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ARTICLE ADDENDUM

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Identification of β-phenylalanine as a non-protein amino acid in cultivated rice, *Oryza sativa*

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Non-protein amino acids, often analogs of the standard 20 protein amino acids, have been discovered in many plant species. Recent research with cultivated rice (*Oryza sativa*) identified (3*R*)-β-tyrosine, as well as a tyrosine amino mutase that synthesizes (3*R*)-β-tyrosine from the protein amino acid (2*S*)-α-tyrosine. Gas chromatography-mass spectrometry (GC-MS) assays and comparison to an authentic standard showed that β-phenylalanine is also a relatively abundant non-protein amino acid in rice leaves and that its biosynthesis occurs independently from that of β-tyrosine.

Keywords: rice, aminomutase, ammonia lyase, β-amino acid, β-tyrosine, β-phenylalanine

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Introduction

A variety of non-protein amino acids, many of which have defensive functions against herbivores, pathogens, and even other plants, are found sporadically in the plant kingdom.[1] Well-studied examples of such defensive non-protein amino acids include 2-amino-4-(guanidinooxy)butyric acid (canavanine) in the seeds of several legume species,[2] β-methylamino-L-alanine in cycads[3] and azetidine-2-carboxylic acid in lily of the valley (*Convallaria majalis*;[4]). However, despite the existence of likely several hundred different non-protein amino acids,[5] biosynthetic enzymes are known for relatively few of these plant metabolites. This is largely due to the fact that only a small number of non-protein amino acids have been identified in model plants like *Arabidopsis thaliana* or in well-studied crop plants with sequenced genomes.

In a targeted search for non-protein amino acids in cultivated rice (*Oryza sativa*), we recently identified (*R*)-β-tyrosine as an abundant metabolite in all tested plant parts.[6] Genetic mapping of natural variation in the abundance of β-tyrosine among different rice varieties identified the *OsTAM1* gene, which encodes a tyrosine amino mutase (TAM) that converts the common protein amino acid (2*S*)-α-tyrosine into (3*R*)-β-tyrosine. Whereas rice and other tested grass species are quite resistant to β-tyrosine, tomato, cabbage, and other dicot plant species are sensitive at low micromolar concentrations. This suggests that (3*R*)-β-tyrosine may contribute to the well-documented allelopathic potential of some rice varieties.[7] Here we show that rice cultivar Nipponbare produces not only (3*R*)-β-tyrosine but also β-phenylalanine.

Results and Discussion

A total of 18 amino acids were identified in rice leaf extracts by gas chromatography-mass spectrometry (GC-MS) in derivatized rice leaf extracts (*O. sativa* cv. Nipponbare). In addition to known protein amino acids and β-tyrosine, this assay showed the presence of other likely non-protein amino acids. A compound corresponding to a previously unknown rice amino acid was shown to co-elute with authentic β-phenylalanine (**Fig. 1A**). The fragmentation pattern of the rice amino acid was identical to that of authentic β-phenylalanine, with an M^+ ion at m/z 265 (11%), the base ion at m/z 178 (100%), and with diagnostic ions at m/z

192 (98%), 104 (58%), and 220 (5%) (**Fig. 1B and C**). This mass spectrum was distinct from that of the standard protein amino acid, α-phenylalanine. The

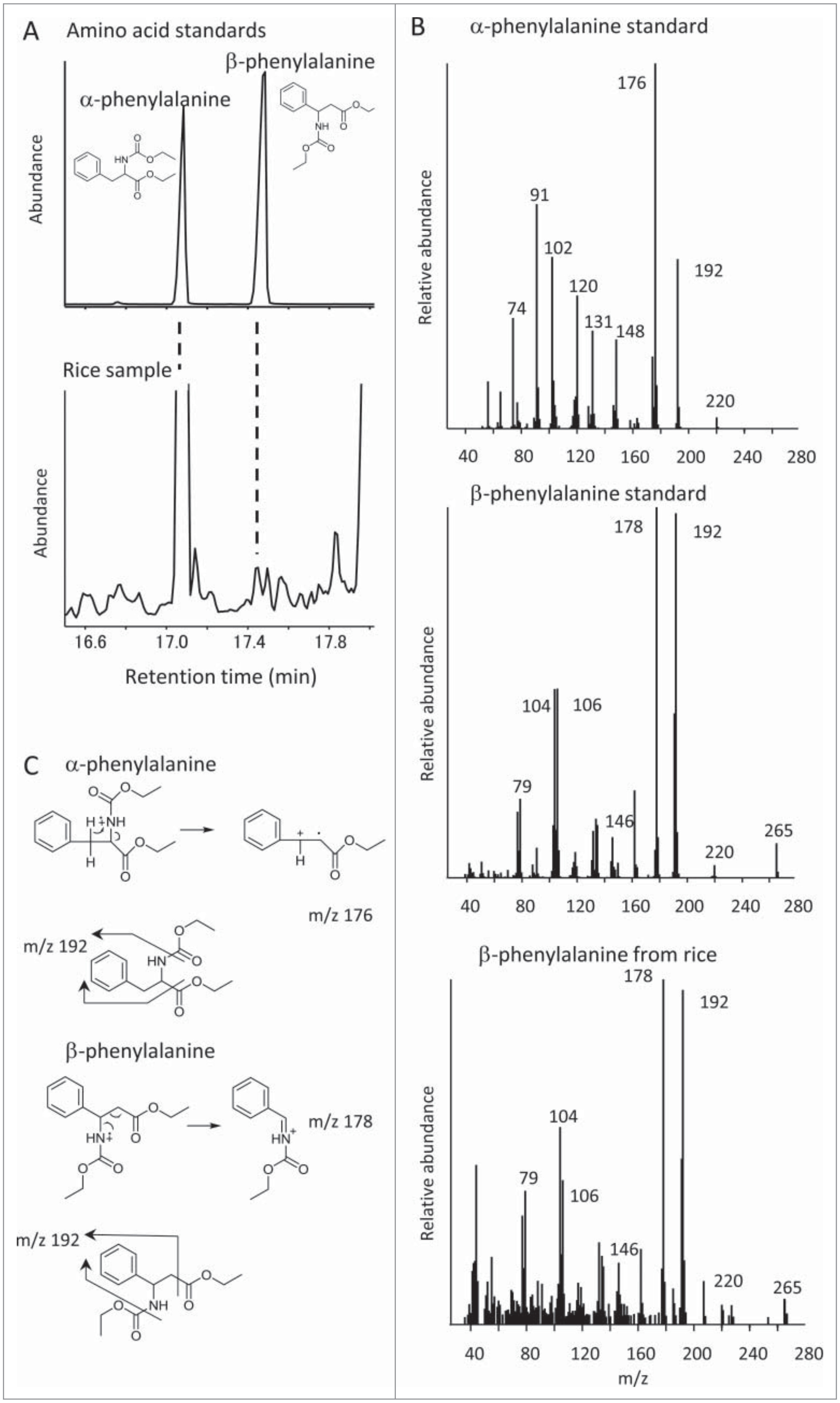

Figure 1. Identification of β-phenylalanine in rice leaf extracts. **(A)** GC-MS total ion chromatograms of a mixture of α- and β-phenylalanine standards (upper panel) and rice leaf extract (lower panel). **(B)** Mass spectra of the α-phenylalanine standard, the β-phenylalanine standard, and the rice sample. **(C)** Predicted fragmentation patterns of α-phenylalanine (m/z 176; m/z 192) and β-phenylalanine (m/z 178; m/z 192).

stereochemistry at the β-position of rice β-phenylalanine could not be determined due to the low abundance of the compound in our samples. By comparison to a β-alanine internal standard, we estimated that β-phenylalanine is present at 10 to 20 ng/mg fresh rice leaf tissue.

Several microorganisms and plants are known to form β-amino acids from α-amino acids using aminomutases that belong to the 3,5-dihydro-5-methylidene-4H-imidazol-4-one (MIO)-dependent phenylalanine ammonia lyase (PAL) family. The Nipponbare-based rice genome database shows 9 members of the PAL gene family.[8] One of these is LOC_Os12g33610, which we identified as a tyrosine aminomutase (*OsTAM1*;[6]). To highlight key regions and amino acids differentiating the rice PAL family, the alignment of the 9 rice PAL-like proteins with 2 previously identified aminomutases, TAM SgcC4 from *Streptomyces globisporus* and the phenylalanine aminomutase TcPAM from *Taxus canadiensis*,[9,10] as well as the tyrosine ammonia lyase RsTAL from *Rhodobacter sphaeroides*,[11] is shown in **Figure 2**. The rice proteins OsTAM1 and LOC_Os11g48110, which have 95% overall identity at the amino acid sequence level, nevertheless show polymorphisms in 2 regions (designated by circles) that are known to contribute to substrate specificity and product stereochemistry.[12] In particular, a 10 amino acid deletion in OsTAM1 may be critical because residues indicated by the box in **Figure 2** are key determinants of substrate specificity. For example, it is known that histidine of SgcC4 has such a role in *S. globisporus*.[13] Other reports show that the histidine of RsTAL is a substrate selectivity switch; replacing it with a phenylalanine completely changes the substrate selectivity from tyrosine to phenylalanine.[14,15] In previous research we showed that *OsTAM1* encodes tyrosine aminomutase but not phenylalanine aminomutase activity in Nipponbare rice. Although tyrosine is not used as an aminomutase substrate by very similar LOC_Os11g48110 protein,[6] phenylalanine or other amino acids might be.

So far, TAM genes have been found only in the genomes of microorganisms. Thus, is uncertain how a higher plant like rice obtained this enzymatic activity. To investigate the evolutionary origins of OsTAM1, we made an interspecific phylogenetic tree based on the amino acid sequences of PAL family proteins. PAL proteins in 3 well-studied grass species, rice, *Sorghum bicolor*, and *Zea mays*, show high sequence diversity. All of the PAL family members of microorganisms are clustered into one group, within which the TAMs of microorganisms form their own clade. In contrast to the *Taxus* spp PAM proteins (TbPAM and TcPAM in **Fig. 3**), which cluster more closely with the microbial proteins, OsTAM1, LOC_Os11g48110, and Sb01g014020 form a distinct clade. This observation suggests that OsTAM1 and LOC_Os11g48110 in rice are derived from the PAL family of the Poaceae, rather than having their origins in a lateral gene transfer from microorganisms.

β-Phenylalanine has been studied extensively as a constituent of bacterial polyketide antibiotics[12] and the anti-cancer drug taxol that has been isolated from the bark of yew trees (*Taxus* sp;[10]). Thus, it is unusual and interesting that rice accumulates β-phenylalanine as a free amino acid. β-Tyrosine has defensive functions in rice[6] and it is possible that β-phenylalanine plays a similar role. Future research will determine how β-phenylalanine is synthesized in rice and whether it has defensive functions as a free amino acid or as a component of more complex rice metabolites.

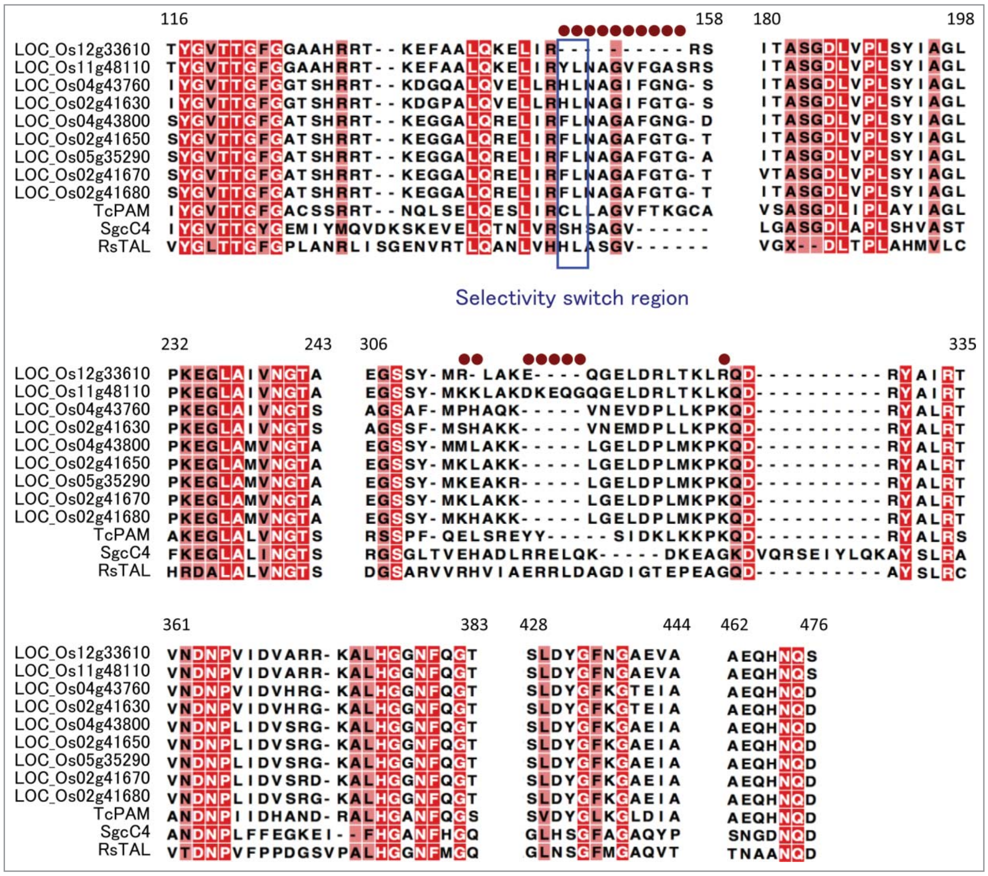

Figure 2. Sequence comparison of highly conserved regions in MIO-dependent aminomutases and ammonialyases. Residue numbers corresponding to LOC_Os12g33610 (OsTAM1) are shown above the alignment. Circles above the sequences indicate the positions of polymorphisms between OsTAM1 and LOC_Os11g48110. The box indicates known substrate-switching residues that are directly involved in substrate specificity. Amino acid sequences of rice are from the Michigan State University Rice Genome Annotation Project (http://rice.plantbiology.msu.edu); those of other species are from GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

Methods

Rice seedlings (*Oryza sativa* cv Nipponbare) were grown in water for one week in a growth chamber under fluorescent light with a 16h:8h day:night cycle at 32°C.

About 100 mg of foliar tissue was weighed and ground with 5-mm steel balls using a Beads Crusher μT-12 (Taitec, Japan). Ground tissue was extracted with 500 μL 5N HCl, the extracts were centrifuged at 6000 *g* for 5 min, at 25°C, and the supernatants were passed through a SCX cartridge (Oasis MCX 6 mL 500 mg, Waters). After the removal of interfering compounds by washing with 5 ml 0.1 N HCl, amino acids were eluted with 5 ml of a 50:50 (v/v) mixture of 4 M ammonia and methanol. The obtained solutions were evaporated and dissolved with 60 μL H_2O. For derivatization, 40 μL of ethanol/pyridine (4:4, v/v) and 5 μL of ethyl chloroformate

(ECF) were added to each sample. Samples were vortexed for 5 s and, 5 minutes later, 20 μL of dichloromethane was added and the vial was thoroughly shaken for extraction of the *N*-ethoxycarbonyl ethyl ester (ECEE) derivatives into the organic layer. One μL of the organic phase was injected into a gas chromatography-mass spectrometry system (an Agilent 6890N GC linked to an Agilent 5975B MS, Agilent Technology, CA) with an HP-5MS capillary column (0.25 mm inside diameter × 30 m, 0.25 μm film thickness), with helium carrier gas at 1.0 mL/min in the splitless mode. The oven temperature was held at 60°C (2 min), then raised to 290°C at 10°C/min, and finally kept at 290°C for 5 min. The injector temperature was maintained at 220°C. Quantitative analysis of β-phenylalanine in rice was performed by GC-MS using extracted ion areas (m/z 192 for β-phenylalanine; m/z 116 for β-alanine) with an internal standard (β-alanine).

For the protein sequence alignments and phylogenetic tree construction, individual protein sequences of the OsPAL family were downloaded from the Michigan State University Rice Genome Annotation Project (http://rice.plantbiology.msu.edu). Sequences of PAL-like genes from other organisms were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Gene identifiers for each protein are presented in **Figure 3**. Protein sequence alignment and phylogenetic tree construction were accomplished using CLC Main Workbench (Ver. Six.8.4, CLC Bio, Aarhus, Denmark). Phylogenetic tree construction was performed using a Neighbor-Joining method and bootstrap valueswere computed based on 1,000 iterations.

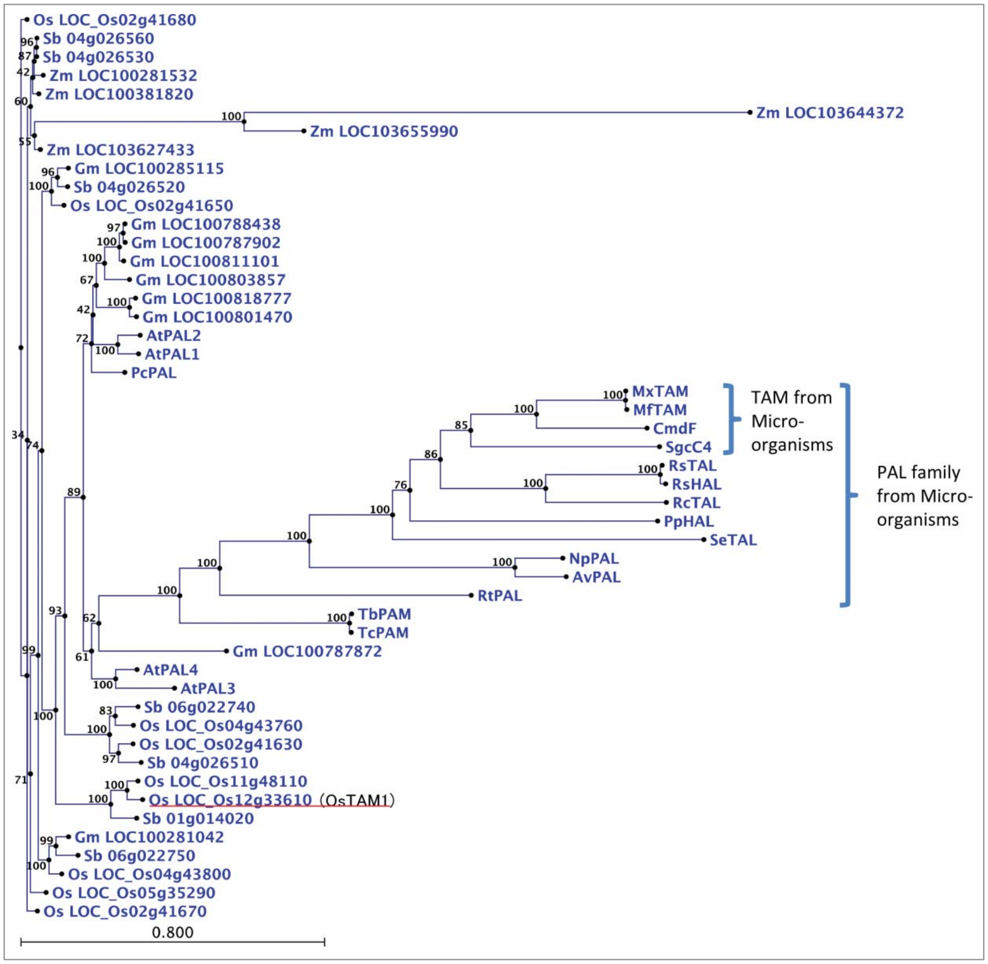

Figure 3. Phylogenetic tree of PAL-family proteins, made using a Neighbor-Joining method. The bootstrap values, based on 1000 iterations, are shown above the branch points. Rice amino acid sequences are from the Michigan State University Rice Genome Annotation Project (http://rice.plantbiology.msu.edu); those of other species are from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Annotation numbers of PAL proteins from *Oryza sativa* L. ssp. Japonica (Os), *Sorghum bicolor* (Sb), *Zea may* (Zm), *Glycine max* (Gm), and *Arabidopsis thaliana* (At) are used as their names. Genbank ID numbers of other proteins are: PcPAL1 (P24481), MxTAM (FM212244), McTAM (AAU01183), CmdF (CAJ46694), SgcC4 (AAL06680), RsTAL (ABA81174), RsHAL (3720675), RcTAL (WP_023923512), PpHAL (P21310), SeTAL (ABC88669), NpPAL (WP_023923512), AvPAL (ABA23593), RtPAL (AAA33883), TbPAM (ADA577030), and TcPAM (AAT47186).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Huang T, Jander G, De Vos M. Non-protein amino acids in plant defense against insect herbivores: representative cases and opportunities for further functional analysis. Phytochemistry 2011; 72:1531-7; PMID:21529857; http://dx.doi.org/10.1016/j.phytochem.2011.03.019
2. Rosenthal GA. *L*-Canavanine: a higher plant insecticidal allelochemical. Amino Acids 2001; 21:319-30; PMID:11764412; http://dx.doi.org/10.1007/s007260170017
3. Rivadeneyra-Dominguez E, Rodriguez-Landa JF. Cycads and their association with certain neurodegenerative diseases. Neurologia 2014; 29:517-22; PMID:23725821; http://dx.doi.org/10.1016/j.nrl.2013.03.005
4. Bach TM, Takagi H. Properties, metabolisms, and applications of (L)-proline analogues. Appl Microbiol Biotechnol 2013; 97:6623-34; PMID:23780584; http://dx.doi.org/10.1007/s00253-013-5022-7
5. Bell EA. 'Uncommon' amino acids in plants. FEBS Lett. 1976; 64:29-35; PMID:1269757; http://dx.doi.org/10.1016/0014-5793(76)80241-4
6. Yan J, Aboshi T, Teraishi M, Strickler SR, Spindel JE, Tung CW, Takata R, Matsumoto F, Maesaka Y, McCouch SR, et al. The rice tyrosine aminomutase TAM1 is required for β-tyrosine biosynthesis. Plant

Cell 2015; 27:1265-78; PMID:25901084; http://dx.doi.org/10.1105/tpc.15.00058

7. Olofsdotter M. Getting closer to breeding for competitive ability and the role of allelopathy - An example from rice (*Oryza sativa*). Weed Technology 2001; 15:798-806; http://dx.doi.org/10.1614/0890-037X(2001)015%5b0798:GCTBFC%5d2.0.CO;2
8. Tonnessen BW, Manosalva P, Lang JM, Baraoidan M, Bordeos A, Mauleon R, Oard J, Hulbert S, Leung H, Leach JE. Rice phenylalanine ammonia-lyase gene *OsPAL4* is associated with broad spectrum disease resistance. Plant Mol Biol 2014; 87:273-86; PMID:25515696; http://dx.doi.org/10.1007/s11103-014-0275-9
9. Christenson SD, Liu W, Toney MD, Shen B. A novel 4-methylideneimidazole-5-one-containing tyrosine aminomutase in enediyne antitumor antibiotic C-1027 biosynthesis. J Am Chem Soc 2003; 125:6062-3; PMID:12785829; http://dx.doi.org/10.1021/ja034609m
10. Walker KD, Klettke K, Akiyama T, Croteau R. Cloning, heterologous expression, and characterization of a phenylalanine aminomutase involved in Taxol biosynthesis. J Biol Chem 2004; 279:53947-54; PMID:15494399; http://dx.doi.org/10.1074/jbc.M411215200
11. Watts KT, Lee PC, Schmidt-Dannert C. Exploring recombinant flavonoid biosynthesis in metabolically engineered *Escherichia coli*. Chembiochem, 2004; 5:500-7; PMID:15185374; http://dx.doi.org/10.1002/cbic.200300783
12. Lohman JR, Shen B.. Four-Methylideneimidazole-5-one-containing aminomutases in enediyne biosynthesis. In Methods in Enzymology, Hopwood DA, ed (Amsterdam: Elsevier), 2012; pp. 299-319.
13. Christianson CV, Montavon TJ, Van Lanen SG, Shen B, Bruner SD. The structure of L-tyrosine 2,3-aminomutase from the C-1027 enediyne antitumor antibiotic biosynthetic pathway. Biochemistry 2007; 46:7205-14; PMID:17516659; http://dx.doi.org/10.1021/bi7003685
14. Watts KT, Mijts BN, Lee PC, Manning AJ, Schmidt-Dannert C. Discovery of a substrate selectivity switch in tyrosine ammonia-lyase, a member of the aromatic amino acid lyase family. Chemistry & biology, 2006; 13:1317-26; PMID:17185227; http://dx.doi.org/10.1016/j.chembiol.2006.10.008
15. Louie GV, Bowman ME, Moffitt MC, Baiga TJ, Moore BS, Noel JP. Structural determinants and modulation of substrate specificity in phenylalanine-tyrosine ammonia-lyases. Chemistry & biology 2006; 13:1327-1338.