Evaluation of *Brachypodium distachyon* L-Tyrosine Decarboxylase Using L-Tyrosine Over-Producing *Saccharomyces cerevisiae*

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**Abstract**

To demonstrate that herbaceous biomass is a versatile gene resource, we focused on the model plant *Brachypodium distachyon*, and screened the *B. distachyon* for homologs of tyrosine decarboxylase (TDC), which is involved in the modification of aromatic compounds. A total of 5 candidate genes were identified in cDNA libraries of *B. distachyon* and were introduced into *Saccharomyces cerevisiae* to evaluate TDC expression and tyramine production. It is suggested that two TDCs encoded in the transcripts Bradi2g51120.1 and Bradi2g51170.1 have L-tyrosine decarboxylation activity. Bradi2g51170.1 was introduced into the L-tyrosine over-producing strain of *S. cerevisiae* that was constructed by the introduction of mutant genes that promote deregulated feedback inhibition. The amount of tyramine produced by the resulting transformant was 6.6-fold higher (approximately 200 mg/L) than the control strain, indicating that *B. distachyon* TDC effectively converts L-tyrosine to tyramine. Our results suggest that *B. distachyon* possesses enzymes that are capable of modifying aromatic residues, and that *S. cerevisiae* is a suitable host for the production of L-tyrosine derivatives.

**Introduction**

Plants produce various kinds of compounds containing aromatic residues via secondary metabolite pathways, such as the phenylpropanoid biosynthesis pathway [1–7]. Although a number of plant genes involved in the modification of aromatic residues have been identified, the majority of plant genomes have not been sequenced due to their large sizes compared to those of microbes and are expected to contain numerous novel and unidentified genes.

*Brachypodium distachyon* is a model plant for cereal crops, such as barley and wheat, and is often used for biological characterization of grass biomass due to its short life cycle, small size, simple transformation procedure and small genome size [8]. Recently, full-length cDNA
libraries of *B. distachyon* were constructed and have been made publically available [9]. However, there are few reports concerning the characterization and application of genes and proteins derived from *B. distachyon*.

The yeast *S. cerevisiae* has been widely studied and is commonly used as a model eukaryote. Various heterogeneous genes have been functionally characterized using *S. cerevisiae* as a host strain [10–13]. Genetically modified *S. cerevisiae* strains have also been used in the fermentation industry to produce various compounds, including fuels and organic acids [13, 14]. *S. cerevisiae* has also been used as host for the biosynthesis of aromatic compounds. For example, Kim et al. [15] reported 2-phenylethanol production via the Ehrlich pathway, and Vannelli et al. [16] demonstrated *p*-hydroxyxinnamic acid production using a cytochrome P-450-expressing strain of *S. cerevisiae*. Koopman et al. successfully produced flavonoid naringenin using genetically engineered *S. cerevisiae* [17].

The shikimate pathway is a metabolic route for the biosynthesis of aromatic amino acid in microorganisms. The first reaction in this pathway involves the stereo-specific condensation of erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP) to 3-deoxy-D-heptulosonate-7-phosphate (DAHP) in a reaction catalyzed by DAHP synthase (Fig 1) [18]. In *S. cerevisiae*, DAHP synthase is encoded by the *ARO3* and *ARO4* genes, and the corresponding proteins, ARO3 and ARO4, are strongly regulated by L-phenylalanine and L-tyrosine, respectively, which are produced in this pathway [19]. According to a report by Helmsstaedt et al. [20], a single serine-to-alanine substitution in ARO4 at position 195 impairs L-tyrosine sensitivity, leading to deregulation of ARO4. The conversion of chorismate to phenylpyruvate (PPA) by chorismate mutase is another step regulating aromatic amino acid productivity in this pathway [18]. *S. cerevisiae* chorismate mutase is encoded by *ARO7* and its activity is inhibited by L-tyrosine and L-tryptophan; however, the substitution of glycine with serine at position 141 generates L-tyrosine-insensitive ARO7 [19]. Although these findings indicate that enzymes involved in amino acid biosynthesis in *S. cerevisiae* can be improved through genetic modification, only a few reports have described the application of the *S. cerevisiae* biosynthesis pathway for aromatic amino acids for chemical production [15, 16, 21].

To demonstrate that the genomes of herbaceous biomass such as *B. distachyon* is a versatile and useful resource for genes involved in the production of aromatic compounds, here, we searched the *B. distachyon* genome for genes encoding L-tyrosine decarboxylase (TDC), which is involved in alkaloid biosynthesis. In *E. coli*, tyramine production pathway was previously reported, and TDC gene derived from *Lactobacillus brevis* JCM1170 was used for tyramine production in that report [22]. Several genes annotated as TDC encoding were identified by screening *B. distachyon* cDNA libraries and were then evaluated using *S. cerevisiae* as a host. TDC-expressing *S. cerevisiae* successfully converted L-tyrosine to tyramine, which is the decarboxylation product of L-tyrosine. By increasing L-tyrosine availability, tyramine productivity by the recombinant *S. cerevisiae* strain expressing TDC derived from *B. distachyon* was 6.6-fold higher than that of the control strain.

### Materials and Methods

#### Plasmid construction and yeast transformation

Polymerase chain reactions (PCR) were performed using PrimeSTAR HS (Takara Bio, Shiga, Japan) and the primer pairs listed in Table 1. PCR cycle conditions were as follows: 98°C for 1 minute followed by 30 cycles of 98°C for 15s, 68°C for 30s, and 72°C for 90s. Plasmids for transformation of *S. cerevisiae* were constructed by PCR amplifying the identified gene fragments encoding TDC homologs using Bradi1g28960.1, Bradi2g51120.1, Bradi2g51170.1, Bradi3g14750.1, or Bradi3g14780.1 as a template with the appropriate primer pairs. Each gene was
identified using GRAMENE (http://www.gramene.org/) (Brachypodium.org is also available (http://www.brachypodium.org/)). PCR cycle conditions were as follows: 98°C for 1 minute followed by 30 cycles of 98°C for 15s, 68°C for 30s, and 72°C for 90s. Each amplified fragment was introduced into the NheI or SalI, and XmaI sites of pGK422 [21], generating plasmids pGK422-tdc60, pGK422-tdc20, pGK422-tdc70, pGK422-tdc50, or pGK422-tdc80. δ-integrative plasmids were constructed by PCR amplifying the gene fragment encoding LEU2 from pRS405 DNA [22] with LEU2d(F)_InF and LEU2d(R)_InF. The obtained fragment was introduced into the XhoI sites of pδU [23], which contained URA3 as a selective marker, using an In-Fusion HD Cloning kit (Takara Bio), generating the plasmid pδL. A gene fragment containing the PGK1 promoter region was amplified by PCR using pGK422 as a template with the appropriate

Fig 1. Proposed biosynthesis pathway for tyramine (ARO3, ARO4; 3-deoxy-D-heptulosonate-7-phosphate synthase: ARO7; chorismate mutase: TDC; L-tyrosine decarboxylase). ARO3, ARO4 and ARO7 are derived from S. cerevisiae, whereas TDC is originated from B. distachyon.

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| Strain, plasmid, primer, or transformant | Relevant features | Source or reference |
|----------------------------------------|-----------------|-------------------|
| **Strains**                            |                 |                   |
| Escherichia coli Nova blue              | endA1 hsdR17(λK12−mK12+) supE44 thi-1 gyrA96 relA1 lac recA1/F′[proAB+ lacIq Z∆M15::Tn10(Tetr)]; used for gene cloning. | Novagene |
| Saccharomyces cerevisiae YPH499         | MATαura3-52 leu2-Δ1 lys2-801 his-Δ200 trp1-Δ63 ade2-101 | ATCC |
| **Plasmids**                           |                 |                   |
| pGK422                                 | Versatile vector containing 2µ ori (long form, from pWI3) and PGK1 promoter in S. cerevisiae; selection marker is ADE2. | [23] |
| pGK422-tdc20                          | Vector for expressing Brad1g51120.1; created from pGK422 | This study |
| pGK422-tdc70                          | Vector for expressing Brad1g51170.1; created from pGK422 | This study |
| pGK422-tdc60                          | Vector for expressing Brad1g28960.1; created from pGK422 | This study |
| pGK422-tdc50                          | Vector for expressing Brad3g14750.1; created from pGK422 | This study |
| pGK422-tdc80                          | Vector for expressing Brad3g14780.1; created from pGK422 | This study |
| p5U                                   | δ-integration vector in S. cerevisiae; selection marker is URA3. | [24] |
| p5U-PGK                               | δ-integration vector in S. cerevisiae; selection marker is LEU2. | This study |
| p5U-PGK                               | Versatile δ-integration vector including PGK1 promoter in S. cerevisiae; created from p5U-PGK | This study |
| pδL-PGK                               | Versatile δ-integration vector including PGK1 promoter in S. cerevisiae; created from pδL-PGK | This study |
| p5U-ARO4fbr                           | Vector for expressing ARO4fbr; created from p5U-PGK | This study |
| pδL-ARO7fbr                           | Vector for expressing ARO7fbr; created from pδL-PGK | This study |
| **Transformants**                      |                 |                   |
| YPH499/p422                            | YPH499 harboring pGK422 | This study |
| YPH499/p422tdc20                       | YPH499 transformant harboring pGK422-tdc20 | This study |
| YPH499/p422tdc70                       | YPH499 transformant harboring pGK422-tdc70 | This study |
| YPH499/p422tdc60                       | YPH499 transformant harboring pGK422-tdc60 | This study |
| YPH499/p422tdc50                       | YPH499 transformant harboring pGK422-tdc50 | This study |
| YPH499/p422tdc80                       | YPH499 transformant harboring pGK422-tdc80 | This study |
| YPH499/6U/δL                           | YPH499 transformant integrated p5U-PGK and pδL-PGK. | This study |
| YPH499/δUARO4fbr                       | YPH499 transformant integrated δu-ARO4fbr. | This study |
| YPH499/δUARO4fbr/δL                    | YPH499 transformant integrated pδU-ARO4fbr and pδL-PGK. | This study |
| YPH499/6U/LARO7fbr                     | YPH499 transformant integrated p5U-PGK and pδL-ARO7fbr. | This study |
| YPH499/6U/LARO7fbr/LARO7fbr            | YPH499 transformant integrated pδU-ARO7fbr and pδL-ARO7fbr. | This study |
| YPH499/6U/Ltdc70                       | YPH499/6U/δL transformant harboring pGK422-tdc70 | This study |
| YPH499/6UARO4fbr/Ltdc70                | YPH499/δUARO4fbr/LδL transformant harboring pGK422-tdc70 | This study |
| YPH499/δUARO7fbr/Ltdc70                | YPH499/δUARO7fbr/LδARO7fbr transformant harboring pGK422-tdc70 | This study |
| **Oligonucleotide primers**            |                 |                   |
| Br28960.1_Fw                           | AAAAGCTAGCATGCGCGATCGAGGAGA |          |
| Br28960.1_Rv                           | GGTTCGGGCGCTACTGTAACAATTTCTTA |          |
| Br51120.1_Fw                           | ACCGGTCGACATGGCCACCAAGTCGATGGA |          |
| Br51120.1_Rv                           | TCCGCCGCGGCTAAACAACGGCGTGAAGA |          |
| Br51170.1_Fw                           | ACCGGTCGACATGGCCACCAAGTCGATGGA |          |
| Br51170.1_Rv                           | TCCGCCGCGGCTAAACAACGGCGTGAAGA |          |
| Br14750.1_Fw                           | AAAAGCTAGCATGCGCGAGATCGAGGAGA |          |
| Br14750.1_Rv                           | GGTTCGGGCGCTACTGTAACAATTTCTTA |          |
| Br14780.1_Fw                           | AAAAGCTAGCATGCGCGAGATCGAGGAGA |          |

(Continued)
primer pair and was then introduced into the PstI and BamHI sites of pδU and pδL using an In-Fusion HD Cloning kit, generating pδU-PGK and pδL-PGK, respectively. The synthetic gene fragments ARO4\textsuperscript{br} and ARO7\textsuperscript{br} were obtained from a commercial source (Invitrogen, San Diego, CA) (see S1 File). A gene fragment encoding ARO4\textsuperscript{br} was PCR amplified using ARO4\textsuperscript{br}_Fw and ARO4\textsuperscript{br}_Rv, and was then introduced into the BamHI sites of pδU-PGK using an In-Fusion HD Cloning kit, generating pδU-ARO4\textsuperscript{br}. The synthetic ARO7\textsuperscript{br} gene fragment was directly introduced into the BamHI sites of pδL-PGK using an In-Fusion HD Cloning kit, generating pδL-ARO7\textsuperscript{br}.

Plasmids were transformed into *S. cerevisiae* using lithium acetate method [24, 25], and the resulting transformants are listed in Table 1. The transformants with the highest tyramine or L-tyrosine productivity were selected and used in subsequent experiments.

### Culture conditions

A single colony of each *S. cerevisiae* transformant was inoculated into a test tube containing 5 mL synthetic dextrose (SD) medium containing 2% glucose without adenine, uracil, or leucine as preculture. To evaluate tyramine or L-tyrosine productivity, preculture broth was seeded into 5 mL SD medium containing 2% glucose to give an initial OD\textsubscript{600} value of 0.1. Test tubes were incubated at 30°C for 72 h with agitation at 180 rpm.

### Analytical methods

The concentration of ethanol and glucose in the culture supernatant was measured using a BF-5 biosensor (Oji Scientific Instruments, Hyogo, Japan).

For estimation of produced L-tyrosine and tyramine, GC-MS was carried out using a GCMS-QP2010 Ultra (Shimadzu, Kyoto, Japan) equipped with a CP-Sil 8 CB-MS capillary column (30 m x 0.25 mm x 0.25 μm; Agilent). Helium was used as carrier gas to maintain a flow rate of 2.1 ml/min. The injection volume was 1 μl with a split ratio of 1:10. The oven temperature was initially held at 150°C for 5 min, raised to 300°C at 10°C/min, and further maintained at 300°C for 5 min. The total running time was 25 min. The other settings were as follows: 250°C interface temperature, 200°C ion source temperature, and electron impact ionization (EI) at 70 eV. Dried residues of tyramine and tyrosine were derivatized for 60 min at 80°C in

### Table 1. (Continued)

| Strain, plasmid, primer, or transformant | Relevant features | Source or reference |
|-----------------------------------------|------------------|---------------------|
| Br14780.1_Rv                           | AATTCCCGGGCTAGTGCTCCGCTTCCTCTA |                      |
| LEU2d(F)_InF                           | ATCGATACCGTCGAGCTCCTCGAGACGTTGAGCCATTTAGTATCAATTTG |                      |
| LEU2d(R)_InF                           | GGTCACGGGCCCCCCCCTCGAGTTTATTTACCAGAAGGCAGAAT |                      |
| PGK_to_delta_Fw                        | CTTGATATCAGAATTTCCCTCGAGAAAAGATGCCGATT |                      |
| PGK_to_delta_Rv                        | CGCTCCTGAAACTAGTCTGTTTTAAGCCAGAAG |                      |
| ARO4\textsuperscript{br}_Fw            | AGGTCGACACTAGTAGAGTAGTATCTCCTCAATGT |                      |
| ARO4\textsuperscript{br}_Rv            | TTCTCTAGACGCGCAGTTAAATGATGGTAGATGAGTATGTTTACCTCTCT |                      |
| RT_ARO4_Fw                             | TTGTCATGTCGCTTGCTTGTC |                      |
| RT_ARO4_Rv                             | CCGTGTGTCCTGGCTTGCTTC |                      |
| RT_ARO7_Fw                             | ATGTCCTTCAGTGTATGAGGCACAC |                      |
| RT_ARO7_Rv                             | TGAAGGCCCATACTCGAAGA |                      |
| RT_PGK1_Fw                             | TTGAGAACCACACACAGACC |                      |
| RT_PGK1_Rv                             | TGAAGCCATACCCACACACCA |                      |

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50 μL N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide (MTBSTFA) and 50 μL N-dimethylformamide prior to analysis [26, 27]. Cycloleucine was used as the internal standard.

Quantification of integrated copy numbers by real-time PCR

The integrated copy number of each recombinant strain was quantified using real-time PCR. Template genomic DNA was isolated from yeast cells cultivated in SD medium for 72 h at 30°C using a GenTLE precipitation carrier (Takara Bio) following the manufacturer’s protocol. The two sets of PCR primers used to detect ARO4 and ARO4br, and ARO7 and ARO7br listed in Table 1. Quantitative real-time PCR was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) with thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan). The normalized gene copy number was calculated by the relative quantification method with the PGK1 gene as the housekeeping gene.

Results

Cloning and functional expression of the gene encoding B. distachyon L-tyrosine decarboxylase in S. cerevisiae

The B. distachyon genome was screened for genes homologous to TDC genes derived from A. thaliana using GRAMENE, and 5 candidate TDC genes (Bradi1g28960.1, Bradi2g51120.1, Bradi2g51170.1, Bradi3g14750.1, and Bradi3g14780.1) were identified. After cloning each candidate gene into multi-copy vector pGK422, the resulting TDC expression vectors were individually introduced into S. cerevisiae YPH499. Each transformant was cultured in SD medium, and the culture supernatant was analyzed by GC-MS. A specific peak derived from tyramine-tyramine-2TBDMS derivatives (m/z = 144) was observed at approximately 17.2 min in GC-MS spectra of the culture supernatants of YPH499/p422tdc20 and YPH499/p422tdc70, but was not detected in the culture supernatants of the control strain, YPH499/p422, or those of YPH499/p422tdc60, YPH499/p422tdc50, and YPH499/p422tdc80 (data not shown). YPH499/p422tdc20 and YPH499/p422tdc70 produced 20 and 25 mg/L tyramine, respectively, in medium containing 2% glucose as the carbon source. The results of these analysis demonstrated that the B. distachyon transcripts Bradi2g51120.1 and Bradi2g51170.1 encoded a gene encoding TDC.

Construction of a L-tyrosine over-producing S. cerevisiae strain

To increase tyramine productivity in S. cerevisiae, we attempted to construct a strain that over-produces L-tyrosine by introduction of the enzymes, ARO4 and ARO7, which regulate L-tyrosine biosynthesis in S. cerevisiae [19, 20], into YPH499. After the construction of YPH499/δUARO4br, the gene encoding ARO7br was introduced into that transformant. Both ARO4 and ARO7 were integrated into the genome of YPH499 using the δ-integration method [24]. YPH499/δU/δL, YPH499/δU/δLARO7br, YPH499/δUARO4br/δL and YPH499/δUARO4br/δLARO7br were cultured in SD medium containing 2% glucose, and the culture supernatants were analyzed by GC-MS to quantify the amount of L-tyrosine produced after 72 h cultivation (Fig 2A). A total of 0.80, 1.99 and 4.84 mg/L tyrosine was found in the culture supernatant of YPH499/δU/δLARO7br, YPH499/δUARO4br/δL and YPH499/δUARO4br/δLARO7br, respectively, whereas no tyrosine was detected in the culture supernatant of the control strain YPH499/δU/δL. The copy number of ARO4br integrated into the genome of YPH499/δUARO4br/δL and YPH499/δUARO4br/δLARO7br, which originated from YPH499/δUARO4br/δL, was estimated by real-time PCR to be 2 in all strains. In contrast, the copy number of ARO7br in the genome of YPH499/δUARO4br/δLARO7br was estimated to be approximately 20, whereas that of YPH499/δU/δLARO7br was approximately 9 (Fig 2B).
Biosynthesis of tyramine using L-tyrosine over-producing *S. cerevisiae*

To evaluate the ability of *B. distachyon* TDC to convert L-tyrosine to tyramine, the gene encoding TDC70 was introduced into strains YPH499/ΔU/ΔL, YPH499/ΔUARO4br/ΔL, YPH499/ΔUARO4br/ΔLARO7br, and YPH499/ΔUARO4br/ΔLARO7br. Fig 3(A) shows the time courses of cell growth of each transformant. Although the cell growth rates of YPH499/ΔUARO4br/ΔL and YPH499/ΔUARO4br/ΔLARO7br were higher than those of YPH499/ΔU/ΔL and YPH499/ΔUARO4br/ΔLARO7br, the maximal level of cell growth was similar among the four transformants. Fig 3(B) and 3(C) show time courses of the glucose consumption and ethanol production rates, respectively, of each transformant. The rates of glucose consumption and ethanol production of YPH499/ΔUARO4br/ΔL and YPH499/ΔUARO4br/ΔLARO7br were higher than those of YPH499/ΔU/ΔL and YPH499/ΔUARO4br/ΔLARO7br. Fig 3(D) shows the time courses of tyramine production by the recombinant strains. The maximal levels of tyramine production, which started after 12 h cultivation, reached by YPH499/ΔU/ΔL, YPH499/ΔUARO7br, YPH499/ΔUARO4br/ΔL and YPH499/ΔUARO4br/ΔLARO7br were 30.4, 44.7, 113, and 200 mg/L, respectively, after 72 h of cultivation.
Plants accumulate large numbers of compounds that contain aromatic residues, such as phenylpropanoids, flavonoids, coumarins, and alkaloids, via secondary biosynthesis pathways [1–3, 28–30]. The structural diversity of aromatic compounds produced in plants is realized through sets of enzyme superfamilies, such as oxygenases, ligases, and decarboxylases [6]. For example, (S)-norcoclaurine, which is an intermediate of the benzylisoquinoline alkaloid
biosynthetic pathway, is synthesized from two molecules containing L-tyrosine modified with hydroxyl groups in the benzene ring through reactions catalyzed by aromatic amino acid decarboxylase and monoxygenase [3]. L-tyrosine derivatives can be converted to various compounds due to the hydroxyl group at the para position, and categorized into important parts in aromatic compounds. As various types of enzymes capable of modifying aromatic residues are found in plants, an increasing number of enzymes involved in the synthesis aromatic compounds will be identified as the genomes sequences of more plants become available.

Recently, the complete genome of B. distachyon was sequenced and used to construct full-length cDNA libraries [9]. To demonstrate that B. distachyon is a useful gene resource, we here focused on the B. distachyon genome for homologs of TDC, which catalyzes the decarboxylation of L-tyrosine and is involved in the production of aromatic compounds [31, 32]. It is suggestive that the transcripts Bradi2g51120.1 and Bradi2g51170.1 encode enzymes with L-tyrosine decarboxylation activity, and the corresponding genes were identified as novel TDC genes of B. distachyon.

The activity of B. distachyon TDC was further evaluated by constructing an L-tyrosine over-producing strain of S. cerevisiae. In the biosynthesis pathway of L-tyrosine in S. cerevisiae, ARO4 and ARO7 (ARO4fr and ARO7fr) are key enzymes that regulate L-tyrosine productivity and are subject to feedback inhibition by the produced L-tyrosine [19, 20]. Here, genes encoding L-tyrosine-insensitive ARO4 and ARO7 mutants were introduced into the genome of S. cerevisiae YPH499 strain using the δ-integration method. Helmstaedt et al. reported L-tyrosine-insensitive ARO4fr [20], whereas ARO7fr was previously constructed by Luttik et al. [19]. As shown in Fig 2(A), the L-tyrosine productivity of YPH499/δUARO4fr/δL and YPH499/δU/δLARO7fr was higher than that of YPH499/δU/δL. Quantitative real-time PCR analysis revealed that 2 copies of ARO4fr were introduced into the genome of YPH499/δUARO4fr/δL, whereas approximately 10 copies of ARO7fr genes were integrated into the YPH499/δU/δLARO7fr genome (Fig 2(B)). Together, these findings indicate that ARO4fr enhances L-tyrosine productivity more efficiently than ARO7fr (Fig 2(A)). This result may be attributed to the low availability of intracellular chorismate in YPH499/δU/δLARO7fr compared to that in ARO4fr-expressing strains. ARO4fr catalyzes the specific condensation of E4P and PEP into chorismate in the first step of the shikimate pathway, and the subsequent dislocation reaction is catalyzed by ARO7fr (Fig 1). As YPH499/δU/δLARO7fr expresses L-tyrosine sensitive ARO4, the formation of chorismate is strongly regulated by the produced L-tyrosine, which would therefore limit the available chorismate in this strain. Consistent with this speculation, the amount L-tyrosine produced by YPH499/δUARO4fr/δLARO7fr reached 4.84 mg/L in the culture supernatant, whereas YPH499/δU/δL did not produce L-tyrosine at detectable levels. We also investigated the correlation between L-tyrosine productivity and the copy number of ARO4fr or ARO7fr. Although the copy number of ARO4fr affected L-tyrosine productivity in the case of ARO4fr (See S2 File), L-tyrosine productivity wasn’t proportional to the copy number of ARO7fr in the case of ARO7fr (See S3 File).

The TDC homolog of B. distachyon encoded by Bradi2g51170.1 was functionally characterized by introduction into YPH499/δUARO4fr/δLARO7fr. YPH499/δUARO4fr/δLARO7fr/tdc70 was cultured using SD medium containing 2% glucose as the carbon source. As shown in Fig 3(D), 200 mg/L tyramine was produced by YPH499/δUARO4fr/δLARO7fr/tdc70, a level that was 6.6-fold higher than that of YPH499/δU/δL/tdc70 as the control strain. Based on the cell density, and protein and amino acid compositions of S. cerevisiae, we estimated the flux distribution rates of L-tyrosine into tyramine and biomass in each transformant after 72 h of cultivation [33, 34]. With increasing L-tyrosine productivity, the ratio of L-tyrosine distributed into tyramine was increased (Table 2). As L-tyrosine was not detected in the culture supernatant of the tyramine-producing strains, free L-tyrosine was thought to be completely converted to tyramine. These findings indicate that one of the rate-limiting steps of tyramine production remains L-
tyrosine availability. Thus, the TDC encoded by Bradi2g51170.1 may be a promising enzyme for the microbial production of aromatic compounds. We also attempted to express a candidate TDC derived from Arabidopsis in Saccharomyces; however, Arabidopsis TDC could not be expressed using our expression system (data not shown). As shown in Fig 3(A)–3(C), the cell growth, glucose consumption, and ethanol production rates of YPH499/ΔUARO4frΔLARO7fr/tdc70 and YPH499/ΔUARO4fr/ΔL/tdc70 were higher than those of YPH499/ΔU/ΔL/tdc70 and YPH499/ΔU/ΔLARO7fr/tdc70. These results may be attributed to the greater carbon flux in the glycolysis pathway resulting from the expression of ARO4fr, which promotes the condensation of PEP and E4P. In this study, we transformed pGK422-tdc70 into two different ARO4fr- and ARO7fr-expressing backgrounds. As a result, tyramine productivity, cell growth rates, glucose consumption rate, and ethanol production rates were almost the same among them (See S4 File). Using YPH499/ΔUARO4fr as the parent strain, TDC20 was also evaluated and compared to TDC70. Tyramine productivity of TDC70 was slightly higher than that of TDC20 (See S5 File).

In conclusion, we screened the genome of Brachypodium distachyon for genes encoding TDC, which is an enzyme involved in the modification of aromatic compounds, and identified two putative genes encoding TDC using Saccharomyces as a host strain. This result implies that Brachypodium distachyon has high potential as a genetic resource for the microbial production of aromatic compounds.

Although aromatic compounds have reportedly been produced using Saccharomyces, the yield of L-tyrosine derivatives, such as alkaloids, was very low [21]. We speculate that the L-tyrosine over-producing strain constructed here may be applicable to the production of L-tyrosine derivatives with complicated structures.

Supporting Information

S1 File. The nucleotide sequences of synthetic ARO4fr and ARO7fr genes (Under lines indicate open reading frame, capital letters indicate the nucleotide sequences substituted in order to deregulate feedback inhibition, and italic characters indicate flag-tag sequence). (DOCX)

S2 File. Correlation between L-tyrosine productivity and the copy number of ARO4fr. YPH499/ΔUARO4frΔL (Y; YPH499 (control), 1; colony 6, 2; colony 8, 3; colony 9).1 copy number of ARO4fr was integrated into the genome of colony 6 and 8, whereas 2 were colony 9, which was adopted for further experiments in this study. (DOCX)

S3 File. Correlation between L-tyrosine productivity and the copy number of ARO7fr. Results of YPH499/ΔUARO4frΔLARO7fr (Y; YPH499/ΔUARO4frΔL (control), 1; colony 3, 2; colony 5, 3; colony 18, 4; colony adopted in this study). Gray bar indicates L-tyrosine productivity per OD600, and black bar indicates normalized integrated copy number of ARO7 and ARO7fr. (DOCX)

|                | YPH499/ΔU/ΔL/tdc70 | YPH499/ΔU/ΔLARO7fr/tdc70 | YPH499/ΔUARO4frΔL/tdc70 | YPH499/ΔUARO4frΔLARO7fr/tdc70 |
|----------------|--------------------|--------------------------|------------------------|-------------------------------|
| Tyramine (mol%) | 51.9 ±1.8          | 61.1 ±2.3                | 80.0 ±1.8              | 87.4 ±0.3                     |
| Biomass* (mol%) | 48.1 ±1.8          | 38.9 ±2.3                | 20.0 ±1.8              | 12.6 ±0.3                     |

*A flux value to tyrosine building biomass was determined from OD600 values and its conversion coefficient to dry cell weight (0.25 g-DCW/L/OD600) by using the composition ratio of L-tyrosine in biomass [33, 34]. The flux was estimated as tyrosine concentration of culture (mmol/L). Flux distributions between tyrosine and biomass from tyrosine were estimated from each concentration.

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S4 File. Culture profiles of transformants in SD medium containing 2% glucose as the carbon source. Time-courses of (A) cell growth, (B) glucose consumption, (C) ethanol production, and (D) tyramine production for YPH499/ΔARO4/fbr/ΔARO7/fbr/tdc70 adopted in the manuscript (closed circles) and YPH499/ΔARO4/fbr/ΔARO7/fbr/tdc70 originated from different ARO4/ARO7 background (open circles). Each data point shows the average of 3 independent experiments, and error bars represent the standard deviation.

(SDOCX)

S5 File. Evaluation of tyramine productivity using YPH499/ΔARO4/fbr/tdc20 and YPH499/ΔARO4/fbr/tdc70 after 96 h cultivation.

(SDOCX)

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Author Contributions

Conceived and designed the experiments: SN TS KM FM AK. Performed the experiments: SN TS SO MO. Analyzed the data: SN TS SO MO. Wrote the paper: SN TS.

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