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Fairy chemicals (FCs), 2-azahypoxanthine (AHX), imidazole-4-carboxamide (ICA), and 2-aza-8-oxohypoxanthine (AOH) are molecules with many diverse actions in plants. The defined biosynthetic pathway for FCs is a novel purine metabolism in which they are biosynthesized from 5-aminoimidazole-4-carboxamide. Here, we show that one of the purine salvage enzymes, hypoxanthine-guanine phosphoribosyltransferase (HGPRT), recognizes AHX and AOH as substrates. Two novel compounds, AOH ribonucleotide and its ribonucleoside which are derivatives of AOH, were enzymatically synthesized. The structures were determined using mass spectrometry, 1D and 2D NMR spectroscopy, and X-ray single-crystal diffraction analysis. This report demonstrates the function of HGPRT and the existence of novel purine metabolism associated with the FCs biosynthesis in rice.

Introduction

"Fairy rings" is a unique natural phenomenon that appears on the lawns, grasslands, and forests worldwide. Its characteristic feature is the rings with excessive or suppressive grown turfgrass, and occasionally with the appearance of mushrooms.[1-3] The name "fairy rings" comes from Western Europe's lore that fairies dance within the ring.[2, 4, 5] This natural phenomenon is caused by approximately 60 species of basidiomycetes inhabiting in the soil that mycelia grow regularly and radially.[2, 3, 6]

In 2010, two plant growth regulators, the promotor, 2-azahypoxanthine (AHX), and the inhibitor, imidazole-4-carboxamide (ICA), were found from the culture broth of a basidiomycetes *Lepista sordida*, one of the major fairy ring-forming fungi.[7, 8] After a while, another plant growth-promoting compound, 2-aza-8-oxohypoxanthine (AOH), was discovered as a metabolite of AHX in rice. Furthermore, the oxidation mechanism of AHX to give AOH has been elucidated in plants.[9] These three compounds (AHX, ICA, and AOH) are probably involved in the formation of fairy rings by mediating the interaction between plants and mushrooms, and they were named fairy chemicals (FCs) after the title of the article in *Nature*.[10] Our subsequent studies have shown that FCs confer tolerance for various stress (temperature, salt, drought, etc.) to plants, and increase grain yields up to 20%.[5, 7-9, 11, 12] In addition to the activity of FCs against plants, the proof of the universal existence of FCs in plants suggested that FCs are a candidate for a new family of plant hormones.[5, 9, 13-16]

Our research has also uncovered biosynthetic and metabolic pathways for FCs in rice (Fig. S1).[15-17] The metabolites of FCs, the glycosides, regulated the homeostasis of FCs in rice. The precursor of purine metabolism, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), is known to branch into 5-aminoimidazole-4-carboxamide (AICA) in addition to the pathway into purine metabolism, and it has been reported that AHX and ICA are biosynthesized from AICA in rice.[9, 13] Besides, the conversion of AHX to AOH is probably catalyzed by xanthine dehydrogenase (XDH), a purine metabolic enzyme which catalyzes the oxidation of hypoxanthine to xanthine and xanthine to uric acid.[9, 13] Concerning the unique 1,2,3-triazine ring in AHX and AOH, we recently proved that that nitrosonium ion (NO^+) or dinitrogen trioxide (N_2O_3), each of which is derived from NO produced by NO synthase (NOS), is directly involved in the formation of AHX in *L. sordida*.[18]

From these findings and the structures of FCs, we estimated that FCs are highly associated with purine metabolism. Purine nucleotides are precursors of purine nucleobases and essential compounds in all organisms. They are involved in many crucial biochemical processes such as the synthesis of nucleic acids, energy sources, and precursors of nucleotide cofactors. Their synthetic pathway has been confirmed in various mammals and plants, and there are two principal routes for the biosynthesis of purine nucleotides: the *de novo* pathways and the salvage pathways. The *de*

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novo pathway produces purine nucleotides from D-ribose-5-phosphate supplied from the pentose phosphate pathway and precursor molecules such as amino acids and carbon dioxide. It consists of more than ten reactions and requires some enzymes and energy. On the other hand, the salvage pathway produces purine nucleotides from purine bases and 5-phosphoribosyl-1-pyrophosphate (PRPP) in a single step by the action of phosphoribosyltransferase.[19, 20]

Hypoxanthine-guanine phosphoribosyltransferases (HGPRT; EC 2.4.2.8) is a key enzyme of the purine salvage pathway. Genes encoding HGPRTs from several organisms have been cloned, sequenced, and overexpressed in *Escherichia coli*, and some recombinant HGPRT enzymes have been characterized kinetically.[21] This enzyme exhibits specificity for 6-oxopurines and catalyzes Mg^{2+}-dependent reversible transfer of the phosphoribosyl group of PRPP to the *N*9 position of 6-oxopurines. Hence, the reversible conversions of hypoxanthine to inosine monophosphate (IMP; hypoxanthine *N*9-ribonucleotide) and guanine to guanosine monophosphate (GMP; guanine *N*9-ribonucleotide) are catalyzed by HGPRT along with the reversible conversion of PRPP to inorganic pyrophosphate.[20, 22] AHX and AOH also have a 6-oxopurine skeleton and their structures are very similar to hypoxanthine. Thus, we deduced that the reversible conversions of AHX and AOH to their *N*9-ribonucleotides were catalyzed by HGPRT, AHX and AOH *N*9-ribonucleotides were involved in the biosynthesis and metabolism for AHX and AOH.

In this paper, we report the cloning of cDNA coding for *Oryza sativa* HGPRT (OsHGPRT) and the activity of recombinant OsHGPRT (rOsHGPRT) expressed in *E. coli* against FCs. Novel purine metabolism for FCs were discussed from the extracted crude enzyme solution. Furthermore, structure determination of a ribotide produced by treatment of AOH with rOsHGPRT is described.

Results and discussion

Cloning, expression, and purification of recombinant OsHGPRT

A rice cDNA library was constructed using total RNA isolated from rice seedlings cultivated for a week. The cDNA coding for OsHGPRT (*Oshgprt*) was amplified by reverse transcription-polymerase chain reaction (RT-PCR) and cloned into the expression vector pET-28a(+). *Oshgprt* has a coding region of 786 nucleotides for a protein of 261 amino acids. The sequence of the construct of pET-28a(+) and *Oshgprt* was identical to the gene encoding OsHGPRT, which was previously determined in the International Rice Genome Sequencing Project.[23] The protein with hexa-histidine tag (His-tag) at the *N*-terminal was purified by Ni^{2+} affinity chromatography. The purified rOsHGPRT appeared as a single band with approximately 33 kDa (theoretically 29.8 kDa) on SDS-PAGE and the yield was 10 mg per 1.0 g of cells (Fig. S2).

Biochemical characterization of OsHGPRT towards purine analogues

To clarify the role of OsHGPRT in the biosynthesis or metabolism of FCs, phosphoribosylation activity of rOsHGPRT was examined toward purine analogues (hypoxanthine, guanine, adenine, xanthine, uric acid, 8-azahypoxanthine, allopurinol, AHX, AOH) and ICA, and AICA (Fig. 1a and Table S1). The reaction products of these compounds were analyzed by LC-MS.

Although hypoxanthine and guanine, the original substrates of HGPRT, were strongly recognized as the substrate, the enzyme showed the highest activity against allopurinol, a structural isomer of hypoxanthine. Allopurinol is known to be a potent inhibitor of xanthine oxidase (XOD) which is one of the purine metabolic enzymes and also used as a hyperuricemia drug, and its ribonucleoside and ribonucleotide were detected from allopurinol-treated soybean and hemolysate of patients who were treated with the drug, respectively.[24, 25] Furthermore, it has been reported that protozoans- and human-derived HGPRTs exhibited the activity against allopurinol, indicating that this compound is recognized as a substrate for HGPRT in a wide variety of species.[26-28] Based on these studies, our results suggested that allopurinol is a substrate of HGPRT in plants.

The enzyme also showed high activity against 8-azahypoxanthine and AHX, and both of them have very similar structures to hypoxanthine and a nitrogen atom instead of a carbon atom in hypoxanthine. It has been reported that phosphoribosyl transfer to 8-azahypoxanthine and AHX is catalyzed by HGPRTs from *Plasmodium lophurae*, *Leishmania donouani*, *Human erythrocytes*, and HEp-2 cells.[28, 29] Thus, the substituted nitrogen atoms in the 6-oxopurine ring are not likely to significantly affect the substrate specificity of HGPRT. In contrast, the conversion rates of AOH and xanthine having two carbonyl groups in the purine ring were 38.0% and 19.9% compared to allopurinol, respectively, while that of uric acid possessing three carbonyl groups in the molecule, was dramatically decreased to 2.9% (Fig. 1a and Table S1). This result suggested that the number of the carbonyl group in the structure has a significant effect on the enzyme activity as a substrate. On the other hand, the catalytic activity of the enzyme was found to be low (~6.0%) for the three compounds without the 6-oxopurine skeleton (ICA, AICA, and adenine). In general, HGPRT recognizes 6-oxopurine as a substrate, and several crystal structures of HGPRT have been reported in which the amino acid residues that determine its substrate specificity have been identified. Based on these studies, interactions with highly conserved amino acid residues might contribute to the substrate specificity of HGPRT: hydrogen bonds between Lys 201 in rice (Lys 164 in human, Lys 133 in *E. coli*, and Lys 133 in *Arabidopsis thaliana*) and the 6-oxo group in the purine base, and p-p stacking interactions between the aromatic side chain of Phe 229 in rice (Phe 187 in human, Phe 156 in *E. coli*, and Phe 171 in *Arabidopsis thaliana*) and the purine base (Fig. S3).[30-32]

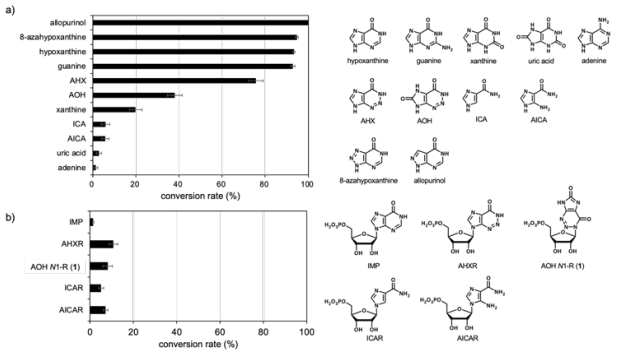

Fig. 1. The conversion rates of phosphoribosylation (a) and dephosphoribosylation (b) for each substrate by rOsHGPRT. After the reaction for 10 min, an equal amount of methanol was added to stop the reaction and subjected to LC-MS analysis. The conversion rate was calculated from the remaining amount of the substrate relative to the amount of negative control (added boiled enzyme solution). Data are presented as means ± S.D. (n=3).

Structure determination of AOH ribonucleotide produced by action of rOsHGPRT

Purine ribonucleotides are intermediates of purine metabolism and are known to be present in living organisms. Therefore, we thought that ribonucleotides of FCs may be involved in the biosynthesis of these compounds in plants. Since AHXR and ICAR already had been chemically synthesized by us, the structures of both the compounds were easily identified by LC-MS/MS analysis.[33, 34] However, chemical synthesis of ribotide of AOH has never been achieved. Therefore, we tried to determine the structures of products obtained by the rOsHGPRT-catalyzed reaction. LC-MS analysis of the reaction mixture indicated that it contained two AOH-ribonucleotides (AOHR; **1** and **3**) and two AOH-ribonucleoside (AOHr; **2** and **4**)(Fig. 2a,b and Fig. S4). The main product **1** was successfully purified by HPLC. We have not isolated them (**3** and **4**) and determine their structures yet, due to the small amounts of them.

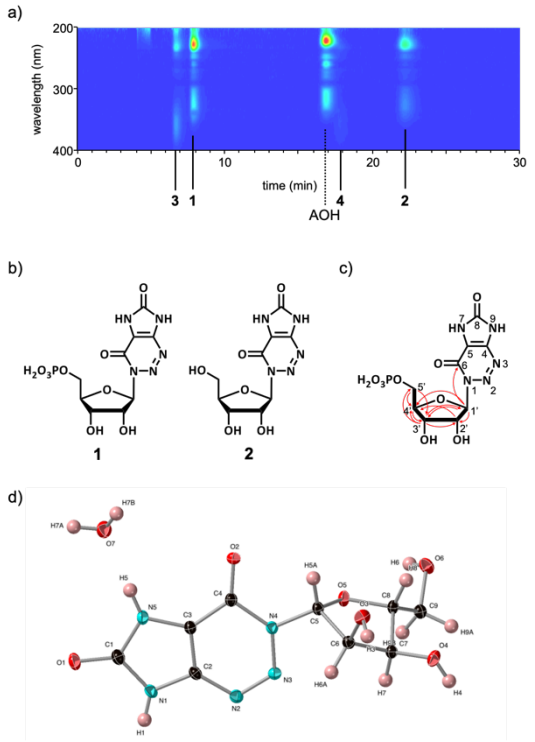

Fig. 2 (a) HPLC profiles of the reaction solution with rOsHGPRT and AOH. Enzyme reaction mixture extracted with methanol was injected into HPLC with a photo-diode array detector. Compounds **1** and **3** with *m/z* 366 $[M+H]^+$ are AOH ribo and compounds **2** and **4** with *m/z* 286 $[M+H]^+$ are AOHr. (b) Structures of **1** and **2**. (c) 2D-NMR correlations of **1**. Red arrows and bold lines indicate the HMBC correlation and DQF-COSY correlation, respectively. (d) ORTEP drawings of **2** with ellipsoids at the 50% probability level.

Compound **1** was obtained as a colorless amorphous material. The molecular formula was determined as $C_9H_{12}N_5O_9P$ by high-resolution electrospray ionization mass spectrometry (HRESIMS) (*m/z* 364.0286 $[M-H]^-$; calcd for $C_9H_{11}N_5O_9P$, 364.0300), indicating the presence of eight degrees of unsaturation in the molecule (Fig. S4a). The structure of **1** was elucidated by the interpretation of nuclear magnetic resonance (NMR) spectra including distortionless enhancement by polarization transfer (DEPT), double quantum filtered correlation spectroscopy (DQF-COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond coherence (HMBC) (Figs. S5-S9). The DEPT experiment indicated the presence of one methylene, four methines and four non-hydrogen-bearing carbons. The ^{13}C NMR data (δ_C 114.0, 142.5, 150.3, 154.9) indicated that **1** had the same skeleton as that of AOH[16]. The ribose part (C-1' to C-5') was constructed by the DQF-COSY correlations (H-1'/H-2'; H-2'/H-3'; H-3'/H-4'; H-4'/H-5') and the HMBC correlations (H-1'/C-2', C-3', C-4'; H-2'/C-4'; H-3'/C-1', C-2', C-4',C-5'; H-4'/C-3'; H-5'/C-3', C-4'). The linkage position between the sugar and AOH was determined from the HMBC cross-peak (H-1'/C-6) (Fig. 2c). The complete assignment of all the proton and carbon signals of NMR was accomplished as shown in Table S2. Compound **1** was a novel compound.

Although it has been reported that HGPRT transfers the phosphoribosyl group of PRPP only to the *N*9 position of 6-oxopurine, compound **1** is *N*1-ribotide. Purine nucleotides in living organisms have a 5'-phosphoribosyl group attached to the *N*9 position, and there is no report of the isolation and detection of purine nucleotides with a 5'-phosphoribosyl group connected to the *N*1 position from nature. To support the structural determination of this extremely rare enzyme product **1** by NMR, we attempted to determine the structure of **1** by X-ray crystallography. However, **1** did not crystallize. Therefore, **1** was treated with alkaline phosphatase (ALP; EC 3.1.3.1) from *E. coli* that catalyzes the hydrolysis of a wide variety of phosphate monoesters. As a result, **1** was dephosphorylated, giving the corresponding riboside **2** which was easily crystallized (Fig. S10). X-ray crystallographic analysis of **2** determined the structure as AOH *N*1-ribonucleoside (Fig. 2 and Table S3).

Dephosphoribosylation activity of rOsHGPRT towards purine ribonucleotides

Since the synthesis of AOHR by rOsHGPRT was successful, the reverse reaction (dephosphoribosylation) activity of the enzyme was examined for ribonucleotides of FCs, AICA, and hypoxanthine; AHXR, AOH *N*1-R (**1**), ICAR, AICAR, and IMP. As a result, all the substrates had a conversion rate of less than 10.8% (Fig. 1b and Table S4). The crucial role of phosphoribosyltransferase been defined as the efficient biosynthesis of purine ribonucleotides in the salvage pathway, hence, they have higher activity towards purine nucleobase than purine ribonucleotide.[19] The ratio of phosphoribosylation/dephosphoribosylation activity of this enzyme was high for hypoxanthine, AHX, and AOH, while it was close to 1 for AICA and ICA. In particular, the dephosphoribosylation activity of rOsHGPRT against IMP was significantly weaker than that against hypoxanthine in phosphoribosylation, hypoxanthine was scarcely produced in the reaction for 10 min. This tendency was also observed in the reaction of AHX or AOH, which has a 6-oxopurine skeleton, suggesting that HGPRT is involved in their salvage pathway in rice.

Metabolism and biosynthesis of AHX in rice

Plants synthesize purine nucleotides and metabolize them into purine nucleobases via purine nucleosides. Most of the nucleobases are catabolized to release nitrogen(s) in the ring and some are used in salvage reactions for recycling into nucleotide pool.[20, 35]

Considering the cascade of purine ribonucleotide, purine ribonucleoside, and purine base, AHX and AOH may also have the cycles in which their ribonucleotides are metabolized to them via ribonucleosides by several enzymes, and used for resynthesis of ribonucleotides by HGPRT. Then, LC–MS/MS analysis was first performed utilizing chemically synthesized AHXR and AHXr, and AOHR (**1**) and AOHr (**2**) as authentic standards to investigate their endogeny in rice. However, these compounds were not detected in rice seedlings by the analysis using previously established highly sensitive detection methods.[13] Under culture conditions where FCs are detected, those compounds may be further metabolized and accumulated in various forms in the plant. To understand the metabolism in plants, rice seedlings treated with a large amount of AHXR (100 µM) were extracted, partially purified and subjected to LC-MS/MS analysis. Although AHXR and AHXr were not detected in the AHXR-treated plants, AHXR was also not detected even in the medium after incubation with rice seedlings. On the other hand, approximately 80% of AHXR still remained in the medium without seedlings (control). These results suggested the ribotide was very easily metabolized in rice (Fig. S11).

Since AHXR is readily and rapidly metabolized in rice, metabolites were examined using crude enzymes from rice cells. When the enzymes were incubated with AHXR, almost all of the ribotide was converted to another compound within 30 min (Fig. S12). The LC-MS/MS analysis indicated a molecular weight (*m/z* 268.0688 $[M-H]^-$) and a product ion (*m/z* 240) consistent with AHXr only in the reaction solution (Fig. S13). The results indicate that AHXR was enzymatically converted to AHXr by the treatment.

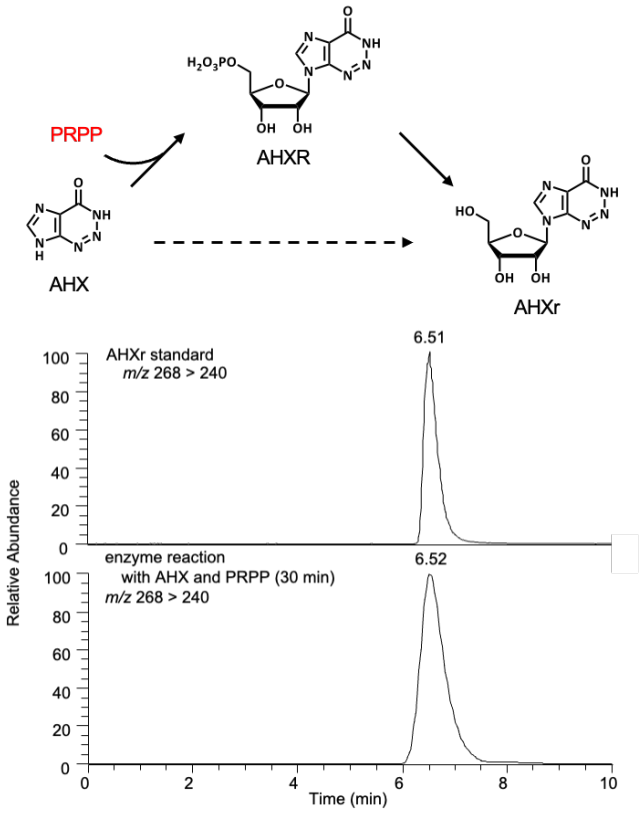

Fig. 3 Detection of AHXr in the reaction solution with enzymes from rice by LC-MS/MS. The DEAE fraction eluted with 0.2 M NaCl was used for the assay and the reaction was carried out at 30°C for 12 h. AHXr was identified by the characteristic transition at *m/z* 268 > 240 by MS/MS analysis

In addition, phosphorylation activity in the crude enzyme solution against AHX was investigated. Although AHXR was not detected in the reaction mixture, AHXr was detected in the reaction mixture (Fig. 3). These results led us to think that the crude enzyme solution converted AHX to AHXR, and then to AHXr. As expected, the production of AHXr did not proceed in the absence of PRPP, implying that AHXr was formed from AHX via AHXR produced by phosphoribosyltransferase (Fig. S13). Notably, AHXr was detected 30 min after the reaction started and then disappeared 12 h after (Figs. 3 and S13). These results suggest that AHXR and AHXr are rapidly metabolized, which may be one reason why these compounds are not detected in plant extracts.

Although the possibility that AHXR and AHXr are biosynthesized in plants has been demonstrated, the pathway remains unclear. On the other hand, the biosynthetic pathway of AHX in rice has already been proven with reference to the chemical synthesis of AHX (Fig. S14).[9, 13, 36] That is, it has been revealed that NOS synthase-derived NO is directly involved in 1,2,3-triazine formation of AHX with AICA in *L. sordida*.[18] NO is a molecular messenger that possesses a wide range of physiological functions and is produced in plants by nitrate reductase and non-symbiotic hemoglobin or by non-enzymatic sources.[37, 38 39]Furthermore, this route can be extended to AICAR and AICA, therefore, AICA, AICAr, and AICAR were incubated with an NO donor (NOC7) in the distilled water, respectively. As a result, HPLC analysis showed that all the compounds reacted with the donor and turned into the corresponding triazine products (AHXR, AHXr, and AHX). In addition, an NO scavenger cPTIO completely inhibited the construction of the triazine ring (Fig. S15). These results suggested that endogenous NO in plants is involved in the conversion of AICA skeleton (AICAr and AICAR) to AHX skeleton (AHXr and AHXR) (Fig. 4).

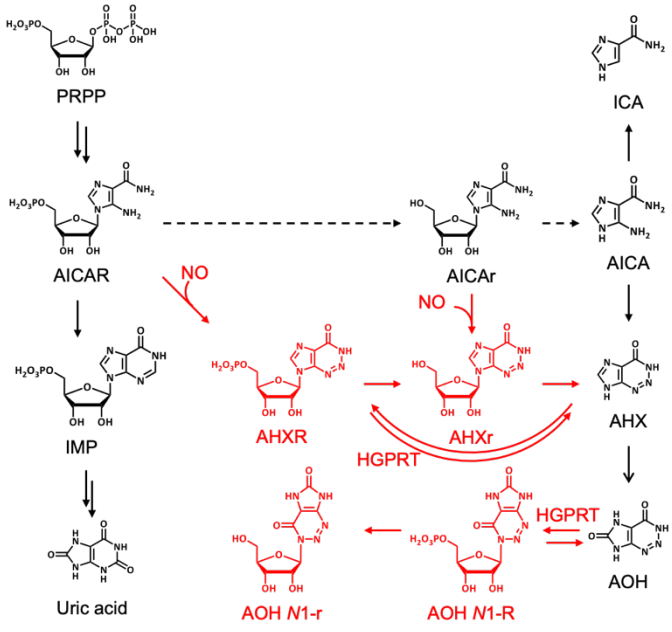

Fig. 4 Novel purine metabolism in rice including the routes which this paper revealed. New metabolites and routes are indicated in red. AHXR, AHX ribonucleotide; AHXr, AHX ribonucleoside; AOH *N*1-R, AOH *N*1-ribonucleotide; AOH *N*1-r, AOH *N*1-ribonucleoside.

Conclusions

We reported a novel purine metabolism related to AHX biosynthesis in rice which may be common in plants. HGPRT not only recognizes AHX and AOH as substrates but also produces. AOHR links to ribose at N-1 of the purine ring, and its binding position to the sugar is extremely unique in the purine metabolic pathway (Fig. 4). In addition, crude enzymes from rice converted AHX to AHXR and then AHXR to AHXr. Despite the fact that purine metabolism has been extensively studied, the findings of this study suggest that there are still veiled routes and metabolites in the purine pathway. Moreover, elucidating the biosynthesis or metabolism of FCs in plants will provide new physiological basis for plant growth regulation.

Author Contributions

H.K. conceived the project and designed outlines most of the experiments. H.T., J.-H.C., and K.F. performed the experiments. H.O., and M.I. synthesized authentic AHXR. J.-H.C., R.N., J.W., M.K., D.N., T.S., H.H., and H.K. assisted with the experiments and contributed to discussion. H.T., J.-H.C., and H.K. wrote the manuscript. ‡H.T., J.-H.C. contributed equally to this research.

Conflicts of interest

There are no conflicts to declare.

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