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Biosynthetic Mechanism of 2-Acetyl-1-pyrroline and Its Relationship with Δ^1-Pyrroline-5-carboxylic Acid and Methylglyoxal in Aromatic Rice (*Oryza sativa* L.) Callus

TZOU-CHI HUANG,[†] CHIA-SHIH TENG,[†] JIA-LIN CHANG,[‡] HUI-SU CHUANG,[‡] CHI-TANG HO,[§] AND MEI-LI WU*,[†]

Department of Food Science, National Pingtung University of Science & Technology; Institute of Biotechnology, National Pingtung University of Science & Technology, 912, Pingtung, Taiwan; and Department of Food Science, Rutgers University, 65 Dudley Road, New Brunswick, New Jersey 08901-8520

2-Acetyl-1-pyrroline (2-AP) was identified as the major flavor compound in aromatic rice varieties Tainung 71 and 72. In order to understand the mechanism of 2-AP biosynthesis in aromatic rice, we studied the formation of putative precursors, Δ^1-pyrroline-5-carboxylic acid and methylglyoxal. The endogenous Δ^1-pyrroline-5-carboxylic acid contents of Tainung 71 and 72 calli reached 191 to 276%, compared to nonaromatic rice Tainung 67. In addition, calli of Tainung 71 and 72 contained 1.30- and 1.36-fold, respectively, higher methylglyoxal levels than that of Tainung 67. Specific enzyme activities of Δ^1-pyrroline-5-carboxylic acid-synthetic enzyme including Δ^1-pyrolline-5-carboxylic acid synthetase (P5CS) and ornithine aminotransferase (OAT) increased significantly in aromatic rice varieties. The expression levels of *P5CS1* and *P5CS2* genes were found to be significantly higher in aromatic rice than nonaromatic rice. Results of a tracer experiment with ^{15}N-labeled glutamic acid revealed that the nitrogen atom of 2-acetyl-1-pyrroline was derived from glutamic acid. Upregulation of *P5CS* in aromatic rice Tainung 72 may contribute to the increase of Δ^1-pyrroline-5-carboxylic acid level and thus leads to the accumulation of an extra amount of 2-acetyl-1-pyrroline.

KEYWORDS: Aromatic rice; Δ^1-pyrroline-5-carboxylic acid; methylglyoxal; 2-acetyl-1-pyrroline; biosynthetic pathway

INTRODUCTION

Aromatic rice (*Oryza sativa* L.), with a "popcorn"-like aroma, gains wide popularity in Asia and the Middle East and commands higher prices than nonaromatic varieties in international rice markets. In recent years, there is an increasing demand for this variety especially in Europe and America (*1*). Among the volatile compounds found in aromatic rice, 2-acetyl-1-pyrroline (2-AP) has been identified as a key odor-active component (*2*). 2-Acetyl-1-pyrroline has also been found in callus and the vegetative organs of rice (*3*).

Fragrance in rice is a highly heritable trait; the genes controlling aroma trait in rice have been studied by genetic analysis and molecular mapping. However, the results have shown that there appears to be genetic differences among aromatic rice varieties. Siddiq et al. (*4*) stated that two recessive aroma genes were located on chromosomes 5 and 9 in an India variety, T3. Pinson (*1*) determined the genes of controlling aroma in six cultivars and found that the scent of aromatic rice was under the control of one or two recessive genes, dependent upon the cultivar studied. Tomar and Prassad (*5*) identified a dominant aroma gene on chromosome 11 in rice landraces. In addition, a single recessive aroma gene (*fgr*) has been located on chromosome 8 in many different rice varieties, including a Bangladesh native variety, Surjarmkhi (*6*), an American variety, Della (*7*), and a Philippines variety, Azucena (*8*).

Little is known about the biosynthetic pathway of 2-acetyl-1-pyrroline; however, amino acids including proline and ornithine have been implicated as precursors for 2-acetyl-1-pyrroline production in a heat-treated yeast extract (*9*). Romanczyk et al. (*10*) demonstrated that significant amount of 2-acetyl-1-pyrroline could be produced by *Bacillus cereus* strain 35 on plate count agar containing proline, ornithine, and glutamate. Recently, L-proline was found to enhance the in vitro synthesis of 2-acetyl-1-pyrroline in a semidifferentiated callus culture of *Pandanus amaryllifolius* (*11*). Additionally, Suprasanna et al. (*12*) reported

* To whom correspondence should be addressed. Phone: 886-87740524. Fax: 886-87740213. E-mail: mlwu@mail.npust.edu.tw.
[†] Department of Food Science, National Pingtung University of Science & Technology.
[‡] Institute of Biotechnology, National Pingtung University of Science & Technology.
[§] Rutgers University.

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that L-proline and hydroxyproline are possible precursors for 2-acetyl-1-pyrroline formation in Basmati rice callus. Yoshihashi et al. (*3*) showed that proline, glutamic acid, and ornithine are the precursors of 2-acetyl-1-pyrroline in rice seedling and callus. By a tracer experiment using ^{15}N-proline, the result also demonstrated that the nitrogen source of 2-acetyl-1-pyrroline can be derived from proline. Collectively, these existing data indicated that proline, ornithine, and glutamate are the most probable precursors for 2-acetyl-1-pyrroline biosynthesis.

In higher plants, ornithine, proline, and glutamate can be converted to a common metabolite, Δ^1-pyrroline-5-carboxylic acid, via three distinct enzymes: ornithine aminotransferase (OAT), proline dehydrogenase (PRODH), and Δ^1-pyrolline-5-carboxylic acid synthetase (P5CS). Δ^1-Pyrolline-5-carboxylic acid seems to play a role in programmed cell death and has some effect on stress-related gene expression in organisms. Iyer and Caplan (*13*) reported that some osmotically responsive genes were induced in rice by treatment with Δ^1-pyrolline-5-carboxylic acid. In addition, Hellmann et al. (*14*) suggested that proline-induced toxic effects in *Arabidopsis thaliana* are associated with the production of a catabolic intermediate Δ^1-pyrroline-5-carboxylic acid, which might mediate apoptotic cascade. They also hypothesized that γ-glutamyl semialdehyde, which is in spontaneous equilibrium with Δ^1-pyrroline-5-carboxylic acid, contains a reactive aldehyde group, which might result in toxicity by interaction with other cellular components. In yeast *Saccharomyces cerevisiae*, Δ^1-pyrroline-5-carboxylic acid induces the generation of reactive oxygen species and therefore causes cell death (*15*). Furthermore, Δ^1-pyrroline-5-carboxylic acid is capable of inhibiting cell growth and inducing apoptosis of human tumor cell lines (*16*).

In our previous study (*17*), a model system composed of methylglyoxal and Δ^1-pyrolline-5-carboxylic acid biosynthesized via PRODH from *Bacillus subtilis* ssp. *natto* was set up to investigate the formation of 2-acetyl-1-pyrroline. We have shown that Δ^1-pyrolline-5-carboxylic acid could react with methylglyoxal to form 2-acetyl-1-pyrroline. We therefore hypothesized that Δ^1-pyrroline-5-carboxylic acid and methylglyoxal might be precursors for 2-acetyl-1-pyrroline biosynthesis. In our current study on the biosynthetic mechanism of 2-acetyl-1-pyrroline in aromatic rice, we focused on the formation of putative precursors Δ^1-pyrolline-5-carboxylic acid and methylglyoxal. The concentrations of endogenous 2-acetyl-1-pyrroline, Δ^1-pyrolline-5-carboxylic acid, and methylglyoxal were measured in aromatic and nonaromatic rice calli. The enzymatic activities and expression patterns of Δ^1-pyrolline-5-carboxylic acid-synthetic enzymes including OAT, PRODH, and P5CS were compared in nonaromatic and aromatic rice calli. The pathway leading to 2-acetyl-1-pyrroline biosynthesis in rice callus is proposed.

MATERIALS AND METHODS

Plant Material. *O. sativa* L. cv Tainung 67, a japonica variety, was obtained from District Agricultural Research and Extension Station Council of Agriculture, Kaohsiung, Taiwan. Japonica type aromatic rice varieties Tainung 71 and 72 were obtained from the Agricultural Research Institute, Taichung and Chiayi, Taiwan. Dehusked seeds were surface-sterilized with 75% ethanol for 30 s and 5% sodium hypochlorite for 1 h and then washed several times with sterilized distilled water. The sterilized seeds were inoculated on a callus induction medium 2N6, consisting of N6 salt (*18*) supplemented with 0.2% 2,4-dichlorophenoxyacetic acid and 5% sucrose. Rice callus was incubated at 25 °C with a 16 h photoperiod and was maintained by subculture every week. The callus was harvested at selected intervals for the analysis of Δ^1-pyrroline-5-carboxylic acid.

Preparation of Ethanol Extract. For quantitative analysis, 1 g of fresh weight rice callus was ground into powder with liquid nitrogen and resuspended in 1 mL of 100% ethanol. The mixture was homogenized with a sonicator for 15 min. After centrifugation at 8000*g* for 30 min, the supernatant was filtered through a 0.45 μm filter membrane. 2,4,6-Trimethylpyridine (10 μL, 100 ppm; Sigma-Aldrich, St. Louis, MO) was used as an internal standard in quantitative analysis and was added into 90 μL of the supernatant prior to analysis.

Gas Chromatography−Mass Spectrometry (GC-MS). Volatile compounds were injected into a Agilent 6890 gas chromatograph with a HP5973 mass selective detector (Agilent Technologies, Palo Alto, CA) equipped with a 30 m × 0.25 mm i.d., 0.25 μm, (5%-phenyl)-methylpolysiloxane nonpolar stationary phase HP-5MS fused silica capillary column (Agilent Technologies) following the method of Huang et al. (*17*). The sample was injected in a splitless mode, and the flow rate of helium was 1 mL/min. The injection port temperature was 200 °C. The temperature of the HP-5MS column was programmed to start at 50 °C after injection. After 1 min, the temperature was increased at incremental rates of 5−250 °C and held for 15 min. The temperature of the mass transfer line was set at 280 °C. The mass spectrometer was operated in an electron impact (EI) ionization mode with an electron energy of 70 eV and an electron multiplier voltage of 1832 V. The temperature of the ion source was set at 250 °C. The mass spectra were obtained by scanning from *m*/*z* 40 to 250 with a scan rate of 0.68 s/scan. Identifications of volatile compounds were performed by comparing the mass spectra with those of the Wiley 275.L computed database and by determination of the retention indices (Kovats retention indices).

Quantitative Analysis of Δ^1-Pyrolline-5-carboxylic Acid by High-Performance Liquid Chromatography. A modified method of O'Donnell et al. (*19*) was used to analyze the endogenous contents of Δ^1-pyrroline-5-carboxylic acid in rice callus. Fresh weight callus (1 g) was ground into powder with liquid nitrogen and resuspended in 1 mL of extraction solution containing 50 mM Tris-HCl (pH 8.0), 10% glycerol, 1% Triton X-100, and 1% β-mercaptoethanol. The mixture was votexed for 1 min and kept in an ice bath for 2 min. The above procedure was repeated five times, and then, the mixture was allowed to stand for 30 min. After centrifugation at 14 000*g* for 30 min at 4 °C, a 450 μL aliquot of the supernatant or Δ^1-pyrolline-5-carboxylic acid standard (Sigma-Aldrich, St. Louis, MO) was added to a mixture containing 500 μL of 10% trichloroacetic acid and 50 μL of 0.1 M *o*-aminobenzaldehyde. The mixture was kept at room temperature for 25 min and then centrifuged for 15 min at 14 000*g*. The supernatant was removed and then immediately filtered through a 0.45 μm filter membrane. The filtrate was subjected to quantitative analysis by reverse-phase HPLC on a L-6200A HPLC system (Hitachi, Tokyo, Japan) equipped with a 250 mm × 4.6 mm i.d., 10 μm, Inertsil ODS2 C_{18} column (GL Sciences Inc., Tokyo, Japan) and a UV detector at 254 nm. The injection volume was 20 μL, and the mobile phase was methanol/water (33/67) at a flow rate of 1.5 mL/min.

Quantitative Analysis of Methylglyoxal by High-Performance Liquid Chromatography. A modification of the method described by Cordeiro and Ponces Freire (*20*) was used to determine methylglyoxal concentration in rice callus. Callus (1 g) was ground to a fine powder in liquid nitrogen and resuspended in 1 mL of 0.5 N perchloric acid (Sigma-Aldrich, St. Louis, MO). The mixture was homogenized with a sonicator for 5 min and then centrifuged at 8000*g* at 4 °C for 20 min. The supernatant was neutralized with saturated potassium carbonate solution and kept at room temperature for 15 min. The supernatant (1 mL) was collected after centrifugation at 8000*g* for 10 min and mixed with 200 μL of 7.2 mM 1,2-diaminobenzene derivatizing agent (Sigma-Aldrich, St. Louis, MO), 100 μL of 10 mM 2,3-dimethylquinoxaline as internal standard (Sigma-Aldrich, St. Louis, MO), 200 μL of 5 M perchloroacetic acid, and 500 μL of deionized water. After incubation at 4 °C for 24 h, the sample was passed through a Sep-Pak C_{18} Cartridge (Waters, Milford, MA), which was pretreated with 8 mL of acetonitrile and 8 mL of 10 mM KH_2PO_4 (pH 2.5). After washing with 2 mL of 10 mM KH_2PO_4 (pH 2.5), the cartridge was eluted with 1.5 mL of acetonitrile. The eluates were subjected to HPLC analysis. The quinoxaline derivative of methylglyoxal and the 2,3-dimethylquinoxaline internal standard were measured using a L-6200A HPLC system

Table 1. List of Primer Sequences Used in This Study

gene	primer pair	sequence (5′ to 3′)	product size (bp)
OAT	OsOAT-F	CTGGAGCTGAAGGAGTGGAAACAGC	187
	OsOAT-R	GATGGCCAGGAACCAATGGG	
P5CS1	OsP5CS1-F	GCAATCTGAACCAAGGCATCAGG	734
	OsP5CS1-R	TTTAGCAGGACTGTTGGCACTGG	
P5CS2	OsP5CS2-F	TGCTTCCTTGGAACAACCCCACTGG	893
	OsP5CS2-R	TCGCGAAGGCGGACGTGGAGAACAC	

(Hitachi, Tokyo, Japan) equipped with a 250 mm × 4.6 mm i.d., 5 μm, Mightysil-C_{18} column (Kanto Chemicals, Tokyo, Japan) and a UV detector at 320 nm. The mobile phase employed was a 32/68 (by volume) mixture of acetonitrile/KH_2PO_4 (10 mM, pH 2.5) at a flow rate of 1.0 mL/min; the injection volume was 20 μL.

Measurement of Activity of Δ^1-Pyrolline-5-carboxylic Acid-synthetic Enzymes. For measurement of the activities of P5CS, OAT, and PRODH, the total protein was extracted from rice callus incubated in a medium for 30 days. Rice callus (1 g) was ground to a fine powder with liquid nitrogen. Total protein was isolated from the callus powder using 1 mL of extraction solution containing 50 mM Tris-HCl (pH 8.0), 10% glycerol, 1% Triton X-100, and 1% β-mercaptoethanol. After centrifugation, the protein extract was subjected to enzyme assay. PRODH activity was determined with the spectrophotometric method described by Scarpulla and Soffer (*21*). The reaction was carried out at 25 °C in 50 mM Tris-HCl (pH 7.5) containing 100 mM L-proline, 10 mM KCN, 10 μM FAD, 0.2 mM phenazine methosulfate, 0.2 mM *p*-iodonitrotetrazolium (Sigma-Aldrich, St. Louis, MO), 0.75% Triton X-100, and enzyme extract. One unit of PRODH activity was defined as the amount of activity yielding reduction of 1.0 μmol of *p*-iodonitrotetrazolium per minute under the assay condition using the extinction coefficient 11.5/(mM·cm). P5CS and OAT activities were determined by measuring the formation of Δ^1-pyrroline-5-carboxylic acid using *o*-aminobenzaldehyde reagent. Δ^1-Pyrolline-5-carboxylic acid concentration was determined following the method of Mezl and Knox (*22*). OAT activity was assayed in 1 mL of assay mixture containing 50 mM Tris-HCl (pH 8.0), 35 mM L-ornithine, 5 mM α-ketoglutarate, 0.05 mM pyridoxal phosphate, 5 mM *o*-aminobenzaldehyde, and an appropriate amount of enzyme extract. The P5CS assay was carried out in 1 mL of 100 mM Tris-HCl (pH 7.3) containing 25 mM methylglyoxal, 5 mM ATP, 0.4 mM NADPH, 75 mM L-glutamate, and enzyme extract. The reaction mixtures were incubated at 37 °C for 20 min then stopped by adding 0.5 mL of 10% trichloroacetic acid. After centrifugation, the absorbance was measured at 440 nm. The concentration of Δ^1-pyrolline-5-carboxylic acid was determined using the extinction coefficient 2.58/(mM·cm). One unit of enzyme activity was defined as the amount of enzyme that liberates 1 μmol of product per minute under the assay condition used.

Reverse Transcriptase PCR (RT-PCR) Analysis. Rice callus incubated in a medium for 30 days (1 g) was put into liquid nitrogen and ground to a fine powder in a mortar. RNA was extracted by the addition of 1 mL of TRIzol Reagent (Invitrogen, Carlsbad, CA). The homogenate was extracted with 200 μL of chloroform and then precipitated with an equal volume of isopropanol at −70 °C for 1 h. The rice pellet was washed with 70% ethanol, dried, and dissolved in H_2O. RNA concentration and purity was determined according to the absorbances at 260 and 280 nm. First-strand cDNA synthesis was performed with SuperScript II reverse transcriptase (Invitrogen Co., Carlsbad, CA) according to the manufacturer's instructions using 3 μg of total RNA as the template and 0.5 μg of oligo-$(dT)_{12-18}$ as the primer in a final volume of 20 μL. On the basis of the cDNA sequence, the specific primers for RT-PCR were designed for each target gene (**Table 1**). PCR was carried out at 15−35 cycles to determine the linear range of PCR amplification for each target gene. The PCR reaction conditions were as follows: predenaturing at 94 °C for 5 min, followed by 28−35 cycles at 94 °C for 1.0 min, 63 °C for 2.0 min, and 72 °C for 1.0 min, with final extension at 72 °C for 10 min. The RT-PCR products were analyzed on 1% agarose gels containing 0.5 μg/mL ethidium bromide. The expression level of target genes was quantified by densitometric analysis of gels using Image J software (NIH, Bethesda, MD).

Labeling Experiment with ^{15}N-Glutamic Acid. A labeling experiment using ^{15}N-glutamic acid followed the method of Yoshihashi et al. (*3*). Rice callus (3 g; Tainung 72) were incubated in a 50 mL flask containing 20 mL of 2N6 medium with 1.2 mM ^{15}N-glutamic acid (CIL, Andover, MA). After incubation at 25 °C in darkness for 48 h, rice callus were rinsed with distilled water and then subjected to 2-AP analysis.

Statistical Analysis. The data were analyzed using the general linear model (GLM) procedures of the SAS program (SAS Institute Inc.). The Duncan's multiple range tests were used to compare the differences among means. Values for $P < 0.05$ were considered to be statistically significant.

RESULTS

2-Acetyl-1-pyrroline in Rice Callus. From rice callus, 2-acetyl-1-pyrroline was extracted with ethanol and analyzed by GC-MS. A peak with retention time (t_R) at 7.6 min was characterized as 2-acetyl-1-pyrroline. The mass spectrum of 2-acetyl-1-pyrroline consists of molecular ion at *m*/*z* 111 (27) and fragmentation ions at 43 (100), 41 (60), 83 (43), 68 (27), 42 (23), 69 (21), 96 (13), 55 (3.6), 52 (2.2), 67 (2.9), and 54 (1.5). By using 2,4,6-trimethylpyridine as an internal standard, concentrations of 2-acetyl-1-pyrroline in aromatic rice varieties Tainung 71 and Tainung 72 were estimated to be 180 and 340 ppb, respectively. 2-Acetyl-1-pyrroline was not detectable in nonaromatic rice variety Tainung 67.

Δ^1-Pyrolline-5-carboxylic Acid and Methylglyoxal Contents in Calli of Three Rice Cultivars. To evaluate the relationship between 2-acetyl-1-pyrroline formation and putative precursors Δ^1-pyrolline-5-carboxylic acid and methylglyoxal, the concentrations of Δ^1-pyrolline-5-carboxylic acid and methylglyoxal were determined in aromatic and nonaromatic rice calli. The endogenous Δ^1-pyrolline-5-carboxylic acid contents of Tainung 71 and Tainung 72 calli reached 191 and 276%, compared to that in Tainung 67 callus, after 30 days cultivation (**Figure 1A**). As shown in **Figure 1B**, the levels of methylglyoxal in calli of Tainung 71 and 72 contained 1.30- and 1.36-fold, respectively, higher levels than that of Tainung 67. Interestingly, the calli with higher levels of 2-acetyl-1-pyrroline contained higher amounts of Δ^1-pyrolline-5-carboxylic acid and methylglyoxal.

Activity Analysis of Δ^1-Pyrolline-5-carboxylic Acid-synthetic Enzymes. In higher plants, Δ^1-pyrolline-5-carboxylic acid can be synthesized via three distinct enzymes including OAT, PRODH, and P5CS. To examine the relationship between endogeneous levels of Δ^1-pyrolline-5-carboxylic acid and the activities of Δ^1-pyrolline-5-carboxylic acid-synthetic enzymes, the enzyme activities of OAT, PRODH, and P5CS were detected in callus of three rice cultivars. As shown in **Table 2**, the specific activities of P5CS are higher in aromatic rice variety Tainung 71 (114%) and Tainung 72 (123%), with respect to that of nonaromatic rice variety Tainung 67. The OAT activities were 2-fold and 3.3-fold higher in aromatic rice varieties Tainung 71 and Tainung 72 as compared to that of nonaromatic rice variety Tainung 67. There were no significant differences in the activities of PRODH among the calli of these three rice cultivars. The results indicate a positive relationship between Δ^1-pyrolline-5-carboxylic acid content and the enzyme activities of P5CS and OAT in rice callus.

Relative mRNA Levels of Δ^1-Pyrroline-5-carboxylic Acid-synthetic Enzymes. To further explore whether the transcription is the major control point for regulating the expression of P5CS and OAT, the mRNA levels of *OAT*, *P5CS1*, and *P5CS2* genes were detected in the callus from three rice varieties using RT-PCR. The mRNA levels for *P5CS1* were significantly higher

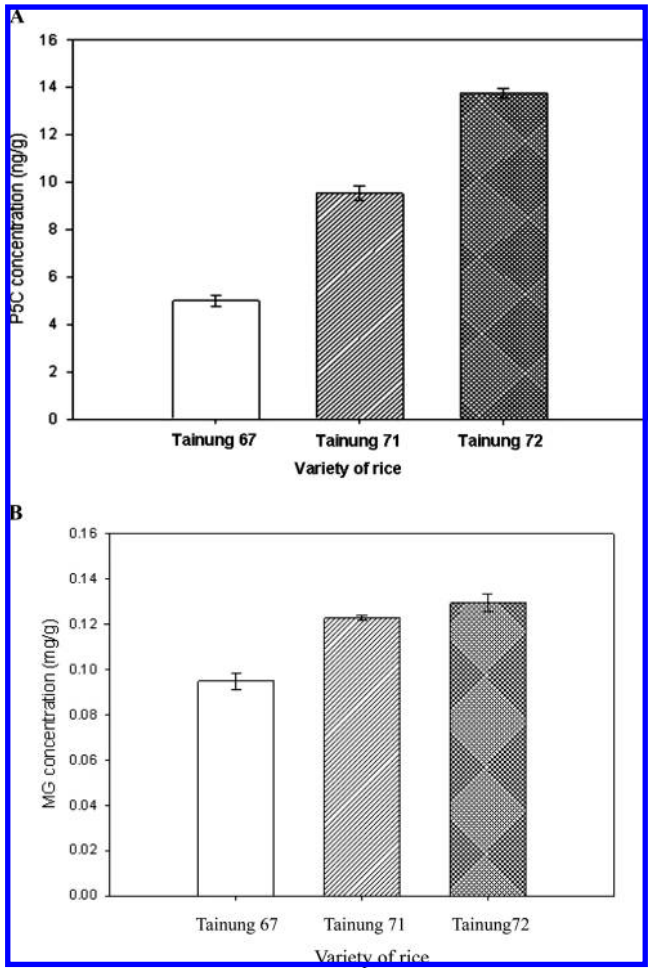

Figure 1. Endogeneous Δ^1-pyrolline-5-carboxylic acid (**A**) and methylglyoxal (**B**) levels of Tainung 67, 71, and 72 rice callus after cultivation on 2N6 media for 30 days.

Table 2. Catalytic Activities of P5CS, PRODH, and OAT Enzymes in Tainung 67, 71, and 72 Rice Calli after Cultivation on 2N6 Media for 30 Days

	enzyme activity (U/mg, $\times 10^{-3}$)[a]		
enzyme	Tainung 67	Tainung 71	Tainung 72
P5CS	3.4 ± 0.27 a	3.9 ± 0.30 b	4.2 ± 0.1 b
PDH	22.5 ± 0.40 a	22.7 ± 0.40 a	23.0 ± 0.17 a
OAT	0.28 ± 0.04 a	0.56 ± 0.01 b	0.93 ± 0.02 c

[a] Data are presented as mean ± SEM from three independent experiments. Total protein was extracted from rice callus, and the enzyme activities were measured as described in the Materials and Methods. Means annotated with different letters in the same row were significantly different ($P < 0.05$).

in aromatic rice Tainung 71 (135%) and Tainung 72 (137%) compared with nonaromatic rice Tainung 67 (**Figure 2**). The expression level of *P5CS2* genes was found to be significantly higher in aromatic rice Tainung 72 (162%) than nonaromatic rice Tainung 67 (**Figure 2**). However, the mRNA level of *OAT* gene did not show any significant difference among these three rice varieties. These results suggest that the increase in the activity of P5CS in aromatic rice appears to be due to an increase in the relative level of mRNA.

Tracer Experiment with ^{15}N-Glutamic Acid in Tainung 72 Rice Callus. To clarify the precursor responsible for 2-acetyl-1-pyrroline synthesis, a tracer experiment with ^{15}N-labeled glutamic acid was conducted in rice callus (Tainung 72). The volatile compounds from the rice callus were extracted with

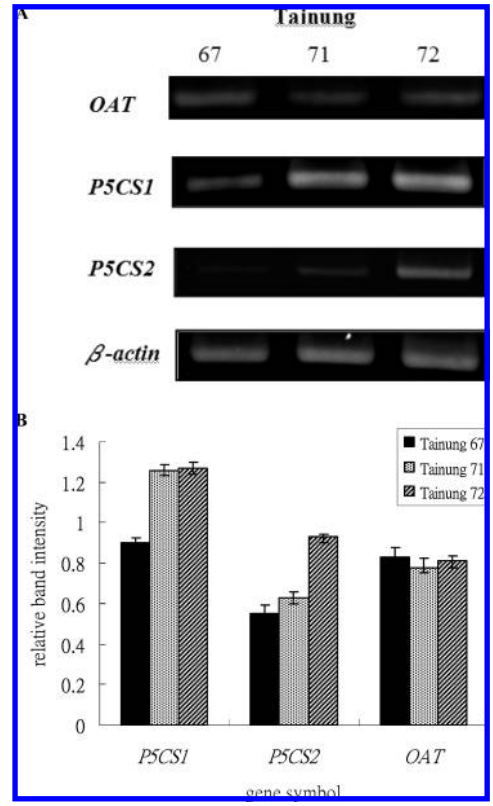

Figure 2. Expression analysis of *P5CS1*, *P5CS2*, and *OAT* from Tainung 67, 71, and 72 rice calli after cultivation on 2N6 media for 30 days.

ethanol and analyzed by GC-MS. A major peak with the mass spectrum having a base peak at *m*/*z* 43 followed by 69, 84, 97, 111, and 112 fragment ions was noted (**Figure 3**). The breakdown of a methyl moiety, a methylene moiety, and an acetyl moiety leads to the formation of the *m*/*z* 97 and *m*/*z* 84 fragments and the *m*/*z* 69 pyrroline ring, respectively. These data indicated that the pyrroline group of 2-acetyl-1-pyrroline contains the ^{15}N atom from the ^{15}N-glutamic acid. As a result, we demonstrated that the pyrroline ring of 2-acetyl-1-pyrroline was indeed derived from glutamic acid. It might be due to the conversion of L-glutamic acid by overexpressed P5CS in Tainung 72 rice callus.

DISCUSSION

The data presented in this report suggests the possible biosynthetic pathway of a major flavor component, 2-acetyl-1-pyrroline, in aromatic rice. The endogenous contents of putative precursors Δ^1-pyrroline-5-carboxylic acid and methylglyoxal for 2-acetyl-1-pyrroline biosynthesis have been measured in calli of nonaromatic rice variety Tainung 67 and aromatic rice varieties Tainung 71 and 72. The differential levels of Δ^1-pyrolline-5-carboxylic acid accumulation observed among these three varieties are positively correlated to the level of 2-acetyl-1-pyrroline.

In aromatic rice Tainung 71 and 72, the increases in Δ^1-pyrolline-5-carboxylic acid content have positive correlations with increases in the enzyme activities of P5CS and OAT. The P5CS and OAT enzymes have been shown to be involved in the formation of proline, which contributes to the increase of osmotolerance in plants. In rice, glutamate is converted by bifunctional enzyme P5CS to glutamic γ-semialdehyde (GSA), which cyclizes spontaneously to Δ^1-pyrolline-5-carboxylic acid (*22*). Δ^1-Pyrolline-5-carboxylic acid is then reduced into proline by P5C reductase. Two P5CS genes, *P5CS1* and *P5CS2*, have

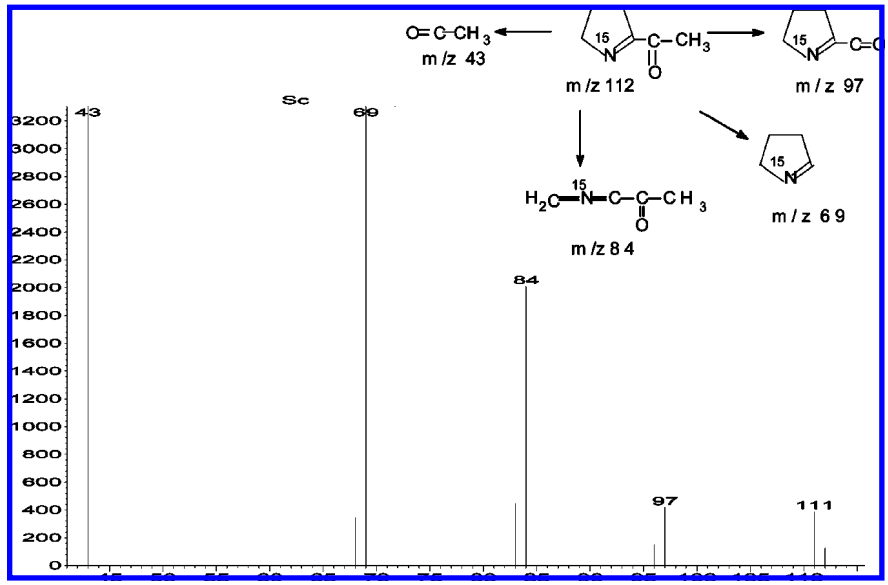

Figure 3. Mass spectrum and fragmentation pattern of ^{15}N-labeled 2-acetyl-1-pyrroline.

been identified in rice (*23*). Here, upregulation of P5CS enzyme paralleled the transcription levels in the aromatic rice varieties Tainung 71 and 72, since RT-PCR indicated an increase in the amounts of *P5CS1* and *P5CS2* transcripts. In rice, expression of *P5CS1* gene was stimulated by the treatment of high salt, dehydration, cold, and ABA (*24*), while *P5CS2* was induced by salt and cold stress (*25*). Δ^1-Pyrolline-5-carboxylic acid is also synthesized through transamination of ornithine catalyzed by OAT. The activity of OAT enzyme in aromatic rice was markedly higher than that in nonaromatic rice Tainung 67, while the level of mRNA expression remained nearly unchanged. The results suggest that the upregulation of OAT appears not to be mediated by transcriptional control in aromatic rice Tainung 71 and 72. Osmotic stress had some effect on 2-acetyl-1-pyrroline accumulation in aromatic rice. Yoshihashi et al. (*26*) reported that the aromatic rice variety Khao Dawk Mali 105 grown under drought conditions could accumulate a higher level of 2-acetyl-1-pyrroline. During osmotic stress such as drought and salt, a high level of proline is biosynthesized via intermediate Δ^1-pyrolline-5-carboxylic acid by glutamate and ornithine pathways (*27*). In aromatic rice, drought conditions might result in an increase of endogenous Δ^1-pyrolline-5-carboxylic acid and thus lead to the formation of 2-acetyl-1-pyrroline.

In vitro model system has demonstrated that methylglyoxal was one of the main precursors for 2-acetyl-1-pyrroline synthesis (*17*). Methylglyoxal might react directly with Δ^1-pyrolline-5-carboxylic acid, derived from L-proline by purified recombinant *B. subtilis* ssp. *natto* PRODH, and lead to the formation of 2-acetyl-1-pyrroline. Similarly, methylglyoxal was found to react with 1-piperideine-6-carboxylic acid and incorporate into 2-acetyltetrahydropyridine as the acetyl group (*28*). The overproduced Δ^1-pyrolline-5-carboxylic acid may be detoxified by methylglyoxal following a similar mechanism proposed by our previous study (*17*) and results in the biosynthesis of 2-acetyl-1-pyrroline.

The results indicate that the increase of 2-acetyl-1-pyrroline seems to be due to the higher level of Δ^1-pyrolline-5-carboxylic acid and methylglyoxal accumulated in aromatic rice varieties, and the level of Δ^1-pyrolline-5-carboxylic acid was directly related to the enzyme activities of OAT and P5CS. A hypothetical pathway for the formation of 2-acetyl-1-pyrroline from 1-pyrroline and methylglyoxal has been proposed by Schieberle (*29*). Our data showed that Δ^1-pyrolline-5-carboxylic acid, derived from L-glutamic acid by P5CS, might react directly with methylglyoxal and lead to the formation of 2-acetyl-1-pyrroline. On the other hand, Δ^1-pyrolline-5-carboxylic acid may also degrade to 1-pyrroline and condense with methylglyoxal to form 2-AP (**Figure 4**). The results of this study support our proposed pathway for 2-acetyl-1-pyrroline biosynthesis in aromatic rice in which 2-acetyl-1-pyrroline is synthesized from ornithine and glutamate via the intermediate Δ^1-pyrolline-5-carboxylic acid. In conclusion, Δ^1-pyrolline-5-carboxylic acid and methylglyoxal are important precursors for 2-acetyl-1-pyrroline biosynthesis in aromatic rice.

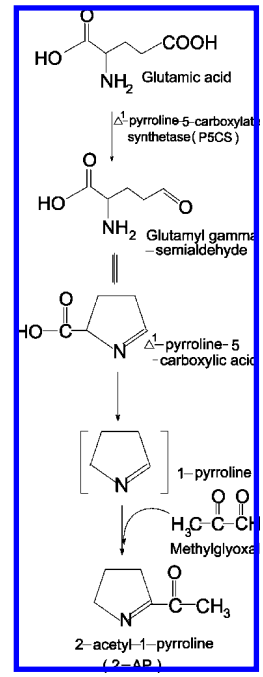

Figure 4. Proposed mechanism for 2-acetyl-1-pyrroline formation in Tainung 72 rice callus.

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