Plant Cell Rep (2007) 26:2009–2015
DOI 10.1007/s00299-007-0405-9

PHYSIOLOGY AND BIOCHEMISTRY

Characterization of tryptamine 5-hydroxylase and serotonin synthesis in rice plants

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Received: 27 March 2007 / Revised: 20 June 2007 / Accepted: 25 June 2007 / Published online: 17 July 2007

Abstract Serotonin is a well-known pineal hormone that in mammals plays a key role in mood. In plants, serotonin is implicated in several physiological roles such as flowering, morphogenesis, and adaptation to environmental changes. However, its biosynthetic enzyme in plants has not been characterized. Therefore, we measured the serotonin content and enzyme activity responsible for serotonin biosynthesis in rice seedlings. Tryptamine 5-hydroxylase (T5H), which converts tryptamine into serotonin, was found as a soluble enzyme that had maximal activity in the roots. The maximal activity of T5H was closely associated with the enriched synthesis of serotonin in roots. Tetrahydropterine-dependent T5H activity was inhibited by tyramine, tryptophan, 5-OH-tryptophan, and octopamine, but remained unaltered by dopamine in vitro. The tissues of rice seedlings grown in the presence of tryptamine exhibited a dose-dependent increase in serotonin in parallel with enhanced T5H enzyme activity. However, no significant increase in serotonin was observed in rice tissues grown in the presence of tryptophan, suggesting that tryptamine is a bottleneck intermediate substrate for serotonin synthesis.

Keywords 5-OH-tryptophan · Octopamine · Serotonin · Tryptamine · Tryptamine 5-hydroxylase

Communicated by D. Somers.

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Abbreviations

AADC	Aromatic L-amino acid decarboxylase
FW	Fresh weight
T5H	Tryptamine 5-hydroxylase
TDC	Tryptophan decarboxylase
TPH	Tryptophan hydroxylase
Trp	Tryptophan

Introduction

Serotonin (5-hydroxytryptamine) is a well-known neurotransmitter that regulates mood, sleep, and anxiety in mammals, as well as alcoholism in humans (Veenstra-VanderWeele et al. 2000). In plants, serotonin has been found in more than 42 species (Roshchina 2001) since it was first identified in plant fruit in 1954 (Bowden et al. 1954). Fruits and seeds are the major tissues in which serotonin occurs abundantly. Its quantities in plants vary greatly among species and tissues, ranging from 0.007 μg g^{-1} in fresh leaves to 2,000 μg g^{-1} in seeds of *Griffonia simplicifolia* (Fellows and Bell 1970). Diverse physiological roles of serotonin in plants have been postulated. Serotonin may act as a growth regulator stimulating the growth of roots (Csaba and Pal 1982) and the hook of oat coleoptiles (Niaussat et al. 1958). It also stimulates the germination of both radish seeds (Roshchina 2001) and the pollen of *Hippeastrum hybridum* (Roshchina and Melnikova 1998). Furthermore, serotonin is implicated in the exudation of xylem sap from roots, flowering, ion permeability, and in a protective role as an antioxidant (Odjakova and Hadjiivanova 1997; Roshchina 2001).

In animals, serotonin is synthesized from tryptophan in two successive steps involving tryptophan hydroxylase

(TPH; EC 1.14.16.4) and aromatic L-amino acid decarboxylase (AADC; EC 4.1.1.28) in which tryptophan hydroxylase acts as the rate-limiting enzyme (Veenstra-VanderWeele et al. 2000; Fig. 1). In plants, serotonin is purportedly synthesized in a different manner whereby tryptophan is first catalyzed into tryptamine by tryptophan decarboxylase (TDC; EC 4.1.1.28), followed by the catalysis of tryptamine by tryptamine 5-hydroxylase (T5H) to form serotonin (Schröder et al. 1999). However, in St. John's wort (*Hypericum perforatum*), serotonin synthesis was reported to occur via 5-hydroxytryptophan as in mammals (Murch et al. 2000).

Although the serotonin content in many plant species has been quantified (Badria 2002; Kang and Back 2006), no studies have reported the levels of its biosynthetic enzyme T5H. In particular, the enzymatic features, including substrate specificity of T5H, a member of the cytochrome P450 family (Schröder et al. 1999), have not yet been characterized. Our objective was to investigate the level of serotonin in rice plants in parallel with T5H enzyme activity and to characterize the substrate specificity of T5H. In addition, we measured the serotonin content and T5H activity upon exogenous application of tryptophan and/or tryptamine to determine whether the precursor or substrate affects serotonin biosynthesis and T5H enzyme activity in rice seedlings.

Materials and methods

Plant growth and substrate treatment

Seeds of wild-type rice (*Oryza sativa* cv. Dongjin) were husked and sterilized with 70% ethanol for 1 min and then with 2% sodium hypochlorite for 50 min. The seeds were further washed three times with sterilized water and sown on 1.5% agar plates supplemented with or without various concentrations of substrates. These were incubated at 25°C under 12 h light/12 h dark for 10 days. The tissues were then frozen using liquid nitrogen and kept at –80°C for further analysis.

Preparation of microsomal and soluble proteins

Rice seedlings (0.1 g) were harvested and ground in homogenization buffer (0.1 M HEPES, pH 7.5; 25 mM mercaptoethanol; 1 mM dithiothreitol) using a mortar and pestle. The homogenate was filtered through two layers of Miracloth (CalBiochem, La Jolla, CA, USA) and centrifuged for 10 min at 3,000×*g*. The supernatant was centrifuged for 60 min at 100,000×*g* to obtain a crude microsomal pellet. The pellets were dissolved in homogenization buffer and used for microsomal fractions.

Measurement of T5H enzyme activity

Tissue samples (0.2 g) were harvested and ground in homogenization buffer (0.1 M HEPES, pH 7.5; 25 mM mercaptoethanol; 1 mM dithiothreitol) using a mortar and pestle. The supernatants were cleared by centrifugation at 18,000×*g* for 20 min and incubated in a reaction mixture containing 5 mM tryptamine, 50 mM HEPES (pH 7.0), 5 mM dithiothreitol, 0.01 mM $Fe(NH_4)_2(SO_4)_2$, 2 mM NADH, and 0.5 mM tetrahydropterine at 37°C for 30 min (Vrana et al. 1993; Schröder et al. 1999). After the addition of 20-µl of 16.7 M acetic acid to halt the reaction, the volume of the mixture was increased to 500 µl using methanol, and a 20-µl aliquot was subjected to high-per-

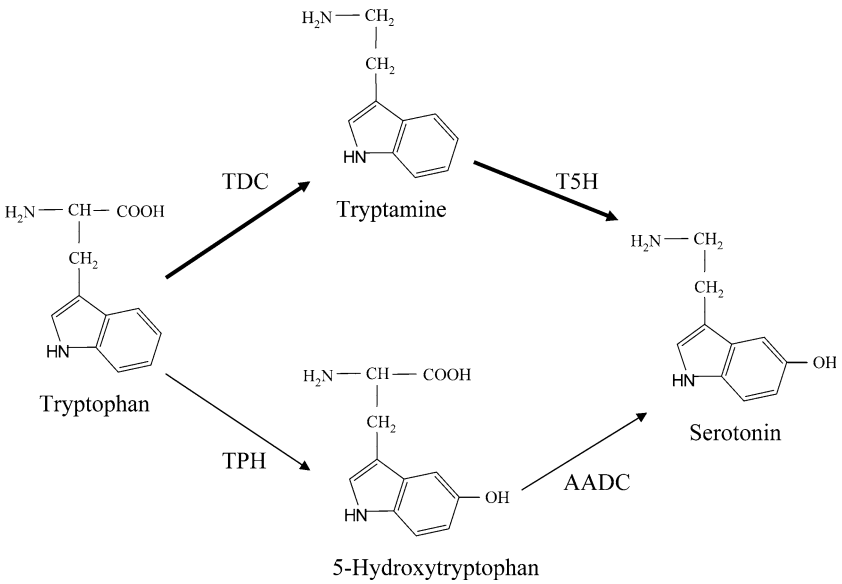

Fig. 1 Biosynthetic pathway of serotonin in plants. Serotonin is biosynthesized from tryptophan through sequential catalysis by two enzymes, tryptophan decarboxylase (*TDC*) and tryptamine 5-hydroxylase (*T5H*). In animals, serotonin is synthesized by tryptophan 5-hydroxylase (*TPH*) and aromatic L-amino acid decarboxylase (*AADC*; Groβe 1982; Veenstra-VanderWeele et al. 2000)

formance liquid chromatography (HPLC). The enzymatic product of serotonin was analyzed at 280 nm under the same HPLC conditions described for serotonin detection.

Serotonin and tryptamine analysis

Serotonin was analyzed as follows. Tissues (0.3 g) were ground with 6 ml (20× vol) of methanol and immediately centrifuged at 13,500×*g* for 5 min. The supernatant was filtered through Millex-LG (Waters, Milford, MA, USA), and 1.5 ml (1/4 vol) of distilled water was added to the filtered solution. The sample was passed through a Sep-pak C18 (Waters) cartridge, and the cartridge was washed with 7.5 ml (i.e., same volume as sample) of 80% methanol. The passing and washing solutions were combined. The mixture was evaporated to dryness and dissolved in 0.5 ml of 50% methanol. This solution was analyzed using reversed-phase HPLC (Waters 2690 system) using a Waters Atlantis C18 column (3.9 × 150 mm^2) with isocratic elution of 5% (v/v) methanol in water containing 0.3% trifluoroacetic acid at a flow rate of 0.8 ml min^{-1}. The eluates were detected at 280 nm.

To measure tryptamine, 1 ml of methanol was added to 0.1 g of the various tissues. The mixture was further homogenized for 5 min at room temperature. The homogenate was centrifuged for 15 min at 12,000×*g*. The supernatant was collected, and the residue was extracted with 50% methanol. The mixture was evaporated to dryness and dissolved in 200 µl of 50% methanol. This solution was analyzed using reversed-phase HPLC as described above. Serotonin and tryptamine standards were purchased from Sigma (St. Louis, MO, USA).

Results

Activities of soluble and microsomal T5H in rice seedlings

Protein extracts from rice seedlings were divided into soluble and microsomal fractions to determine whether cytochrome P450 T5H enzyme activity was associated with the membrane fraction. We used protein extracts from rice roots for this analysis because the roots showed high T5H enzyme activity. Microsomal T5H activity had a low specific activity of approximately 75 pkat mg^{-1} protein, whereas soluble T5H showed four times greater activity than microsomal T5H activity (Fig. 2). The relatively low level of microsomal T5H activity suggests that the T5H enzyme may be only somewhat associated with membranes either by binding to anchor proteins or by electrostatic binding to the lipid bilayer. The purity of the soluble

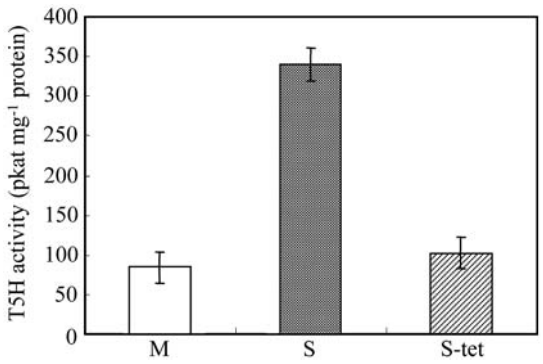

Fig. 2 Specific activity of T5H. Rice roots were homogenized and centrifuged for 60 min at 100,000×*g* to obtain a crude microsomal pellet. The pellets were dissolved in 100 µl of homogenization buffer and used as microsomal fractions. The supernatant extracts from the centrifugation were used as soluble protein fractions. The soluble (*S*), membrane-bound fractions (*M*), and soluble in the absence of tetrahydropterine (*S-tet*) were assayed in 10-µl aliquots as described in the "Materials and methods". Data represent the mean ± standard deviation of two replicate samples

fraction was confirmed by measuring tyramine *N*-hydroxycinnamoyltransferase (THT), which was only found in the soluble fraction of rice cell extracts (Lee et al. 2007). The soluble fraction from rice roots had high THT activity of up to 15 pkat mg^{-1} protein, but the microsomal fraction showed no detectable THT activity. These data clearly suggest that the presence of soluble T5H activity was not due to contamination by the microsomal fraction. In addition, T5H activity decreased by 70% in the absence of tetrahydropterine (Fig. 2), indicating that the T5H enzyme belongs to the family of amino acid hydroxylases such as phenylalanine hydroxylase, tyrosine hydroxylase, and tryptophan hydroxylase because the amino acid hydroxylases require tetrahydrobiopterin as a co-substrate for their enzymatic reactions (Fitzpatrick 1999).

Effect of various substrates on T5H activity

The substrate specificity of T5H was examined by measuring the conversion of tryptamine to serotonin in the presence of several compounds derived from aromatic amino acids (Fig. 3). Dopamine, which is a decarboxylated form of dihydroxyphenylalanine, had no effect on T5H activity in the presence of both a tryptamine substrate (5 mM) and a series of dopamine concentrations ranging from 0.5 to 10 mM. However, octopamine, which is a 5-hydroxylated form of tyramine, began to inhibit T5H at 0.5 mM and inhibited T5H by 35% at 1 mM. Tryptophan also inhibited T5H activity by 30% at 0.5 mM; 5-OH-tryptophan showed a similar inhibitory effect on T5H activity. However, tyramine, which is a decarboxylated form of tyrosine, inhibited T5H by 15% at 0.5 mM.

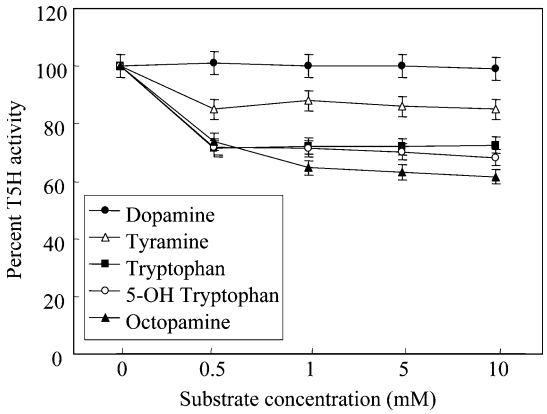

Fig. 3 Effects of various substrates on T5H activity. Assays were performed in the presence of nonspecific substrates. At the indicated concentrations of the nonspecific substrates, T5H was assayed and expressed as a percentage relative to the activity measured in the absence of the nonspecific substrates. Data are the mean ± standard deviation of three replicate samples

This level of T5H inhibition was 50% of that achieved by tryptophan or tryptophan-derived substrates such as 5-OH-tryptophan and octopamine. These data indicate that the T5H enzyme displays a broad range of substrate specificity, although tryptamine is predominantly accepted as the major substrate of the T5H enzyme. However, the definite conclusion requires further in-depth study to identify new products generated by T5H enzyme in the presence of other possible competitive substrates shown above.

Analyses of serotonin content and T5H enzyme activity in rice plants

Ten-day-old rice seedlings were dissected into three parts: leaves, stems, and roots. The levels of serotonin were quantified using HPLC (Table 1). The highest level of serotonin was detected in roots at 19.5 μg g^{-1} fresh weight (fw); stems and leaves had five and three times less serotonin, respectively, compared to roots. Furthermore, we quantified the serotonin content of 19-week-old hulls and immature seeds. The hulls contained serotonin levels as high as 3.6 μg g^{-1} fw, which were comparable to those in stems. Seeds had the lowest serotonin levels compared to those found in other tissues.

Table 1 Tissue-specific enzyme activity of tryptamine 5-hydroxylase (T5H) with serotonin levels in rice plants

Tissue	Serotonin (μg g^{-1} fresh weight)	T5H enzyme activity (pkat mg^{-1} protein)
Leaf	6.1 ± 1.4	78 ± 11
Stem	4.3 ± 1.1	134 ± 18
Root	19.5 ± 4.8	343 ± 23
Hull	3.6 ± 0.8	72 ± 12
Seed	0.2 ± 0.1	78 ± 10

Leaf, stem, and root tissues were obtained from 10-day-old rice seedlings grown on 1.5% agar. Hull and seed tissues obtained were from immature seeds 15 days after anthesis. The data represent the mean ± standard deviation of three replicate samples

We measured the specific activity of T5H to determine whether the levels of serotonin in different tissues were correlated with T5H enzyme activity. Similar to the serotonin content, the T5H activity was highest in roots at four times that in other tissues on average. Nineteen-week-old hulls and seeds were still green and immature, respectively, and proteins could be extracted for the enzyme assay. In contrast to the lowest serotonin level of ~0.2 μg g^{-1} fw in seeds, a high level of T5H activity was observed in seeds. The low level of serotonin in rice seeds is in contrast to that in other plants in which serotonin accumulates mainly in the reproductive organs (Roshchina 2001).

Serotonin synthesis upon treatment with exogenous substrates

To determine whether the exogenous application of tryptophan and/or tryptamine in rice seedlings can alter the synthesis of serotonin, tissues of 10-day-old rice seedlings grown on 1.5% agar supplemented with tryptophan and/or tryptamine were extracted with methanol, and the extracts were analyzed to quantify the serotonin content. Upon tryptophan and/or tryptamine supplementation, the growth of rice seedlings was significantly inhibited, and both substrates had detrimental effects, particularly on root growth (Fig. 4). Tryptophan had a greater inhibitory effect than tryptamine on rice root growth. The serotonin levels in leaves, stems, and roots of plants grown in the presence of 1 mM tryptophan were not altered compared to those grown in the absence of tryptophan (Fig. 5). However, 5 mM tryptophan increased the serotonin content in roots by two times to 60 μg g^{-1} fw. In contrast to tryptophan, 1 mM tryptamine resulted in the accumulation of serotonin at 220, 300, and 600 μg g^{-1} fw in leaves, stems, and roots, respectively, whereas 5 mM tryptamine resulted in even greater serotonin synthesis at 522, 1,000, and 6,500 μg g^{-1} fw in leaves, stems, and roots, respectively. In the presence of tryptamine, the highest serotonin contents were detected in roots, followed by stems and leaves, and the levels were well matched by those of T5H-specific activity.

Rice seedlings grown in the presence of 5 mM tryptophan plus 5 mM tryptamine had lower serotonin contents than rice seedlings grown in the presence of 5 mM tryptamine only. For example, leaves and stems of plants grown on 1 mM tryptophan plus 1 mM tryptamine produced 2.7 and 1.7 times less serotonin, respectively, than those of plants grown on 1 mM tryptamine only, but roots exhibited similar serotonin levels of ~700 μg g^{-1} fw between these

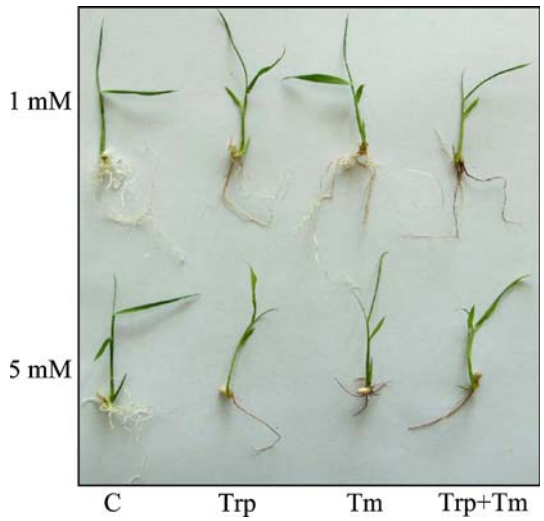

Fig. 4 Phenotypic characteristics of rice seedlings grown in the presence of tryptophan (*Trp*) and/or tryptamine (*Tm*). Seeds were grown on 1.5% agar at 28°C under 12 h dark/12 h light. The photograph was taken 10 days after seeding

two treatments. Striking differences were observed using 5 mM concentrations. Tryptophan plus tryptamine resulted in serotonin levels eight, four, and two times lower in the leaves, stems, and roots, respectively, than those observed with tryptamine treatment alone. These data were consistent with the results of substrate specificity analyses, which showed that T5H activity was negatively affected by the presence of tryptophan (Fig. 3). These data clearly indicate that the level of tryptamine plays a pivotal role in serotonin synthesis and seems to act as a bottleneck intermediate substrate. Furthermore, T5H is constitutively expressed in rice tissues, although the levels vary among the tissues.

T5H enzyme activity and tryptamine content upon treatment with various substrates

To determine why rice seedlings produce low levels of serotonin upon treatment with both 5 mM tryptophan plus 5 mM tryptamine compared to those treated with 5 mM tryptamine only, we analyzed the tryptamine content and T5H enzyme activity in various tissues challenged with tryptamine or/and tryptophan. Of the rice tissues examined, roots showed the most dramatic variation in both T5H enzyme activity and tryptamine content. For example, rice roots grown in the presence of tryptamine and/or tryptophan exhibited highly variable T5H enzyme activity ranging from 171 to 960 pkat mg^{-1} protein, whereas leaves and stems did not exhibit significant variation in T5H activity. In the presence of 5 mM tryptophan, T5H activity decreased by two times in roots compared to the control, whereas in the presence of 5 mM tryptamine, T5H activity increased by 2.8 times compared to the control. This corresponded with data derived from the substrate-specificity analysis of T5H in which T5H activity was inhibited by tryptophan (Fig. 3). In contrast to the 2.8-times increase with 5 mM tryptamine, a slight increase of about 1.2 times occurred in T5H activity in roots treated with 5 mM tryptophan plus 5 mM tryptamine. The increase in T5H activity in the presence of tryptamine leads to a large accumulation of serotonin in roots both in the presence of tryptamine alone and with tryptamine plus tryptophan. In contrast, leaves and stems did not exhibit an increase in T5H activity with tryptamine treatment, but had higher serotonin levels than the control tissues (Fig. 4, Table 2).

To examine whether the increase in serotonin levels was associated with tryptamine levels, we quantified the tryptamine content of the tissues (Table 2). As expected, tryptamine levels were closely coupled to the levels of serotonin in all tissues, with the exception of roots treated with tryptophan. The level of tryptamine was ~23 μg g^{-1} fw in roots in the control, but this level was only 4 μg g^{-1} fw in roots in the presence of 5 mM tryptophan. However, this was in contrast to the levels of serotonin in the control and 5 mM tryptophan treatment. The mechanism by which the serotonin level was doubled in roots in the presence of 5 mM tryptophan is not clear; however, this increase may have been associated with the depletion of the tryptamine pool by six times in roots in the presence of tryptophan via either a simultaneous feedback inhibition of anthranilate

Table 2 Tryptamine 5-hydroxylase (T5H) enzyme activity and tryptamine content after the exogenous application of tryptophan and/or tryptamine

Treatment	T5H enzyme activity (pkat mg^{-1} protein)			Tryptamine content (μg g^{-1} fresh weight)		
	Leaf	Stem	Root	Leaf	Stem	Root
Control	73 ± 5	120 ± 10	343 ± 23	–	–	23
Tryptophan (5 mM)	71 ± 4	118 ± 12	171 ± 12	–	–	4
Tryptamine (5 mM)	79 ± 6	113 ± 8	960 ± 45	20	407	1,203
Tryptophan (5 mM) + tryptamine (5 mM)	78 ± 5	119 ± 3	427 ± 39	4	62	1,172

Tissues were obtained from 10-day-old rice seedlings grown on 1.5% agar supplemented with 5 mM tryptophan and/or tryptamine. The data represent the mean ± standard deviation of two replicate samples

synthase, which is a controlling enzyme in tryptophan biosynthesis (RadWanski and Last 1995), or the inhibition of TDC. Similar levels of tryptamine of ~1.2 mg g^{-1} fw were found in roots in the presence of either tryptamine alone or tryptamine plus tryptophan, whereas stems and leaves contained six times more tryptamine in the presence of tryptamine alone than with tryptamine plus tryptophan. This suggests that the movement of exogenously applied tryptamine from roots to shoots seems to be hindered by tryptophan, but further experiments are required to verify this postulate.

Discussion

Serotonin is a neurotransmitter that plays a key role in mood and liver regeneration in humans (Lesurtel et al. 2006). Its synthesis, regulation, and metabolism have been studied extensively in animals. However, no detailed studies on serotonin biosynthesis have been conducted in plants, although its occurrence in the plant kingdom is relatively well documented (Roshchina 2001). This lack of information on serotonin biosynthesis in plants may be a result of its lesser importance as a plant metabolite, as well as its pharmacological value in human health. Recent studies have shown that serotonin serves as a key substrate for the synthesis of serotonin derivatives such as feruloylserotonin and 4-coumaroylserotonin, which have strong antioxidative activity, chemotherapeutic effects, and atherosclerosis attenuation, in addition to antimicrobial activity against plant pathogens (Tanaka et al. 2003; Koyama et al. 2006).

Serotonin biosynthesis can occur via two metabolic pathways (Fig. 1). One pathway is that found in animals in which tryptophan is first hydroxylated at the C-5 position by the action of TPH, and is then further catalyzed into serotonin by AADC. In the other pathway, tryptophan is first converted into tryptamine, and then serotonin by the two enzymes TDC and T5H. In plants, tryptophan is preferentially decarboxylated into tryptamine and then hydroxylated into serotonin (Groβe 1982; Facchini et al. 2000). Initially, we attempted to measure TPH activity in rice seedlings using ^{3}H tryptophan (Vrana et al. 1993), but found no detectable activity (data not shown), confirming that serotonin is not synthesized via 5-OH tryptophan in rice. The first enzymatic step for serotonin synthesis in rice is catalyzed by TDC, which is present at very low levels in rice tissues (Ueno et al. 2003; Jang et al. 2004). The fact that the highest serotonin levels were found in the rice roots may indicate its possible involvement in root growth and development as mentioned by previous reports (Murch et al. 2001; Hernandez-Ruiz et al. 2005).

T5H is a terminal enzyme responsible for serotonin synthesis using tryptamine as a substrate. The T5H enzyme occurred predominantly as a soluble protein and exhibited broad substrate specificity toward aromatic amino acids and their derived amine compounds such as tyramine and octopamine. However, dopamine derived from phenylalanine had no effect on T5H enzyme catalysis, suggesting that T5H harbors a certain level of substrate specificity among aromatic amino acid derivatives (Fig. 3). T5H enzyme activity was closely associated with the levels of serotonin; roots had the highest T5H activity with maximal accumulation of serotonin (Table 1). The enriched pool of serotonin in rice roots should be closely coupled with the synthesis of various serotonin derivatives such as feruloylserotonin and 4-coumaroylserotonin, catalyzed by serotonin *N*-hydroxycinnamoyl transferase (SHT). SHT and TDC occur preferentially in rice roots (Jang et al. 2004). The peak activities of T5H, TDC, and SHT within roots clearly suggest that rice roots are the major location for the synthesis of serotonin and its derivatives. The levels of serotonin were influenced greatly by tryptamine and little by tryptophan (Fig. 5). The copious production of serotonin at ~6.5 and 1 mg g^{-1} fw in roots and stems, respectively, in the presence of 5 mM tryptamine suggests that the T5H enzyme is highly expressed in roots and that tryptamine absorbed through the roots is able to move to

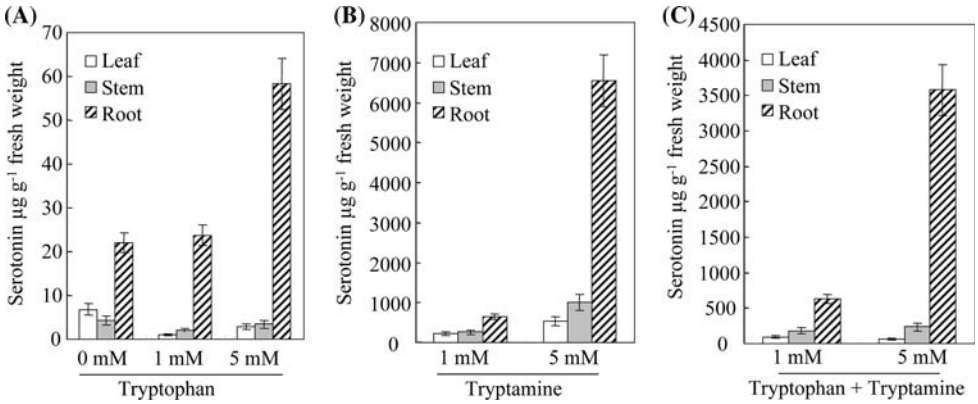

Fig. 5 Serotonin content in various tissues of rice seedlings grown on 1.5% agar supplemented with tryptophan and/or tryptamine. Tissues were harvested from 10-day-old rice seedlings and subjected to high-performance liquid chromatography

the stems, where it is catalyzed into serotonin by T5H expressed in the stems. This also indicates that exogenously applied tryptamine can be readily used by the T5H enzyme in cells. This is consistent with previous studies reporting that tyramine can be transported from roots to stems and leaves of rice seedlings in which it serves as substrate for the synthesis of tyramine derivatives such as feruloyltyramine and 4-coumaroyltyramine (Lee et al. 2007). The presence of tryptophan stimulated serotonin synthesis only in roots treated with 5 mM tryptophan. The enhanced synthesis of serotonin in response to tryptophan treatment is in contrast to the twofold reduction in T5H enzyme activity. In addition, exogenous tryptophan had a depleting effect on the endogenous tryptamine content (Table 2). It is conceivable that excess tryptophan inhibits not only anthranilate synthase, which is the first enzyme in tryptophan biosynthesis (Yao et al. 1995; Tsai et al. 2005), but also various enzyme(s) responsible for serotonin catabolism or the synthesis of serotonin derivatives. However, this phenomenon remains to be clarified. Our results show that serotonin synthesis occurs actively in rice seedlings and requires tryptamine as a direct substrate for the T5H enzyme. It is therefore highly probable that the tryptamine content is tightly coupled with that of serotonin.

Acknowledgments This study was supported by the SRC program from the Korea Science and Engineering Foundation (KOSEF) through the Agricultural Plant Stress Research Center Program grant no. R11-2001-09203001-0.

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