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A nonproteinogenic amino acid, β-tyrosine, accumulates in young rice leaves via long-distance phloem transport from mature leaves

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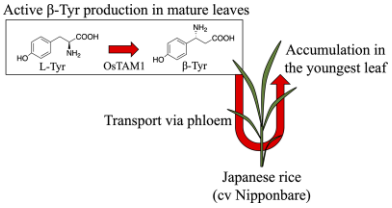
ABSTRACT

Oryza sativa L. ssp. *japonica* cv. Nipponbare produces a nonproteinogenic amino acid (3R)-β-tyrosine from L-tyrosine by tyrosine aminomutase (OsTAM1). However, physiological and ecological function(s) of β-tyrosine have remained obscure. Often an improved understanding of metabolite localization and transport can aid in design of experiments to test physiological functions. In the current study, we investigated the distribution pattern of β-tyrosine in rice seedlings and found that β-tyrosine is most abundant in the youngest leaves. Based upon observations of high TAM1 activity in mature leaves, we hypothesized that β-tyrosine is transported from mature leaves to young leaves. Patterns of predominant mature synthesis and young leaf accumulation were supported by stable isotope studies using labeled β-tyrosine and the removal of mature leaves. Stem exudate analyses was also consistent with β-tyrosine transport through phloem. Thus, we identify young leaves as a key target in efforts to understand the biological function(s) of β-tyrosine in rice.

Graphical Abstract



Japonica rice cultivar Nipponbare actively produces β-tyrosine in mature leaves and accumulates it in the youngest leaf via long-distance phloem transport.

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Keywords: *Oryza sativa* L. ssp. *japonica*, β-tyrosine, transport

For environmental adaptation, plants have developed a variety of physical and chemical defense systems against biotic and abiotic stresses. For chemical defenses, plants produce a vast number of specialized metabolites, ranging from those widely distributed in the plant kingdom to those that are species specific. Defense-related specialized metabolites commonly show distinctive localization patterns in plant tissues according to their functions in the protection of valuable and vulnerable parts (McKey 1974; Ohnmeiss and Baldwin 2000). Some defensive biochemicals are highly localized in the same cells where they are synthesized. For instance, 9-lipoxygenase-derived cyclopentenones and cyclopentanones in maize (*Zea mays*) accumulate in fungal-infected tissue and act as cytotoxic phytoalexins and transcriptional mediators of defense genes (Christensen *et al.* 2015). Sulforaphane in Arabidopsis (*Arabidopsis thaliana*) is also synthesized by leaf tissues undergoing pathogen-induced hypersensitive responses and induces cell death (Andersson *et al.* 2015). In contrast, other compounds localize to specific parts by reallocation from biosynthetically active organs through long-distance transport. For example, nicotine, which poisons acetylcholine receptors of all heterotrophs with neuromuscular junctions, is biosynthesized in roots and transported to damaged leaves via xylem in tobacco (Dawson 1942; Baldwin 1989; Baldwin 2001). Berberin, a strong antimicrobial in *Coptis japonica*, is also synthesized in lateral roots and transported to rhizomes (Fujiwara *et al.* 1993; Iwasa *et al.* 1998; Shitan *et al.* 2003). Furthermore, nonproteinogenic amino acid L-canavanine, the analog of L-arginine, in jack bean (*Canavalia ensiformis* (L.) DC. [Leguminosae]) is transported from cotyledons to aerial parts (Rosenthal and Rhodes 1984). L-Canavanine displays both significant toxicity to a wide range of organisms due to misincorporation into proteins and also functions in a nitrogen storage (Rosenthal 1972; Nakajima, Hiradate and Fujii 2001; Huang, Jander and de Vos 2011).

Predictably, rice (*Oryza sativa* L.) also produces a rich and diverse array of defense compounds. Recently, we identified the nonproteinogenic amino acid β-tyrosine, an analog of L-tyrosine, in most of *japonica* rice varieties as a new biochemical defense in the plant kingdom that is positively regulated following jasmonic acid treatment (Yan *et al.* 2015; Jander *et al.* 2020). β-Tyrosine in rice is formed through transposition of an α-amino group of L-tyrosine to β-position by tyrosine aminomutase (OsTAM1) with *R* enantioselectivity of 94% enantiomeric excess (e.e.), which is much higher than that of TAM from bacteria *Chondromyces crocatus* with *R* enantioselectivity of 70% e.e. (Wanninayake and Walker 2013; Yan *et al.* 2015; Walter, King and Walker 2016). In *C. crocatus*, β-tyrosine is incorporated into chondramides, depsipeptides with antifungal and cytostatic properties (Kunze *et al.* 1995; Rachid *et al.* 2007). Similarly, β-tyrosine is a component of antibiotics produced by microorganisms, such as edeine A and B in *Bacillus brevis* and myxovalargin in *Myxococcus fulvus* (Parry and Kurylo-Borowska 1980; Krug and Müller 2009). However, the predominant physiological and ecological function(s) of β-tyrosine in rice remain less clear. The aim of this study is to create an improved foundational knowledge β-tyrosine distribution in rice, identify mechanism(s) underlying the observed patterns, and discern potential insights into functional role(s). Here, we investigated tissues that are biosynthetically active in the production of β-tyrosine, tissues displaying predominant accumulation, and predictable functional location of this nonproteinogenic amino acid in *japonica* rice cultivar Nipponbare. Collectively our results are consistent with β-tyrosine transport between the youngest leaves (the most newly developed) and mature leaves (previously developed) based on tracking labeled β-tyrosine stable isotopes and the removal of mature leaves. Analyses of excised stem exudates further supported the phloem as involved in leaf-to-leaf transport.

Materials and methods

Plant growth conditions

Husked rice seeds (*Oryza sativa* cv. Nipponbare) were shaken for 1 min in 70% ethanol, shaken for 20 min in 33% antiformin (Fujifilm Wako Pure Chemical Co., Osaka, Japan), and rinsed with tap water 5 times. The seeds were transferred to a Petri dish (9 cm i.d.), soaked in tap water, and incubated at 30 °C overnight. Two germinated seeds were planted in soil Bonsol No. 2 (Sumitomo Kagaku Kogyo Co., Ltd, Osaka, Japan) per plastic pot (6 × 6 × 5 cm) separated by a plastic board. The pots were placed in a growth chamber LPH-410S (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) at 30 °C with a 16 h/8 h light/dark cycle and watered from below.

β-Tyrosine and L-tyrosine localization

Three leaves, the youngest, the second youngest, and the third youngest leaves, from the fifth, the sixth, and the seventh leaf stage seedlings were weighed and placed in 2 mL tubes. The leaves were homogenized with 2 or 3 steel beads (5 mm) for 3-9 min depending on leaf ages at 3000 strokes/min by beads crusher μT-12 (TAITEC Co., Saitama, Japan) in 80% methanol (5 μL/mg of fresh weight) containing 50 μM D-2-phenylglycine (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) as an internal standard. The homogenates were centrifuged at 3000 × *g* for 5 min, filtered through a DISMIC-13HP syringe filter (0.45 μm, Toyo Roshi Co., Ltd., Tokyo, Japan), and derivatized with 6-aminoquinolyl-*N*-succinimidyl carbamate (AQC), which was synthesized as reported previously (Cohen and Michaud 1993), as follows. Ten microliters of the extracts were mixed with 70 μL of 200 mM borate buffer (pH 8.95) and 20 μL of AQC in acetonitrile (3 mg/mL), immediately vortexed, and incubated at 55 °C for 10 min. After centrifugation at 4300 × g for 5 min with CFM-1300 (Iwaki; Asahi Techno Glass, Chiba, Japan), 1 μL of the supernatant was analyzed using liquid chromatography-mass spectrometry (LC-MS).

TAM1 activity

The fourth, the fifth, and the sixth leaves from the sixth leaf stage seedlings were weighed, frozen in liquid nitrogen in 2 mL tubes, and crushed with 2 steel balls for 10 s at 2500 strokes/min 3 times. Ground tissues were extracted by 10 s vortexing in 200 mM borate buffer (pH 8.8, 10 μL/mg of fresh weight) containing 1% (v/v) 100× Protease Inhibitor Cocktail (Fujifilm Wako Pure Chemical Co.), followed by centrifugation at 12 000 × *g* for 15 min at 4 °C. Two hundred and fifty microliters of the supernatants were mixed with 1000 μL of saturated ammonium sulfate solution (Fujifilm Wako Pure Chemical Co.) and placed at 4 °C for 18 h. The precipitates were collected by centrifugation at 10 000 × *g* for 20 min at 4 °C, washed twice with 80% saturated

ammonium sulfate solution, and dissolved in 250 µL of 200 mM borate buffer (pH 8.8) containing Protease Inhibitor Cocktail and 0.2% (v/v) Triton X-100 (Acros Organics, New Jersey, USA). After centrifugation at 10000 × *g* for 20 min at 4 °C, 50 µL of the supernatants were mixed with 50 µL of 200 mM borate buffer (pH 8.8) containing 2 mM L-Tyr (Nacalai tesque Inc., Kyoto, Japan) as the substrate and incubated at 40 °C for 18 h. The reaction tubes were then cooled on ice, heated at 100 °C for 10 min, and centrifuged at 12000 × *g* for 10 min. Ten microliters of the reaction mixture were mixed with 60 µL of 200 mM borate buffer (pH 8.95), 10 µL of 50 µM D-2-phenylglycine solution, and 20 µL of AQC solution. The reaction mixtures were centrifuged at 15000 × *g* for 15 min, and 1 µL of the supernatant was analyzed using LC-MS.

β-Tyrosine transport

A modified method of Grayson *et al.* was used for the [1, 2-$^{13}C_2$] β-tyrosine synthesis (Grayson, Roos and Osswald 2011). *p*-Hydroxybenzaldehyde (195 mg, 1.60 mmol, Fujifilm Wako Pure Chemical Co.) and ammonium acetate (491 mg, 6.37 mmol, Fujifilm Wako Pure Chemical Co.) were dissolved in 17.5 mL of distilled dry methanol and heated to reflux for 30 min under nitrogen atmosphere. To the solution was added dropwise [$^{13}C_3$] malonic acid (42 mg, 0.39 mmol, Cambridge Isotope Laboratories, Inc., Andover, MA, USA) dissolved in 2.5 mL of distilled dry methanol. The reaction mixture was refluxed for another 24 h, concentrated *in vacuo*, and partitioned between water (pH 3) and ethyl acetate. The aqueous layer was then evaporated, dissolved in 0.1 M HCl, and applied on an Oasis MCX column (500 mg, Waters Corporation, Milford, MA, USA) in 8 separate times. The column was washed with 4 mL of 2% (v/v) formic acid (Nacalai tesque Inc.) and 4 mL of methanol, and β-tyrosine was eluted with 4 mL of 50% methanol containing 4 M NH_3 (Nacalai tesque Inc.). Solvents were evaporated to afford [1, 2-$^{13}C_2$] β-tyrosine ammonium salt (31 mg, yield 39%). ^{1}H-NMR spectra were recorded with acetone at 2.22 ppm as internal standard in deuterium oxide (>99.9% D, Euriso-Top, Saint-Aubin, France) by using a Bruker AV-III NMR spectrometer (400 MHz, Bruker BioSpin K.K., Kanagawa, Japan). δ: 7.34 (d, 2H, J = 8.6 Hz), 6.93 (d, 2H, J = 8.6 Hz), 4.58 (m, 1H), 2.89 (dddd, 1H, $^1J_{CH}$ = 127.4 Hz, J = 16.1 Hz, J = 8.2 Hz, $^2J_{CCH}$ = 6.1 Hz), 2.78 (ddt, 1H, $^1J_{CH}$ = 130.2 Hz, J = 16.2 Hz, $^2J_{CCH}$ and J_{HH} = 6.4 Hz) (Figure S1). ^{13}C-NMR spectra for β-tyrosine standard, β-tyrosine ammonium salt, and synthesized [1, 2-$^{13}C_2$] β-tyrosine ammonium salt were recorded in methanol-d_4 (>99.8% D, Euriso-Top) with 49.15 ppm (CD_3OD) as internal standard by the same spectrometer. Based on observed downfield shifts of carboxyl group, C_α, and C_β of synthesized [1, 2-$^{13}C_2$] β-tyrosine (Figure S2), we determined synthesized [1, 2-$^{13}C_2$] β-tyrosine as ammonium salt (Hagen and Roberts 1969; Batchelor, Feeney and Roberts 1975). β-Tyrosine standard was prepared as follows. Boc-β-Tyrosine (Sigma-Aldrich Co., St. Louis, MO) in dichloromethane: methanol (9:1) was cooled to 0 °C and was added to precooled trifluoroacetic acid (an equal amount to the solvent). The mixture was stirred at 0 °C for 1min and at room temperature for another 5 min. After confirming the consumption of Boc-β-tyrosine with thin layer chromatography (TLC), the solution was evaporated to afford β-tyrosine standard. β-Tyrosine ammonium salt was obtained by adding excess ammonia solution to β-tyrosine standard.

The apical 6 cm of the fifth leaves of the fifth leaf stage seedlings were abraded by the slightly modified procedure of Sovonick, Geiger and Fellows (1974). In brief, the leaves were rubbed with 300 mesh alumina paste (Fujifilm Wako Pure Chemical Co.) 5 times, rinsed with distilled water, and immersed in 0.5% ethanol containing 3 mM [1, 2-$^{13}C_2$] β-tyrosine ammonium salt (Figure S3). Controls were immersed in 0.5% ethanol. The seedlings were allowed to stand for 3 days, and the fourth leaves and the newly expanded sixth leaves were analyzed using LC-MS combined with ion trap and time-of-flight (LC-IT-TOF-MS). β-Tyrosine extraction and derivatization were conducted as described in "*β-Tyrosine localization*," except that leaves were homogenized with the 3 steel balls for 3 min.

Removal of mature leaves

The fourth and fifth leaves were removed from the fifth leaf stage seedlings, and the seedlings were allowed to grow for 3 days. Newly expanded sixth leaves from leaves-excised seedlings and intact seedlings were analyzed using LC-MS as described in "*β-tyrosine transport*."

Analysis of phloem exudates

A method of Jiang *et al.* (2018) was slightly modified to collect phloem exudates. Briefly, stems of the sixth leaf stage seedlings were cut about 4 cm above ground, and some cotton pads in 2 mL tubes were put on the incisions of the seedlings (Figure S4). The seedlings were allowed to stand for 1 h and collected phloem exudates were spin-downed. About 60 µL of the aliquot collected from 3 seedlings were diluted with 140 µL of 0.2 M HCl, applied on an Oasis MCX column, and basic fractions obtained as described in "*β-Tyrosine transport*" were concentrated *in vacuo*. The residues were dissolved in 70 µL of 200 mM borate buffer (pH 8.95), followed by mixing with 10 µL of 50 µM D-2-phenylglycine solution and 20 µL of AQC solution. After centrifugation at 15000 × *g* for 15 min, 5 µL of the supernatants were analyzed using LC-IT-TOF-MS. For quantification of β-tyrosine, 10 µL of phloem exudates collected as described above from one seedling were mixed with 60 µL of 200 mM borate buffer (pH 8.95), 10 µL of 50 µM D-2-phenylglycine solution and 20 µL of AQC solution. After centrifugation at 15000 × *g* for 15 min, 1 µL of the supernatants were analyzed using LC-MS.

Chemical analysis

LC-MS was performed using a Prominence HPLC system (LC-20AD pump, SIL-20AC HT auto sampler, and CTO-20AC column oven; Shimadzu, Kyoto, Japan) coupled with a LCMS-2020 (Shimadzu). Separations were performed with an ODS column (Mightysil RP-18 GP 50 × 2.0 mm ID; Kanto Chemical Co., Inc., Tokyo, Japan) at 40 °C with flow rate at 0.2 mL/min. Solvent A was water containing 0.1% (v/v) formic acid and solvent B was acetonitrile containing 0.1% (v/v) formic acid, and the gradient was 10% B (0-2min), 10%-45% B (2-9 min). The MS was operated with positive electrospray ionization (ESI) in selected ion monitoring (SIM) mode (m/z = 352 for β-tyrosine and 322 for D-2-phenylglycine); nebulizer gas flow, 1.5 L/min; drying gas flow, 15 L/min; detector voltage, 1.85 kV; heat block temperature, 200 °C; desolvation line (DL) temperature, 250 °C.

LC-IT-TOF-MS was conducted with LCMS-IT-TOF (Shimadzu) equipped with the Prominence HPLC system. A column, temperature, flow rate, and solvents were the same as LC-MS. The gradient was 1% B (0-2min), 1%-15% B (2-9 min), and 15%-30% B (9-14 min). MS operating parameters were as follows: ESI positive ion mode; probe voltage, 4.50 kV; detector voltage, 1.73 kV; heat block temperature, 200 °C; curved desolvation line (CDL) temperature, 250 °C; nebulizer gas flow, 1.5 L/min. MS1: mass range,

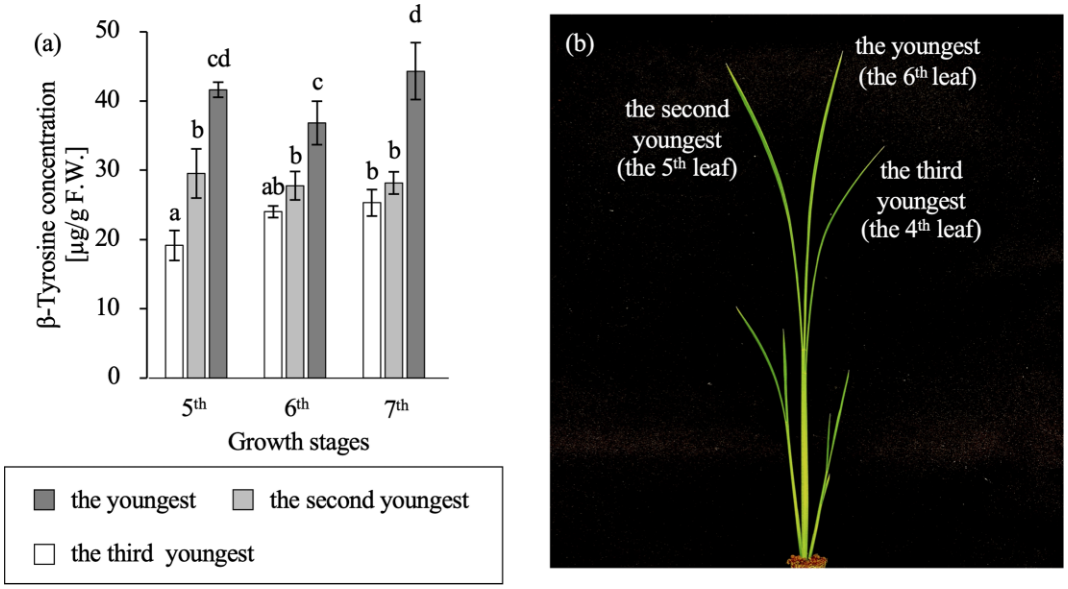

Figure 1. Quantification of β-tyrosine in leaves from different growth stage seedlings (the 5th, 6th and 7th leaf stage seedlings). (a) β-Tyrosine concentrations. Mean ± SD; $n = 4$. Different letters indicate significant differences ($P < .05$) by Tukey–Kramer test. (b) The youngest, the second youngest, and the third youngest leaf of 6th leaf stage seedlings.

m/z 200-400; repeat 2; ion accumulation time, 8 ms. MS2: mass range, *m/z* 50-400; repeat 2; precursor isolation, *m/z* = 352.12 for "*Analysis of phloem exudates*" and *m/z* = 354.10 for "*β-Tyrosine transport*"; precursor isolation range, 0.5 Da; ion accumulation time, 100 ms; energy, 200%; collision gas, 50%; q = 0.251 (45.0 kHz).

Statistical analysis

Tukey–Kramer test and Welch's *t*-test employed in this study were carried out with RStudio ver. 1.2.5033.

Results

To investigate the localization of β-tyrosine in rice shoots, β-tyrosine concentrations in the youngest leaves and mature leaves (the second youngest and the third youngest leaves) from seedlings at 3 different growth stages (5th, 6th, and 7th leaf stage seedlings) were quantified. LC-MS analysis revealed that β-tyrosine is most abundant in the youngest leaves and exists at lower concentrations in mature leaves at every growth stage investigated (5th, 6th, and 7th leaf stage seedlings) (Figure 1). This distribution pattern was also observed for L-tyrosine (Figure S5), implying that β-tyrosine may be involved in defenses in the youngest leaf where proteinogenic amino acids accumulate for leaf development. Furthermore, focusing on the youngest leaf of the fifth leaf stage seedlings and the second youngest leaf of the sixth leaf stage seedlings (both are the 5th leaves), for example, the concentration of β-tyrosine of the latter decreased about 33% when the 6th leaves (the new youngest leaves) were expanded.

It is known as a common pattern in plants that synthesis and storage of defense compounds are often separated into different organs (Jørgensen, Nour-Eldin and Halkier 2015). Based on public transcriptome databases (RiceXPro; http://ricexpro.dna.afrc.go.jp/) (Sato *et al.* 2013), *OsTAM1* is mainly expressed in shoots, but was not known in which leaves the OsTAM1 enzyme is primarily active. Therefore, we analyzed TAM1 activities of the youngest leaf and mature leaves to better understand the basis

Table 1. TAM1 activities of different aged leaves

Leaf age	TAM1 activity (μg h^{-1} g^{-1} FW)
4th	1.9 ± 0.2^a
5th	2.2 ± 0.3^a
6th	1.2 ± 0.3^b

Mean ± SD; $n = 5$. Different letters represent significant differences ($P < .01$) by Tukey–Kramer test.

of preferential β-tyrosine accumulation in the youngest leaves. Our analyses support significantly higher TAM1 activities in mature leaves than the youngest leaf (Table 1), which supports the idea that β-tyrosine is more actively synthesized in mature leaves than in the youngest leaf. Based on high β-tyrosine accumulation in the youngest leaves, and substantial decreases of β-tyrosine concentration and high TAM1 activities in mature leaves, we hypothesized that β-tyrosine is first biosynthesized in mature leaves and then is transported from the mature leaves to the youngest leaves promoting the observed distribution patterns in rice.

To verify our hypothesis, synthetic [1, 2-$^{13}C_2$] β-tyrosine ammonium salt was applied on the fifth leaf of fifth leaf stage seedlings. After incubation for 3 days, the newly expanded 6th leaf (4 replicates) was analyzed by LC-IT-TOF-MS. As shown in Figures 2a and b and S6, [1, 2-$^{13}C_2$] β-tyrosine was detected from the newly expanded leaf of the seedlings treated with [1, 2-$^{13}C_2$] β-tyrosine ammonium salt. These results demonstrate that β-tyrosine is preferentially transported from the mature leaves to the youngest leaf. Labeled β-tyrosine concentrations present in the 6th leaf were about twenty times higher than that of the 4th leaf, indicating that mature leaf β-tyrosine pools appear to be directed to the youngest leaves (6th leaves) (Figure 2c). Next, the newly expanded leaves (the 6th leaves) from seedlings with mature leaves (the 4th and 5th leaves) removed and intact seedlings were analyzed by LC-MS. As a result, the β-tyrosine concentration of the youngest leaf was significantly lower in the mature leaves-removed seedlings than that of intact seedlings (Figure 3).

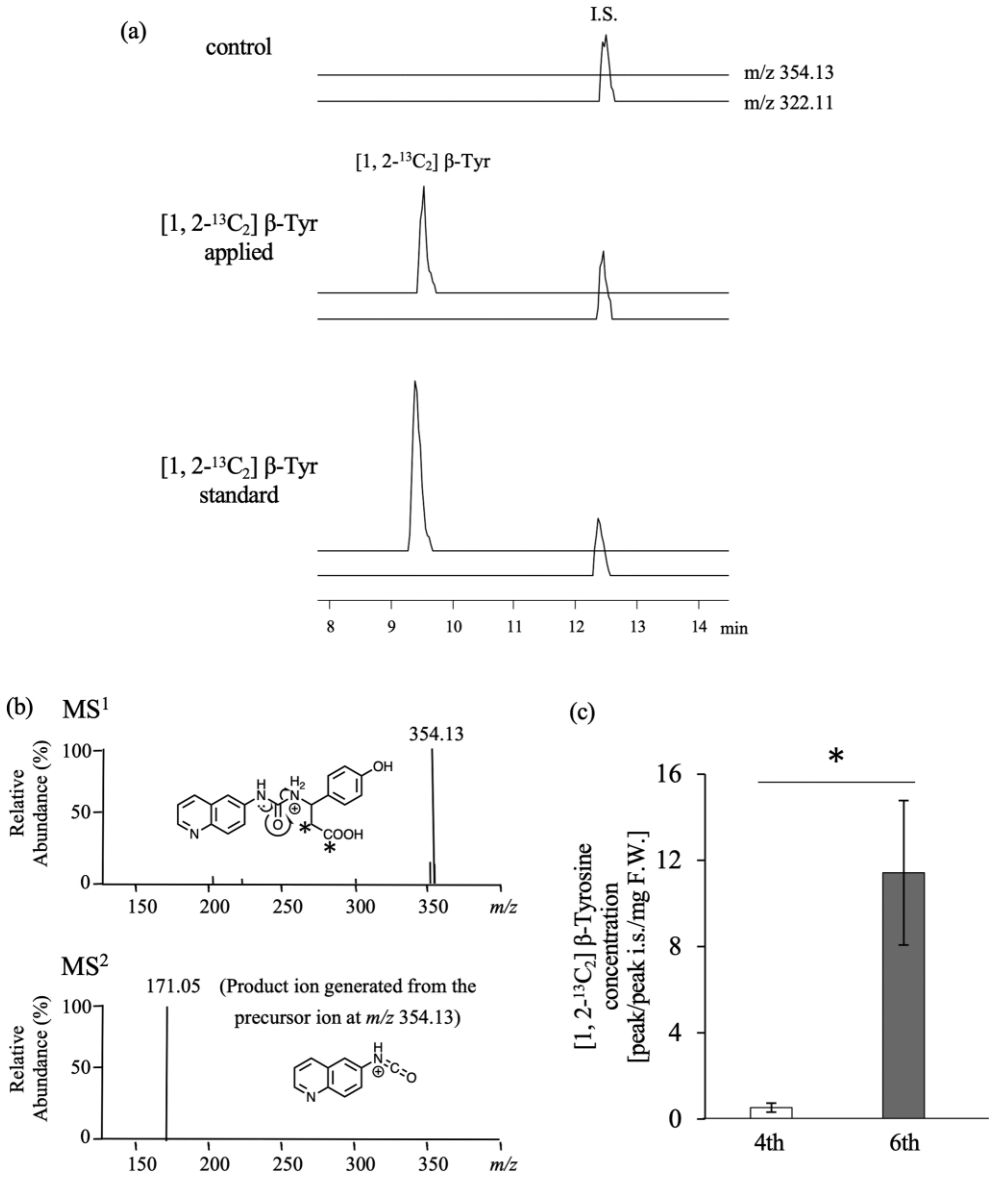

Figure 2. [1, 2-$^{13}C_2$] β-Tyrosine transport from a mature leaf to a young leaf. Labeled β-tyrosine was applied to the 5th leaf and it was preferentially transported to the emergent 6th leaf, compared to the 4th leaf. This experiment was performed with 4 biological replicates. (a) LC-IT-TOF-MS chromatograms (Extracted Ion Chromatograms XIC) of AQC derivatives: control seedlings extracts, [1, 2-$^{13}C_2$] β-tyrosine applied seedling extracts, and [1, 2-$^{13}C_2$] β-tyrosine standard. IS: internal standard (AQC-derivatized D-2-phenylglycine). (b) MS^1 (of the target peak) and MS^2 spectra of AQC-derivatized [1, 2-$^{13}C_2$] β-tyrosine obtained from seedling extracts with their predicted structures. "∗" refers to labeled carbons. (c) [1, 2-$^{13}C_2$] β-Tyrosine concentration of 4th and 6th leaves. Mean ± SD; $n = 4$. Welch's t-test ($P < .01$).

Proteinogenic amino acids synthesized in source tissues are transported to sink tissues through phloem (Tegeder and Masclaux-Daubresse 2018). Therefore, we predicted that β-tyrosine is also transported via phloem. Phloem exudates were collected by cutting the stem of the 6th leaf stage seedlings. LC-IT-TOF-MS and LC-MS analysis revealed that phloem exudates contained β-tyrosine and its concentration was 4 ± 1 ng/μL (Mean ± SD) phloem exudate (Figures 4 and S7).

These data are consistent with the hypothesis that β-tyrosine can be transported from mature leaves to the youngest leaf via phloem.

Discussion

In the current study, we investigated large scale distribution patterns of β-tyrosine in rice (cv Nipponbare) and considered mechanisms underlying the observed β-tyrosine localization to better understand potential physiological and ecological functions. In a series of experiments, we provide evidence that β-tyrosine accumulation in the youngest leaves is partially driven by long-distance transport from mature leaves via phloem.

In long-standing observations, summarized by McKey (1974), biochemical defenses are commonly greater in younger leaves than in mature leaves. Unavoidably, younger leaves have less mechanical protection and damage to them can disrupt the whole growth pattern of plant (McKey 1974). For example, *Cynoglossum officinale* and *Plantago lanceolata* have higher contents of pyrrolizidine alkaloids and iridoid glycosides, respectively, in younger leaves to protect against herbivore attack (Stamp and Bowers 1994; van Dam, Verpoorte and van Der Meijden 1994). In the current study, β-tyrosine concentration

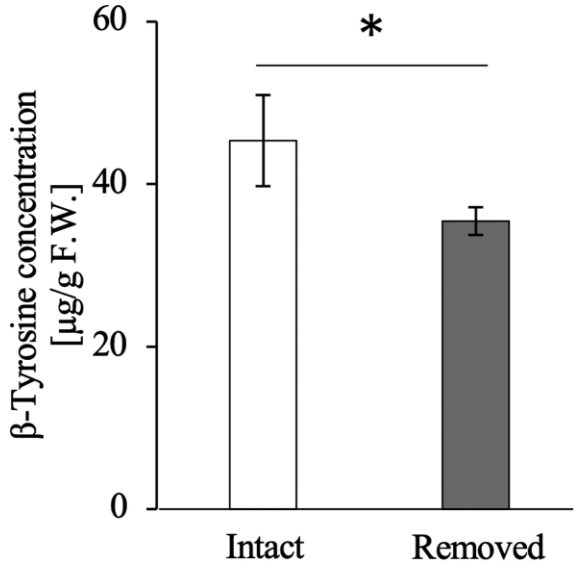

Figure 3. β-Tyrosine concentrations of newly expanded leaves from intact and mature leaf-removed seedlings. Mean ± SD; $n = 6$. Welch's t-test ($P < .01$).

was also the highest in the youngest leaf of 5th, 6th, and 7th leaf stage seedlings of Nipponbare (Figure 1). Given the significant inhibitory activity of β-tyrosine against *Pseudomonas syringae*, a common pathogen of multiple plant species *in vitro* (Yan *et al.* 2015), β-tyrosine appears capable of protecting the youngest leaf from microbial attack. However, further studies are needed to better understand the range of microorganisms, including fungi, that β-tyrosine might inhibit as functional defense.

In the current effort we demonstrated that β-tyrosine preferentially accumulates in the youngest leaves of rice. However, TAM1 activity in the youngest leaf was significantly less than mature leaves (Table 1). We interpreted the relatively higher TAM1 activity in mature leaves as follows. TAM1 belongs to the phenylalanine ammonia lyase (PAL) family (Yokoo *et al.* 2015). It is known that PAL activity is significantly induced in the presence of L-phenylalanine, the substrate of PAL (MacDonald and D'Cunha 2007). In plants, proteinogenic amino acids are synthesized in photosynthetically active mature leaves (Tegeder and Masclaux-Daubresse 2018). Commensurately, mature leaves also have higher PAL transcript accumulation than younger leaves in tobacco (Fukasawa-Akada, Kung and Watson 1996). Given the biosynthetic capacity of mature leaves, we speculate that the higher observed TAM1 activity in mature leaves is due to the abundance of its substrate L-tyrosine actively synthesized there.

Long-distance β-tyrosine transport from mature leaves to the youngest leaf was demonstrated by using stable isotope labeled β-tyrosine (Figures 2a and b and S6). Based on higher TAM1 activity in mature leaves (Table 1) and the decrease of β-tyrosine concentration in the youngest leaf from mature leaves-removed seedlings (Figure 3), transport appears to play a role in coupling mature leaf *de novo* biosynthesis with preferential accumulation in the youngest leaves. We considered that this transport has 2 features that facilitate β-tyrosine accumulation in the youngest leaf. One feature is that β-tyrosine in mature leaves is likely to be recycled in the newly developed youngest leaf. The decrease of β-tyrosine concentrations in the second youngest leaves accompanied with the development of the youngest leaves means that β-tyrosine, which was transported, and *de novo* biosynthesized there when the second youngest leaves were in younger stages, is likely to be transported to newly developed leaf. In this point, β-tyrosine transport systems (from mature leaves to the youngest leaf) found in rice (Figures 2a and b and S6) seem economical in terms of recycling to reduce the cost of *de novo* β-tyrosine synthesis in the youngest leaf. As well as β-tyrosine in rice, younger rosette leaves of *Arabidopsis thaliana* typically have higher glucosinolate concentrations than older leaves (Brown *et al.* 2003). However, unlike β-tyrosine, rosette leaves appear to maintain a fixed concentration during leaf expansion (Brown *et al.* 2003). It means that glucosinolate in *A. thaliana* is not apparently recycled in younger leaves. The other feature is that β-tyrosine in mature leaves is predominantly transported to younger leaves which are classical sink tissues for photoassimilates (Figure 2c). This predominant path also contributes to effective β-tyrosine accumulation in young leaves.

An array of photoassimilates, plant hormones and specialized metabolites are transported through the phloem and analytically demonstratable in phloem exudates (Hoad 1995;

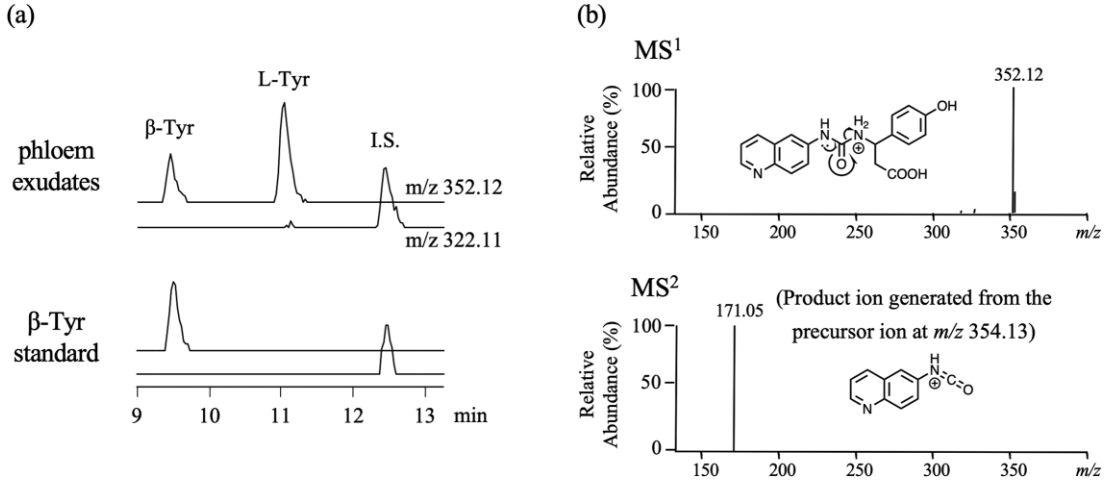

Figure 4. β-Tyrosine in phloem exudates. This experiment was performed with 3 biological replicates. (a) LC-IT-TOF-MS chromatograms (XIC) of AQC-derivatized phloem exudates and β-tyrosine standard. IS: internal standard (AQC-derivatized D-2-phenylglycine). (b) MS^1 (of the target peak) and MS^2 spectra of AQC-derivatized β-tyrosine obtained from phloem exudates with their predicted structures.

Jørgensen, Nour-Eldin and Halkier 2015; Tegeder and Masclaux-Daubresse 2018). Thus, the presence of β-tyrosine in phloem exudates supports the hypothesis that β-tyrosine is also transported via phloem (Figures 4 and S7). Although the current method utilized for phloem exudate collection is considered to be reliable (Ye *et al.* 2010; Jiang *et al.* 2018), the exudate may have also contained xylem exudate due to injuries to the xylem. Considering that xylem transport occurs from root to shoot and transport from source leaves to sinks takes place in the phloem (Tegeder and Masclaux-Daubresse 2018), however, β-tyrosine transport from mature leaves to the youngest leaf must partly occurred in the phloem. Less disruptive methods, such as an analysis of aphid feeding on rice or analysis of phloem exudates obtained by aphid stylectomy, could be used in the future for more exact analysis of β-tyrosine in the phloem.

In current effort, we examined β-tyrosine accumulation in the youngest leaf through long-distance phloem transport from mature rice leaves. Our results parallel common observations in plants where defense-related metabolites are enriched in young developing tissues (McKey 1974). Our data supports the systemic accumulation of β-tyrosine in the youngest leaves of rice and suggests an enhanced protective role in these tissues. Specific transporters have been identified for translocation of defense compounds, including nicotine in tobacco, berberin in *Coptis japonica*, and glucosinolates and GABA in *A. thaliana* (Shitan *et al.* 2003; Meyer *et al.* 2006; Morita *et al.* 2009; Nour-Eldin *et al.* 2012). Such discoveries can reveal not only the molecular basis for the transport but also enable the demonstration of physiological functions. The identification of transporter(s) underlying β-tyrosine cycling and distribution patterns could similarly provide a molecular tool for understanding of physiological and ecological functions of β-tyrosine in rice. We highlight young developing leaves as the key tissue to target for understanding the protective biological functions of this nonproteinogenic amino acids in rice.

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Supplementary material

Supplementary material is available at *Bioscience, Biotechnology, and Biochemistry* online.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

Author contribution

S.S. and N.M. designed research; S.S. performed research; S.S., T.Y., M.T., N.Y., K.O., M.K., Y.O., and N.M. analyzed data; and S.S., E.A.S., and N.M. wrote the paper.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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