior in the metabolic pathways allows the application of a simplified model: the label at C-2 or C-3 could be lost or interchanged during the interconversion between Ser and Gly by the action of serine hydroxymethyltransferase or glycine decarboxylase. It could also be possible that the label in Ser is incorporated into Trp passing through many roundabout steps via the metabolic pathway network. However, the C-1 carbon in Ser is likely to be lost as carbon dioxide during the conversions along the non-photosynthetic pathways, and unlikely to be recovered under the employed experimental conditions. In fact, no double or triple labeled Trp was observed in the present dynamic labeling experiment (data not shown).

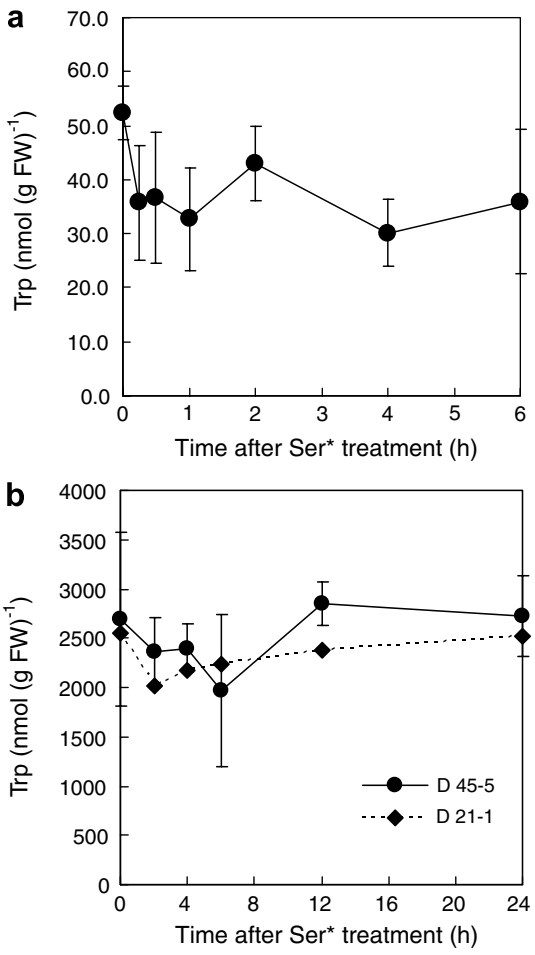

Fig. 4. Concentrations of Trp (C_{Trp}) in untransformed (a) and *OASA1D*-transformed (b) cultured rice cells. Five millimolar of [1-^{13}C]Ser (Ser*) was added in the medium at $t = 0$.

3.2. Construction of the analytical method for the quantification of labeled and unlabeled metabolites

In a dynamic labeling experiment, the amounts of labeled and unlabeled metabolites must be determined separately in order to obtain a value of isotope abundance. Isotope abundance of metabolite X (F_X) is defined as $F_X = [X^*]/([X] + [X^*])$. In this equation, [X] and [X*] represent the amounts of unlabelled (X) and labeled (X*) metabolites, respectively. The quantification of labeled and unlabeled metabolite levels is usually achieved using techniques based on hyphenated mass spectrometry such as GC–MS and LC–MS (Ratcliffe and Shachar-Hill, 2006; Wittmann and Heinzle, 1999). Since the investigation of time-dependent changes in the isotope abundance of target metabolites tends to produce a large number of samples to be analyzed, a high-throughput analytical system would be necessary for dynamic labeling.

For monitoring the isotope labeling in the MFA by MS, an isotopomer of the precursor that has the molecular mass of at least larger by 2 m.u. is generally used, in order to minimize the measurement error due to the naturally occurring isotopomers containing ^{2}H, ^{13}C and ^{15}N. In the present study of the Trp biosynthetic pathway, however, only [1-^{13}C] Ser was available as the labeled precursor. Therefore, we needed a distinction between the [1-^{13}C] Trp (Trp*, MW205) synthesized from [1-^{13}C] Ser and a naturally occurring isotopomer of Trp with MW of 205 that is present with an abundance of 11.3% of total Trp molecules (calculated on the basis of the molecular formula $C_{11}H_{12}N_2O_2$). We dealt with this problem by taking advantage of the multiple reaction monitoring (MRM) detection mode of the triple-stage quadrupole mass spectrometer, as follows. The naturally occurring isotopomer of Trp with MW 205 and the [1-^{13}C] Trp synthesized from exogenously applied [1-^{13}C] Ser are referred to here as Trp$^{\#}$ and Trp*, respectively.

In the MS/MS spectrum of the protonated molecule $[M+H]^+$ of monoisotopic Trp (*m/z* 205), a major fragment ion of *m/z* 146 was observed (Fig. 5a). The MS/MS spectrum of the protonated molecule $[M+H]^+$ of Trp$^{\#}$ (*m/z* 206) showed a fragment ion of *m/z* 147 in addition to that of *m/z* 146. The ratio of the intensities of these two ions (146/147) was 0.25. The fragment ion with *m/z* 146 should be derived from Trp$^{\#}$ bearing ^{13}C at the C-1 carbonyl carbon or at the C-2 methylene carbon (Fig. 5b). On the other

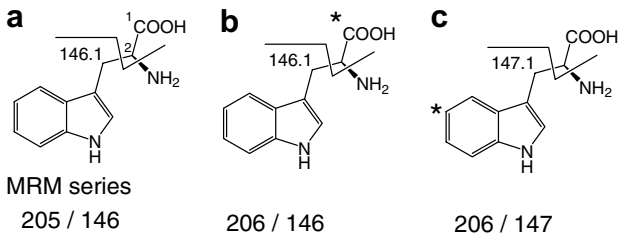

Fig. 5. MS/MS fragmentation patterns of Trp containing no isotope atoms (a), a single ^{13}C atom in the carboxyl or methyne carbon (b), and a single ^{13}C atom in the indole and methylene moieties.

hand, the fragment ion with m/z 147 comes from the isotopes bearing at other positions such as indole ring carbons (Fig. 5c). Thus, Trp$^{\#}$ labeled at the C-1 carbonyl carbon or C-2 methylene carbon is able to be selectively detected by setting a combination of precursor (m/z 206) and product (m/z 146) ions in the MRM detection mode. Defining the Trp$^{\#}$ labeled at C-1 or C-2 as Trp$^{\#C\text{-}1,2}$ and that labeled at other positions as Trp$^{\#Cothers}$, the isotope abundance of the C-1 and C-2 position of Trp observed (F_{Trp}^{obsed}) can be calculated using the following equation:

$$F_{Trp}^{obsed} = [Trp^{\#C\text{-}1,2}]/([Trp^{\#C\text{-}1,2}] + [Trp^{\#Cothers}] + [Trp])$$

In this equation, [Trp] represents a peak area of monoisotopic Trp detected by using the MRM series listed in Table 2.

The actual isotope abundance of the C-1 and C-2 position of naturally occurring Trp ($F_{Trp}^{natural}$) was determined to be 2.3%, which was in good agreement with the theoretical value (2.1%). Based on this, the isotope abundance of Trp* (F_{Trp}) was determined with the following equation:

$$F_{Trp} = F_{Trp}^{obsed} - F_{Trp}^{natural}$$

3.3. Mathematical modeling of the labeling dynamics of the target pathway

A mathematical model consists of differential equations describing the isotope labeling dynamics of the target metabolite in the pathway. Metabolic flux is determined by fitting the model to the time-dependent curves of isotope abundance obtained in the dynamic labeling experiment. In the case of a simple model derived from a one or two-step pathway, metabolic flux can easily be determined by a non-linear (Matsuda et al., 2003) or linear (Okazaki et al., 2004) regression method using the integrated equations of the model. On the other hand, a complex model dealing with a multi-step metabolic pathway requires a more sophisticated method and much computing to optimize parameters for fitting the metabolic model to the data obtained (Heinzle et al., 2007).

In this study, we employed the two-step pathway model to analyze the Trp biosynthetic pathway (Fig. 6). The first step represents the incorporation of exogenously applied [1-^{13}C] Ser (Ser*) into the rice cells. The second step is the conversion of Ser to Trp by TS. The concentrations of Ser and Trp in the rice cells (C_{Ser} and C_{Trp}) were assumed to be constant during the experimental period, by which the three metabolic flux values, influx to the Ser pool in the cell (J_1), conversion flux from Ser to Trp (J_2), and outflux from Trp (J_3), become the same (J). The assumption appeared valid enough in the case of Trp, as shown in Fig. 4.

After the Ser in the suspension medium is labeled at the beginning of the experiment ($t = 0$), Ser* is incorporated into the intracellular Ser pool with isotope abundance F_{Ser}^{max}. Then, the time-dependent changes in the amount of labeled Ser and Trp in the intracellular pool ($F_{Ser}C_{Ser}$ and $F_{Trp}C_{Trp}$, respectively) are expressed as a difference between these inputs (JF_{Ser}^{max} and JF_{Ser}) and outputs (JF_{Ser} and JF_{Trp}) by the following equations (Sims and Folkes, 1964):

$$\frac{dF_{Ser}C_{Ser}}{dt} = JF_{Ser}^{max} - JF_{Ser}$$

$$\frac{dF_{Trp}C_{Trp}}{dt} = JF_{Ser} - JF_{Trp}$$

F_{Trp}^{max} represents the maximum isotope abundance of Trp. The differential equations were integrated to;

Table 2
Multiple reaction monitoring (MRM) series for the detection of unlabeled and labeled Ser and Trp using high performance liquid chromatography-triple-stage tandem mass spectrometry (LC–MS/MS)

Compounds	MRM series	
	Q_1 (m/z)	Q_3 (m/z)
Trp	205.1	146.1
Trp$^{\#C\text{-}1,2}$	206.1	146.1
Trp$^{\#Cothers}$	206.1	147.1
Ser	106.1	60.1
Ser$^{\#C\text{-}1}$	107.1	60.1
Ser$^{\#Cothers}$	107.1	61.1

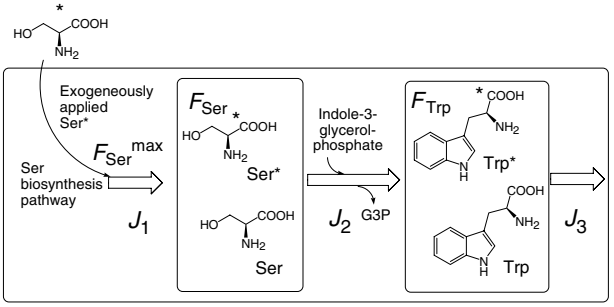

Fig. 6. Two-step model for the determination of the metabolic flux of Trp biosynthesis (J_{1-3}) based on the dynamic labeling experiment. The first step is the incorporation of exogenously applied Ser* into the rice cell. The second step corresponds to the conversion of Ser and Ser* to Trp and Trp* by the activity of tryptophan synthase (TS), respectively. The formed Trp and Trp* are further metabolized into other components including proteins and secondary metabolites. The letters F_{Ser}^{max}, F_{Ser}, and F_{Trp} represent isotope abundances of Ser influx (J_1), the Ser pool, and Trp pool, respectively.

$$F_{Ser} = F_{\mathrm{Ser}}^{\max}(1 - \mathrm{e}^{-t\tau_{\mathrm{Ser}}}) \quad (1)$$

$$F_{\mathrm{Trp}} = F_{\mathrm{Trp}}^{\max}\frac{\tau_{\mathrm{Trp}}(1 - \mathrm{e}^{-t\tau_{\mathrm{Ser}}}) - \tau_{\mathrm{Ser}}(1 - \mathrm{e}^{-t\tau_{\mathrm{Trp}}})}{\tau_{\mathrm{Trp}} - \tau_{\mathrm{Ser}}} \quad (2)$$

Here, τ_X (turnover constant) is defined as,

$$\tau_X = \frac{J}{C_X} \quad (3)$$

The value of C_{Trp} was determined using the HPLC system coupled with a fluorescent detector.

3.4. Estimation of parameters by fitting the metabolic model to the time-course data of the isotope abundance of target metabolites

F_{Ser} was determined based on Eq. (1), and then the value was incorporated into Eq. (2) to determine $F_{\mathrm{Trp}}^{\max}$ and τ_{Trp}. Eq. (1) was fitted to the time-dependent curves of F_{Ser} shown in Fig. 3 by using the non-linear regression function of GraphPad Prism ver3.0 (GraphPad Software, San Diego, CA, USA). Very high (0.96–0.98) correlation coefficients (R^2) were obtained for untransformed cells as well as transformants expressing *OASA1D*. From the regression analysis, the values of $F_{\mathrm{Ser}}^{\max}$ and τ_{Ser} were determined as shown in Table 3. In this model, the assumption that the level of Ser (C_{Ser}) is constant during the experimental period is not correct in a strict sense, because the actual level of Ser in rice cells is likely to be increased by the application of Ser*. The level of Ser in rice cells was not determined in this study. However, the value of F_{Ser} rapidly increased to the maximum level ($F_{\mathrm{Ser}}^{\max}$) at 0.5 h after the Ser* treatment and was kept constant. This suggests that the size of Ser pool in rice cells was kept almost constant during the experimental period after a specific amount of increase by the Ser* treatment. Additionally, the very good fitting of Eq. (1) to the observed curve of F_{Ser} suggests that the error caused by the assumption is not serious and therefore we did not examine other more complex models.

Next, in order to determine $F_{\mathrm{Trp}}^{\max}$ and τ_{Trp}, the non-linear regression was applied to fit Eq. (2) to the time-course of

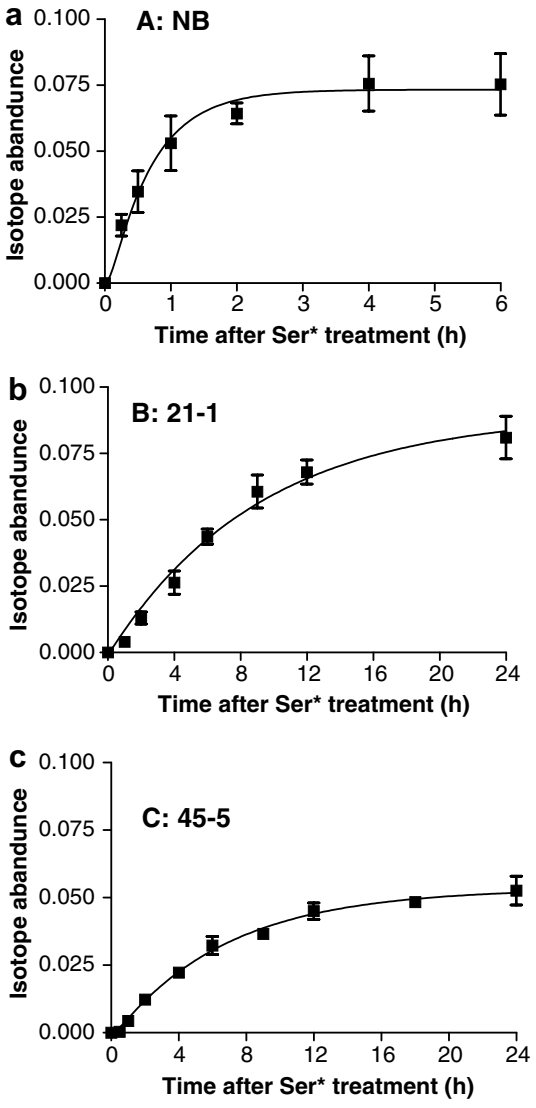

Fig. 7. Time-dependent increases in isotope abundance of Trp (F_{Trp}) in untransformed (NB, a) and *OASA1D*-transformed cultured rise cells (21-1, b and 45-5, c). Five millimolar of [1-^{13}C]Ser (Ser*) was added in the media at $t = 0$.

Table 3
Result of the metabolic flux analysis of Trp biosynthesis (J) in cultured rice cells using the dynamic labeling experiment

Callus line	Experiment number	$F_{\mathrm{Ser}}^{\max}$	τ_{Ser}	R^2(Ser)	$F_{\mathrm{Trp}}^{\max}$	τ_{Trp}	R^2(Trp)	C_{Trp} (nmol g FW^{-1})	J (nmol g FW^{-1} h^{-1})
NB	Exp. 1	0.63	5.46	0.99	0.092	1.12	0.96	39.3	6.4
	Exp. 2	0.72	2.48	0.98	0.146	0.63	0.90	50.2	6.4
	Exp. 3	0.75	6.98	0.98	0.168	0.54	0.91	58.2	7.0
	Exp. 4	0.80	2.93	0.94	0.160	0.44	0.81	49.8	4.4
	Average ± SD								6.0 ± 1.1
21-1	Exp. 1	0.73	8.93	0.99	0.090	0.109	0.94	2246	30.2
	Exp. 2	0.72	6.38	0.95	0.099	0.089	0.85	2476	30.3
	Exp. 3	0.70	4.55	0.99	0.142	0.088	0.84	2435	43.5
	Average ± SD								34.6 ± 7.7
45-5	Exp. 1	0.61	2.00	0.93	0.110	0.061	0.87	2653	29.4
	Exp. 2	0.68	4.30	0.96	0.116	0.089	0.86	1742	26.4
	Exp. 3	0.67	3.25	0.99	0.049	0.174	0.96	2469	31.4
	Average ± SD								29.0 ± 2.5

F_{Trp} shown in Fig. 7 using the τ_{Ser} values obtained above as constants (Table 3). The correlation coefficients ranged from 0.81 to 0.96, depending on the experiments. The F_{Trp}^{max} values were relatively small and varies among the experiments (0.049–0.168) although the experimental conditions were strictly controlled.

3.5. Validation of the metabolic model based on the data obtained from the dynamic labeling experiment

Since the regulation of metabolism depends on various factors including unknown ones *in vivo*, the data obtained by dynamic labeling will not necessarily fit the metabolic model. In such a case, a revision of the model based on the data obtained is essential to determine the appropriate value of metabolic flux. In the model for the dynamics of Trp biosynthesis expressed by Eqs. (1) and (2), the maximum value of F_{Trp} (F_{Trp}^{max}) should be the same as F_{Ser}^{max}. However, the F_{Trp}^{max} values obtained were much smaller than the F_{Ser}^{max} values, suggesting the presence of some unknown factor(s) that were not reflected in the metabolic model used in this study. There are three possible explanations for the smaller F_{Trp}^{max} as follows.

The first is the compartmentalization of Ser in the rice cell. It has been suggested that the biosynthesis of Trp occurs in the plastid. If the level of F_{Ser} in the plastid was kept low for some reason, F_{Trp}^{max} could be reduced. In order to test this hypothesis, we conducted a double labeling experiment using anthranilate-d_4 in addition to Ser*. F_{Ser} in the plastid can be estimated from the fraction of [1-^{13}C] labeling in the total of anthranilate-d_4-incorporated Trp in the rice cells treated with anthranilate-d_4 and Ser*, since Trp labeled with anthranilate-d_4 is synthesized *de novo* in the plastid.

Three hours after being fed 5 mM of Ser*, the rice cells were treated with 0.1 mM anthranilate-d_4, by which Trp-d_4 and double-labeled Trp ([1-^{13}C] Trp-d_4) were considered to be synthesized *de novo* from anthranilate-d_4. Following the incubation for two hours, Trp was extracted from the rice cell and analyzed using LC–MS/MS. The isotope abundance of double-labeled Trp in the Trp pool synthesized from anthranilate-d_4 was determined from the peak area of Trp-d_4, Trp$^{\#C\text{-}1,2}$-d_4, and Trp$^{\#Cothers}$-d_4 using the following equation derived on the basis of the consideration described in the Subsection 3.2:

$$F_{\text{double-labeled Trp}} = [\text{Trp}^{\#C\text{-}1,2}\text{-}d_4]/([[\text{Trp}^{\#C\text{-}1,2}\text{-}d_4] + [\text{Trp}^{\#Cothers}\text{-}d_4] + [\text{Trp-}d_4])$$

The obtained isotope abundance $F_{\text{double-labeled Trp}}$ was very close to that of F_{Ser} observed (data not shown), which suggested that the isotope abundance of F_{Ser} in the plastid was almost identical with the observed F_{Ser} in the experiments. Hence, the compartmentalization of Ser was unlikely to account for the smaller F_{Trp}^{max}.

The second possibility is the presence of the storage form of Trp in the rice cell. It has been presumed that Trp synthesized in the plastid is transported to the cytosol and used for the biosynthesis of protein. A part of Trp could be stored in a cell compartment such as the vacuole by an unknown mechanism. In this regard, the accumulation of *N*-malonyl-Trp has been documented in the water-stressed tomato leaves, which may represent one of the storage forms of Trp in plants (Liu et al., 1995). In the case of rice cells, however, no marked accumulation of *N*-malonyl-Trp nor other derivatives of Trp was observed. Hence, Trp is likely to be stored in intact form, if any. If there was a compartment for Trp storage somewhere in the rice cell and only a limited amount of Trp* was accumulated in the storage compartment during the experimental period, the observed F_{Trp}^{max} would be lowered. Under these situations, the maximal isotope abundance of Trp in the available pool (out side of the storage compartment) is considered to be equal to F_{Ser}^{max}, since Trp in this pool originates from Ser in the plastid. Then, the ratio of the amount of Trp in the available pool to the total amount of Trp in the cell is represented as $F_{Trp}^{max}/F_{Ser}^{max}$, and the amount of Trp in the available pool is expressed as $(F_{Trp}^{max}/F_{Ser}^{max})\ C_{Trp}$. Hence the metabolic flux of Trp biosynthesis (J) in the presence of a Trp pool for storage can be calculated from the following equation:

$$J = \frac{F_{Trp}^{max}}{F_{Ser}^{max}} C_{Trp}\tau_{Trp} \quad (4)$$

The third possible explanation is the tentative contribution of other pathways to Trp biosynthesis. For example, a significant level of protein degradation might have occurred under the experimental conditions in this study, resulting in the generation of a large amount of unlabeled Trp. The occurrence of the biosynthesis of unlabeled Trp is substantially the same as the existence of stored Trp in the model for the dynamic labeling experiment. Therefore, the metabolic flux of the Trp biosynthetic pathway in this case is also able to be determined using the Eq. (4).

It should be noted that the calculation of Trp biosynthetic flux using Eq. (4) is based on the assumption that the effect of bi-directional exchange between stored and available Trp pool is ignorable. Even if such an exchange did occur, the isotope abundance of Trp would increase and finally attain a level close to the F_{Ser}^{max} level as long as the exchange was rapid enough. The assumption appears to be appropriate in the case of untransformed rice cells, since the isotope abundance of Trp clearly reached to the maximum (F_{Trp}^{max}, Fig. 7). In the case of transformed rice cells, however, the increase of isotope abundance was slower than that in untransformed cells and not reached to a maximum level at 24 h after the Ser* treatment. This suggests that there is some effect of the exchange between stored and available Trp. It is currently difficult to estimate such effect quantitatively since the levels of stored and available Trp levels in the cells are unable to be determined separately. Nevertheless, the good fit of the observed time-course of the isotope abundance to the labeling dynamics

expressed by Eq. (2) as shown in Fig. 7 indicates that the occurrence of bi-directional exchange is unlikely to have a critical effect on the result of MFA. The more detailed metabolic flux analysis based on improved labeling dynamic models that take the intra-tissue compartmentation of Trp into consideration is now in progress.

3.6. Determination of the flux of Trp biosynthesis

In this study, the biosynthetic flux of Trp in rice cell cultures was determined with Eq. (4) taking into consideration the above discussion. The analyses of each cell line were repeated three to four times. The results of all experiments are listed in Table 3. The biosynthetic flux of Trp in untransformed cultured cells was estimated to be 6.0 ± 1.1 nmol g FW^{-1} h^{-1}. On the other hand, the biosynthetic flux of Trp (J) in the 21-1 and 45-5 transformed cell lines was determined as 34.6 ± 7.7 and 29.0 ± 2.5 nmol g FW^{-1} h^{-1}, respectively, where the amounts of Trp in the *OASA1D*-transformed cells were increased 45-fold and reached about 2300 nmol g FW^{-1} (Table 1).

The metabolic profiling analysis of transgenic rice calli expressing *OASA1D* showed that the composition of UV-active metabolites in the transformed calli was very similar to that of untransformed calli, except for the marked accumulation of Trp (Morino et al., 2005). The secondary metabolism derived from Trp did not appear to be active in these calli. It was also very likely that the amounts of Trp used for the protein biosynthesis were almost the same between transformed and untransformed cells, because the growth rates of both types of cell lines were essentially unchanged. These results suggested that the increased *de novo* synthesis of Trp in transformed rice cells only resulted in its accumulation in the free form, without further conversion into other metabolites.

The metabolic flux analysis demonstrated that an approximately sixfold increase in the biosynthetic flux of Trp in transgenic rice cells expressing the *OASA1D* transgene resulted in a 45-fold increase in the level of Trp. The concentration of Trp in an untransformed rice cell can be estimated to be about 50 μM (50 nmol g FW^{-1}) from the data in Table 1, assuming that Trp is evenly distributed in rice cells. According to the inhibition curve of AS activity by Trp (Fig. 2a), the activity of AS in the presence of 50 μM of Trp is about 0.5 pkatal mg $protein^{-1}$. The increase in the biosynthetic flux of Trp by sixfold caused by the expression of *OASA1D* suggests that the activity of AS in the transformed cells goes up to 3.0 pkatal mg $protein^{-1}$, if other factors affecting the activity of AS such as the concentration of substrates are the same. In the inhibition curves of AS by Trp in the 45-5 transgenic line (Fig. 2b), a level of catalytic activity of 3.0 pkatal mg $protein^{-1}$ is maintained in the presence of about 2000 nmol g FW^{-1} of Trp, which is almost the same as the observed level of Trp in the 45-5 transgenic line. So this estimation roughly explains the feed-back control of metabolic flux of tryptophan biosynthesis by Trp in a quantitative manner. These results clearly demonstrate that the introduction of a mutant AS gene encoding an enzyme less sensitive to feed-back control increased the amount of Trp in a plant by elevating the metabolic flux of biosynthesis.

4. Concluding remarks

In conclusion, we would like to discuss the perspective behind the dynamic labeling strategy for the MFA of plant metabolic pathways in terms of label selection, analytical method, and data analysis.

4.1. Label selection

The labeling precursors used for the dynamic labeling approach are not restricted to organic compounds. The metabolic flux of the assimilation pathway of inorganic nutrition was analyzed using stable isotope-labeled nitrogen (^{15}N) or sulfur (^{34}S) (Sims and Folkes, 1964). Moreover, application of the dynamic labeling approach using $^{13}CO_2$ as a labeling precursor (Hoon Yang et al., 2006; Yang et al., 2006) allows for the MFA of the primary metabolic pathway in the photosynthesizing organs (Shastri and Morgan, 2005).

4.2. Analytical method

In addition to mass spectrometry, ^{13}C NMR has been used in the MFA of steady state approach as a tool for the determination of the isotope ratio of each carbon atom that makes up a metabolite (Ettenhuber et al., 2005; Glawischnig et al., 2001; Sriram et al., 2004). NMR is able to deal with a metabolite in intact tissue without an extraction process (Ratcliffe and Shachar-Hill, 2001; Ratcliffe et al., 2001). Such potential provides opportunities to apply dynamic labeling approach to the MFA of complex plant metabolic network.

4.3. Data analysis

It has been shown that dynamic labeling has applications other than to metabolic flux analyses (McNeil et al., 2000a; McNeil et al., 2000b; Heinzle et al., 2007; Wang and Hatzimanikatis, 2006a). Heinzle et al. (2007) demonstrated that, in addition to metabolic flux, the activity of enzymes and the levels of intermediates within the target pathway can be estimated from the dynamic labeling data based on the construction of an elaborate metabolic network model. Moreover, they also calculated values of the flux control-coefficient of each step of the pathway using a computer simulation technique. The flux control-coefficient values obtained by the metabolic control analysis (MCA) indicate the quantitative expression of a so-called ''rate-controlling step'' in the metabolic pathway (Kacser and Burns, 1973; Fell, 1997). Although several pioneer MCA works have dealt with plant metabolic pathways

(Stitt et al., 1985; Stitt and Heldt, 1985; Ramli et al., 2005; Thomas et al., 1997), the importance of estimating the flux control-coefficients in the study of plant metabolism should be more considered in order to provide fundamental information for the rational manipulation of metabolic pathways (Nielsen, 1998). Advances in data analysis techniques based on computer simulation will facilitate the MCA as well as MFA of more complex metabolic networks using the dynamic labeling method, which will for sure unveil the regulatory mechanisms of metabolic pathways in plants.

5. Experimental

5.1. General experimental procedures

The transformation of rice calli (*Oryza sativa* L. cv. Nipponbare) was performed by the *Agrobacterium* transfection method with a plasmid vector containing a mutant rice AS α-subunit gene (*OASA1D*) under the control of the maize ubiqutin promoter (Tozawa et al., 2001; Yamada et al., 2004). Two lines of transformed calli (45-5 and 21-1) were subcultured at intervals of 3 weeks on 2N6 medium, consisting of N6 (Chu et al., 1975) supplemented with 2,4-dichlorophenoxy acetic acid (2 mg l^{-1}), casamino acids (1 g l^{-1}), 0.2% Gelrite (Wako, Osaka, Japan) and 150 μM 5-methyltryptophan (5MT). Prior to the analysis, calli were transferred on 2N6 medium without 5MT and subcultured more than two times. Untransformed rice calli were cultured on the 2N6 medium without 5MT. They were maintained in an incubator at 28 °C with a 16-h photoperiod. Calli maintained for 2 weeks after subculture were used for the metabolic flux analyses.

5.2. Anthranilate synthase (AS) activity assay

AS was extracted from rice calli and assayed by a method described previously (Matsuda et al., 2005b; Tozawa et al., 2001).

5.3. Metabolic flux analysis

Rice calli maintained on 2N6 plates for 2 weeks after subculture (0.85 g) were transferred to a sterilized screw vial (50 by 19 mm) and suspended in 3.0 ml of liquid 2N6 medium which did not contain Gelrite and 5MT. The sealed vials were shaken at 28 °C for 6 h at 200 rpm under light condition in a rotary shaking incubator. Following removal of 300 μl of the medium, 300 μl of 50 mM L-[1-^{13}C] serine in water was added to the cell suspension and incubated under the same conditions. About 50 mg of the suspended cells were collected at 0, 0.5, 1, 2, 4, 6, 9, 12, 18, and 24 h after the treatment with L-[1-^{13}C]serine. The cells were washed with 500 ml of distilled water three times to remove the excess L-[1-^{13}C]serine, and immediately frozen in liquid nitrogen. The samples were stored at −80 °C until the extraction procedure. The cells were weighed and extracted with 1.0 ml of water containing 2% acetic acid at 100 °C for 15 min. The extracts were centrifuged at 15,000*g* for 10 min and the supernatants were subjected to LC–MS/MS. For the determination of isotope abundance of Ser and Trp, the sample extract (5 μl) was analyzed with an HPLC system (Agilent 1100 series) coupled with a triple-stage tandem mass spectrometer (API3000; Applied Biosystems/MDS Sciex, Ontario, Canada). Chromatography was performed with a Cadenza CD-C18 column (30 by 2.0 mm; particle size, 3 μm). The column was eluted with a gradient of a mixture of acetonitorile and 0.1% formic acid in water (from 10:90 to 80:20, v/v, over 5 min) at a flow rate of 0.2 ml/min at 35 °C. The mass spectrometer was operated using the turbo ion spray interface in the positive ion mode under the following conditions: nebulizer gas flow (NEB), 14 l/min; curtain gas flow (CUR), 12 l/min; collision gas flow (CAD), 6 l/min; ion spray voltage (IS), 5000 V: and temperature (TEM), 550 °C: declustering potential (DP), 26 V; focusing potential (FP), 290 V; entrance energy (EP), 10 eV; collision energy (CE), 25 eV; and collision cell exit potential (CXP), 10 V. The detection mode of positive multiple reaction monitoring (MRM) was used. The MRM series for the detection of labeled and unlabeled metabolites are shown in Table 1. Peak areas were calculated using Analyst 1.3 software (Applied Biosystems).

5.4. Determination of Trp concentrations

For the determination of Trp levels in rice calli extracts, an aliquot of sample (1–5 μl) was analyzed with an HPLC system (Hitachi L7000 series) coupled with a fluorescence detector (Hitachi L7480). Chromatography was performed with a Cadenza CD-C18 column (75 by 4.6 mm; particle size, 3 μm; Imtakt, Kyoto, Japan); metabolites were eluted at a flow rate of 1.2 ml/min and 35 °C with a gradient of a mixture of acetonitrile and 0.1% acetic acid in water (5:95, v/v, over 3.55 min, and from 5:95 to 80:20 over 3.5 min). Excitation and emission wavelengths of the fluorescence detector were set at 285 and 345 nm, respectively, for the sensitive detection of Trp. The area of each peak was calculated with Smart Chrom software (KYA TECH Corporation, Osaka, Japan).

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