Uptake of adenine by purine permeases of *Coffea canephora*

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Abstract

Purine permeases (PUPs) mediate the proton-coupled uptake of nucleotide bases and their derivatives into cytosol. PUPs facilitate uptake of adenine, cytokinins and nicotine. Caffeine, a purine alkaloid derived from xanthosine, occurs in only a few eudicot species, including coffee, cacao, and tea. Although caffeine is not an endogenous metabolite in Arabidopsis and rice, AtPUP1 and OsPUP7 were suggested to transport caffeine. In this study, we identified 15 PUPs in the genome of *Coffea canephora*. Direct uptake measurements in yeast demonstrated that CcPUP1 and CcPUP5 facilitate adenine—but not caffeine—transport. Adenine uptake was pH-dependent, with increased activity at pH 3 and 4, and inhibited by nigericin, a potassium–proton ionophore, suggesting that CcPUP1 and CcPUP5 function as proton-symporters. Furthermore, adenine uptake was not competitively inhibited by an excess amount of caffeine, which implies that PUPs of *C. canephora* have evolved to become caffeine-insensitive to promote efficient uptake of adenine into cytosol.

Keywords; adenine, caffeine, *Coffea canephora*, purine permease

Introduction

Purine permeases (PUPs) constitute a large family of transporter molecules that are localized at the plasma membrane in plants, where they facilitate proton-coupled uptake of nucleotide bases and their derivatives, including adenine, cytokinins, and nicotine [1,2]. The first PUP member, AtPUP1 of *Arabidopsis thaliana*, was identified as the gene that complemented a yeast mutant in adenine uptake [3]. The protonophore carbonyl cyanide *m*-chlorophenyl hydrazine inhibits the adenine uptake activity of AtPUP1, suggesting that this PUP functions as a proton symporter [4]. In addition, the adenine uptake activity of AtPUP1 is competitively inhibited by purine derivatives including cytokinins and caffeine [4], thus suggesting that AtPUP1 mediates the uptake of a broad range of substrates.

Although Arabidopsis contains 23 PUP members in its genome, only four *PUP* genes have been analyzed to date. Of these, both AtPUP1 and AtPUP2 have similar substrate specificity for the uptake of adenine and cytokinins, but their tissue-specific expression patterns differ: *AtPUP1* is expressed mainly in leaf hydathode tissue and the stigmatic surface, whereas *AtPUP2* in expressed predominantly in vascular tissues [4,5]. Furthermore, AtPUP1 reportedly also facilitates the uptake of pyridoxine [6].

Another of the AtPUPs characterized to date, *AtPUP3*, is expressed in pollen, but the protein did not demonstrate any transport activity in a yeast system [4]. Through its cytokinin uptake activity, AtPUP14 is involved in the spatiotemporal distribution of cytokinin in the meristem and thus in plant morphogenesis [7]. Rice (*Oryza sativa*) contains 12 PUP members in its genome, but only one member, OsPUP7, was characterized as being involved in plant growth and development, possibly mediating cytokinin transport [8]. Although direct transport activity was not measured, OsPUP7 conferred sensitivity to caffeine in yeast, suggesting that OsPUP7 may take up the caffeine [8].

In addition to adenine and cytokinins, derivatives of nucleotide bases include the alkaloids nicotine and caffeine. The ability of a PUP to take up nicotine was first identified in *Nicotiana tabacum* [9,10], in which NtNUP1 acquires nicotine from the apoplast, particularly in root tips. The suppression of *NtNUP1* expression in tobacco hairy roots decreased the nicotine content in the tissue [10], and measurement of direct

uptake activity in yeast showed that NtNUP1 is an uptake transporter of—in addition to nicotine—pyridoxamine, pyridoxine, and anatabine [9]. Although the results of competitive inhibition and yeast growth assays have suggested that PUPs in Arabidopsis, and rice potentially transport caffeine [3,4,8], their caffeine uptake activity has not been measured directly, nor have the PUPs in any caffeine-synthesizing species been characterized. Caffeine is synthesized by only a few eudicot plants, such as coffee (*Coffea* spp.), cacao (*Theobroma cacao*), and tea (*Camellia sinensis*) [11,12]. Coffee is an important cash crop and is cultivated across more than 11 million hectares [13]. Two species (*C. arabica* and *C. canephora*) account for nearly all coffee bean production. *C. arabica* is an autogamous allotetraploid species originating from a cross between *C. canephora* and *C. eugenioides* [14]. In this report we identified 15 PUP members from *C. canephora*, and characterized the transport activity of CcPUPs in yeast.

Materials and Methods

Chemicals

Chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan), unless otherwise stated.

Identification and sequence analysis of PUP homologs in C. canephora

We used the predicted amino acid sequence of AtPUP1 as a query sequence in a BLASTP search of the publicly available database Coffee Genome Hub (http://coffee-genome.org/) to find the *PUP* genes in *C. canephora*. Using default search parameters, we identified 15 CcPUPs, which we named CcPUP1 to CcPUP15 in order of their loci in the genome.

Construction of plasmids and transformation of yeast

The coding sequences of *CcPUP1* and *CcPUP5* were amplified in 25-μL reaction mixtures containing cDNA of cultured *C. canephora* cells, 0.5 μL of PrimeSTAR GXL DNA Polymerase (Takara, Japan), 5 μL of 5×PrimeSTAR GXL buffer, 16 μL of distilled water, 2 μL of dNTPs (2.5 mM), and 5 pmol of each appropriate primer (*CcPUP1*, 5′-CACCATGCCAGTCAATGAGGAACC-3′ and

5′-TCAGCACAACGAGTCATTAGTAG-3′; *CcPUP5*, 5′-CACCATGGAGAATACTACTCAAGAAATGG-3′ and 5′-TCAAGAAGTCCCTAGGAAAGAA-3′). PCR amplification conditions were: denaturation at 98 °C for 1 min; 30 cycles of 98 °C for 10 s, 60 °C for 15 s, and 68 °C for 1 min. A final extension was conducted for 5 minutes at 58°C. PCR amplicons were purified by using the Wizard SV Gel and PCR Clean-Up System (Promega, San Luis Obispo, CA) according to the manufacturer's protocol. *CcPUP1* and *CcPUP5* cDNAs were ligated into pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA). DNA sequence of *CcPUP7* was synthesized by Genewiz (Kawaguchi, Japan), and ligated into pENTR/D-TOPO. These were then transferred into pYES-DEST52 (Invitrogen) by using Gateway cloning technology (Invitrogen) according to the manufacturer's instructions. The cDNA-carrying pYES-DEST52 vectors were used to transduce an *FCY2*-deleted yeast strain (BY4741, Mata, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0,* YER056C::kanMX4; Invitrogen) according to the manufacturer's protocol; pYES-DEST52 lacking a CcPUP cDNA insert was used as a negative control.

Growth assay

Gene expression in pYES-DEST52-transduced yeast cells can be induced by galactose and repressed by glucose. The basic (control) medium for the growth assays was minimal medium (lacking uracil and glucose); 2% galactose and 1% raffinose were added as carbon sources to induce gene expression. Basic medium containing 2% glucose was used to repress gene expression. Transduced yeast cells were pre-incubated in the inducing medium or repression medium for 2 days at 30 °C, and then were diluted to an OD_{600} of 0.002 by adding sterile water. Then 40 µl of each diluted solution was applied to an agar plate containing caffeine, which was incubated at 30 °C for 5 days, according to the literatures [4, 8].

Measurement of transport of purine derivatives in yeast

For caffeine uptake assays, transduced yeast cells were pre-incubated in repression medium for 2 days at 30 °C. Cells were harvested by centrifugation, washed, and resuspended in inducing medium to a final OD_{600} of 0.2. Yeast cells were incubated at

30 °C for 40 hours and then harvested, washed, and resuspended in 100 mM sodium citrate buffer (pH 3.0) containing 1% glucose to a final OD_{600} of 12. Before initiation of the transport assay, yeast cells (100 µL) were preincubated at 30 °C for 2 min; then assay buffer (105 µL) containing 100 mM citrate buffer, pH 3.0, 1% glucose, 176 Bq μL^{-1} ^{14}C-labeled caffeine (American Radiolabeled Chemicals, St Louis, MO) and unlabeled caffeine was added. A 40-µL sample was withdrawn from the reaction tube after 30, 60, 120, and 180 s; each sample was transferred to 4 mL of ice-cold water, filtered onto glass-fiber filters (GE Healthcare, Chicago, IL), and then washed with 4 mL of water in a manifold (Merck Millipore, Burlington, MA). For adenine uptake assays, the incubation was initiated by adding 103 µL of assay buffer containing 100 mM citrate buffer, pH 3.0, 1% glucose, 718 Bq μL^{-1} ^{3}H-labeled adenine (Moravek Biochemicals, Brea, CA) and unlabeled adenine at the final concentration of 100 µM, according to the literature [4].

To vary the pH of the assay, yeast cells were grown at 30 °C for 40 hours, washed, and resuspended in 100 mM sodium citrate buffer for which the pH was adjusted. For assays involving nigericin, a reaction buffer containing 4.0 mM nigericin was used. For competitive inhibition assays, 94 µL of buffer containing 540 µM caffeine, adenine, or sucrose was added 2 min before the start of the reaction, after preincubation, reactions were initiated by adding 9 µL of buffer containing 8.2 kBq μL^{-1} ^{3}H-labeled adenine.

Results

Identification and expression analysis of purine permeases of C. canephora

In this study, we used AtPUP1 as a query in a BLASTP search of the genomic sequence of *C. canephora* in the public database Coffee Genome Hub (http://coffee-genome.org/) to identify PUPs in this species. We named the 15 PUPs that we obtained as CcPUP1 through CcPUP15, in order of their genomic loci, as follows: CcPUP1, Cc02g25680; CcPUP2, Cc03g11350; CcPUP3, Cc03g13540; CcPUP4, Cc06g15040; CcPUP5, Cc08g01780; CcPUP6, Cc08g11780; CcPUP7, Cc09g04610; CcPUP8, Cc09g08430; CcPUP9, Cc09g09080; CcPUP10, Cc09g09090; CcPUP11, Cc09g09160; CcPUP12, Cc10g06500; CcPUP13, Cc10g06800; CcPUP14, Cc10g15390; CcPUP15, Cc10g15400.

A phylogenetic tree constructed by using the amino acid sequences of CcPUPs and characterized PUPs from Arabidopsis, rice, and tobacco showed that AtPUP1 and OsPUP7, which were 34% homologous at the amino acid level, clustered in different clades (Fig. 1A) even though both proteins have been suggested to transport caffeine.

We then used the RNA-seq data available in Coffee Genome Hub (http://coffee-genome.org/) to summarize the tissue expression of *CcPUPs* (Fig. 1B). Whereas *CcPUP1*, *CcPUP4*, *CcPUP6*, and *CcPUP12* are expressed in most tissues, *CcPUP2* is expressed more specifically in leaves and roots. In addition, *CcPUP7* is strongly expressed in perisperm and endosperm, where caffeine is highly accumulated.

CcPUP1 and CcPUP5 confer sensitivity to caffeine in yeast

Results of yeast sensitivity tests suggest that PUPs transport caffeine [4,8]. We therefore individually cloned *CcPUP* cDNAs into the pYES-DEST52 vector, in which gene expression is regulated by the GAL1 promoter, and used the plasmids to transform yeast mutant *fcy2*, which is deficient in adenine uptake [3]. Yeast transformants expressing *CcPUP1*, *CcPUP4*, *CcPUP5*, *CcPUP6*, *CcPUP7*, and *CcPUP12* were cultured. Compared with that of the vector control, growth of the yeast transformants expressing *CcPUP1* and *CcPUP5* was suppressed on induction medium containing 0.2% caffeine (Fig. 2). No difference in growth was observed for yeast transformants harboring *CcPUP4*, *CcPUP6*, *CcPUP7*, or *CcPUP12* (Supplementary Fig. 1)

Transport assays

Because the results of the yeast growth assay suggested that CcPUP1 and CcPUP5 have caffeine uptake activity, we analyzed their direct transport activity by determining the caffeine contents in yeast transformants after their incubation on caffeine as a substrate. No uptake activity was observed for transformants expressing either *CcPUP1* or *CcPUP5* (Fig. 3A). When the pH condition was modified from pH 3 to 7, there was no caffeine uptake activity in these cells (Supplementary Fig. 2). The uptake activity for adenine was then measured using the same transformants. Higher amount of adenine was transported in yeast transformants expressing CcPUP1 or CcPUP5 than the vector control (Fig. 3B). In addition, the adenine uptake activity of CcPUP1 and CcPUP5 was

higher at a pH of 3 or 4 and decreased when the pH was increased (Fig. 4).

Because *CcPUP7* is highly expressed in the perisperm and endosperm, where caffeine is highly accumulated, the caffeine and adenine uptake were also tested for CcPUP7. The uptake activity was not observed in yeast transformants (Supplementary Fig. 3).

Inhibition of adenine uptake

To analyze whether adenine uptake by CcPUP1 and CcPUP5 utilizes a proton gradient, we measured adenine uptake in the presence of nigericin, an ionophore that exchanges K^+ for H^+ across membranes and thus abolishes a pH gradient. Adenine uptake by CcPUP1 and CcPUP5 was decreased by about 42% and 51%, respectively, in the presence of nigericin compared with control values (Fig. 5). Nigericin also reduced the adenine uptake of AtPUP1 (Fig. 5C).

Caffeine was suggested to competitively inhibit the ability of AtPUP1 to take up adenine [3]. To investigate whether caffeine competitively inhibits adenine uptake by CcPUP1 and CcPUP5, we conducted a transport assay under which the incubation media contained 10-fold more caffeine than adenine; controls for this assay included 10-fold increased amounts of adenine and sucrose. For both CcPUP1 and CcPUP5, the uptake of radioactive adenine was decreased in the presence of excess adenine but was unaffected under conditions of excess caffeine or sucrose (Fig. 5). In contrast to findings for CcPUP1 and CcPUP5, addition of an excess of caffeine diminished the uptake of adenine by AtPUP1 (Fig. 5F), in line with a previous report [3].

Discussion

Purine bases such as adenine and guanine are ubiquitous metabolites that are found in all organisms. In addition to purine nucleotides, several plants synthesize purine alkaloids, including caffeine and theobromine [15]. The caffeine synthesized by *Coffea* spp. accumulates predominantly in seeds and leaves, where caffeine restricts development and growth of other organisms [16] and also stimulates plant defense response by affecting signaling pathways [17,18]. In addition, caffeine is secreted from the roots during germination, when it is thought to modulate interactions with pathogens and mycoparasites [19,20].

Several lines of evidence support the importance of membrane transport in the function of metabolites [21,22], and various families of transporters for nucleotide bases and their derivatives have been characterized [1,23]. In the current study, we analyzed the purine permease family members, which are uptake transporters for various purine bases and their derivatives [2], in *C. canephora*, for which genomic and transcriptomic data are publicly available [11,24]. Among the 15 PUP genes that we discovered in the genome of *C. canephora*, two (*CcPUP1* and *CcPUP5*) were identified as encoding candidate uptake transporters, according to growth assays using caffeine-containing media. Using radioactive substrates in a yeast-based system, we showed that both CcPUP1 and CcPUP5 uptake adenine, possibly in a proton-symport manner. Even though growth assay results suggested that both CcPUP1 and CcPUP5 can uptake caffeine, neither transporter recognized caffeine as a substrate. The apparent sensitivity of CcPUP1 and CcPUP5 transformants to caffeine might merely reflect the growth retardation of yeast expressing a membrane transporter. Even in liquid media without caffeine, the growth of the CcPUP1 and CcPUP5 transformants was suppressed compared with vector controls (Supplementary Fig. 4).

In conclusion, among the 15 PUPs that we identified in *C. canephora*, we found that CcPUP1 and CcPUP5, which are adenine transporters, not inhibited by caffeine. The insensitivity of CcPUPs to caffeine may be physiologically important in *C. canephora*, where these proteins need to distinguish adenine from caffeine to efficiently take up adenine in various cells. We surmise that purine permeases in *C. canephora* have evolved to differentiate adenine from caffeine.

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Author Contributions

HK, NS, SO, KY, and AS conceived and designed the experiments; HK and AS performed the experiments; HK, NS, HK*, KY, and AS analyzed the data; and HK and AS wrote the paper with input from all coauthors.

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Disclosure Statement

No potential conflict of interest was reported by the authors.

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Figure Legends

Figure 1. Phylogenetic analysis of purine permease (PUP) proteins in Arabidopsis (At), rice (Os) and *Coffea canephora* (Cc) and their gene expression levels. (a) A phylogenetic tree was generated by using MEGA 7.0 software [25]. The amino acid sequences of PUPs were aligned by using the MUSCLE program. The maximum likelihood method was used to construct a phylogenetic tree with 1000 bootstrap replications. Bootstrap values (maximum, 100) are shown at nodes generating clades. (b) Heat map according to the number of reads per kilobase per million mapped reads (RPKM) for each gene.

Figure 2. Growth of *CcPUP*-transformed yeast on media containing 0.2% caffeine after incubation at 30 °C for 5 days.

Figure 3. Transport assay using CcPUP1 and CcPUP5-expressing yeast. (a) Time-dependent uptake of caffeine in yeast expressing CcPUP1 and CcPUP5. The final concentration of caffeine was 100 μM. (b) Time-dependent uptake of adenine in yeast transformants expressing CcPUP1 and CcPUP5. The final concentration of adenine was 100 μM. Data are presented as mean ±SD (n = 3); different letters indicate values that are significantly different ($P < 0.05$) according to Tukey's Honestly Significant Difference test.

Figure 4. Effect of pH on adenine uptake by CcPUP1 and CcPUP5. Adenine uptake was determined after 3 min of incubation in sodium citrate buffer; the final concentration of adenine was 100 μM. Data are presented as mean ± SD of three replicates; different letters indicated values that are significantly different ($P < 0.05$) according to Tukey's Honestly Significant Difference test.

Figure 5. Inhibition of adenine uptake. Adenine uptake by (a) CcPUP1, (b) CcPUP5, and (b) AtPUP1 after 3 min of incubation in the presence of nigericin. Adenine uptake by (d) CcPUP1, (e) CcPUP5, and (f) AtPUP1 after 3 min of incubation in the presence of excess amounts of adenine, caffeine, and sucrose. The final concentration of each

substrate was 25 μM. Data are given as mean ± SD (n = 3); different letters indicate values that are significantly different ($P < 0.05$) according to the Student t-Test (A–C) or Tukey's Honestly Significant Difference test (D–F).

Supplementary Figure 1. Growth of *CcPUP*-expressing yeast on media containing caffeine after incubation at 30 °C for 5 days.

Supplementary Figure 2. Effect of pH on caffeine uptake by CcPUPs. Caffeine uptake was determined after 3 min of incubation in sodium citrate buffer. The final concentration of caffeine was 100 μM. Data are presented as mean ±SD of three replicates.

Supplementary Figure 3. Transport assay using CcPUP7-expressing yeast. (a) Uptake of caffeine in yeast expressing CcPUP7. The final concentration of caffeine was 100 μM. (b) Uptake of adenine in yeast transformants expressing CcPUP7. The final concentration of adenine was 100 μM. Data are presented as mean ±SD of three replicates.

Supplementary Figure 4. Growth of yeast in liquid media. The OD_{600} of each culture was measured over 24 hours. Data are presented as mean ± SD of three replicates.

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