

Review

Are fairy chemicals a new family of plant hormones?

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Abstract: 2-Azahypoxanthine (AHX, **1**) and imidazole-4-carboxamide (ICA, **2**) were isolated from a fairy-ring-forming fungus *Lepista sordida*. AHX was converted into a metabolite 2-aza-8-oxo-hypoxanthine (AOH, **3**) in plants. It was found out that these three compounds, named as fairy chemicals (FCs), endogenously exist in plants and are biosynthesized via a new purine metabolic pathway. FCs provided tolerance to the plants against various stresses and regulated the growth of all the plants. In addition, FCs increased the yield of rice, wheat, and other crops in the greenhouse and/or field experiments.

Keywords: fairy rings, 2-azahypoxanthine, imidazole-4-carboxamide, 2-aza-8-oxo-hypoxanthine, plant growth regulator

Introduction

This study began with my very personal experiences more than ten years ago. At that time, I lived in a staff dormitory located at the Shizuoka University campus. One day, I noticed that some of the grass adjacent to the lodging became deeply colored compared with the surroundings and drew an arc. The color was too vivid, so I thought that someone scribbled it. The colorful arc became unnoticeable in the winter, and I forgot its existence. However, in the spring of the following year, the color vividness was lost compared with the previous year, but this time the arc that flourished from the surroundings and the diameter became larger than the previous year (Fig. 1). Then, a kind of mushroom appeared on the arc. This mushroom was identified as an edible mushroom *Lepista sordida*. I had not known this phenomenon at that time, and the phenomenon upon literature review turned out to be “fairy rings” (Fig. 1). In the legend of the West, it is known that the fairy makes a circle and dances in it. Since the first paper on “fairy rings” was published

in 1675 and the subsequent papers were introduced in Nature in 1875, the real identity of the fairy (the cause of the turf growing up) has been a mystery, although there was an established theory for some time.¹⁾ The established theory is that through the saprophytic action of the fungus mycelium, the protein portion of the nonliving organic matter in the soil is decomposed to ammonia. The ammonia combines with other compounds or is used as a substrate by successive bacteria to generate nitrites and nitrates. The resulting accumulation of nitrogen in the soil, in a form readily available to higher plants, causes the typical growth pattern of conspicuous bands of taller, darker green plants, that is, the growth stimulation is due to the nitrogen fertilizer produced by the mycelia.^{1)–6)} However, I was apprehensive about this dogma, and thought that the fungus produces specific plant growth stimulator(s); therefore, we started searching the fairy (the stimulator) from a fairy ring-forming fungus.

Disclosure of the “fairy”

Approximately, 60 kinds of fungi, causing this phenomenon are known, and naturally I chose *Lepista sordida* for our research. In artificial cultures, microorganisms often do not produce substances that are produced in nature. Therefore, we confirmed that the strain that we used actually promoted the growth of turfgrass (Fig. 2). In order to purify the fairy, this strain was mass cultured in a liquid medium, divided

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Abbreviations: AHX: 2-Azahypoxanthine; AOH: 2-aza-8-oxohypoxanthine; FCs: fairy chemicals; ICA: imidazole-4-carboxamide.

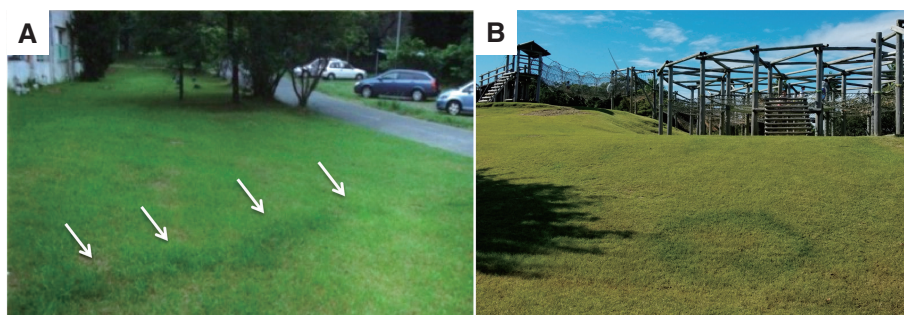


Fig. 1. **A.** Fairy ring that appeared on the campus of Shizuoka University. **B.** Fairy ring that appeared on turfgrass in a park in Hamamatsu City, Japan.



Fig. 2. Effect of the mycelia of *L. sordida* cultivated for 3 weeks on bentgrass. T1 and T2 represent bentgrass inoculated with 0.5 g and 1.5 g (fresh weight) of the mycelia, respectively.

into culture broth and mycelia, and each was extracted with various organic solvents. The effect of each extract on the growth of turfgrass seedlings was examined in a petri dish. The ethyl acetate extract (soluble part) of the culture broth was found

to promote the growth. Being guided by the results in the bioassay, advanced fractionation of the ethyl acetate-soluble part was carried out, and we succeeded in the isolation of the fairy by repeated chromatography and recrystallization. Structure determination of the fairy was not easy because the compound gave only one signal in the $^1\text{H-NMR}$ and four ones in the $^{13}\text{C-NMR}$ (Fig. 3A). Fortunately, this compound was obtained as a crystal, and the structure was determined as 2-azahypoxanthine (AHX, **1**) by X-ray crystallography analysis (Fig. 3A and 4).⁷ This compound had been already synthesized, but it was the first discovery from nature (Fig. 5).⁸

Sporadically, fairy rings appear as rings with suppressed growth. We tried to isolate the growth-suppressing component and succeeded in the isolation and identification of the inhibitor. The

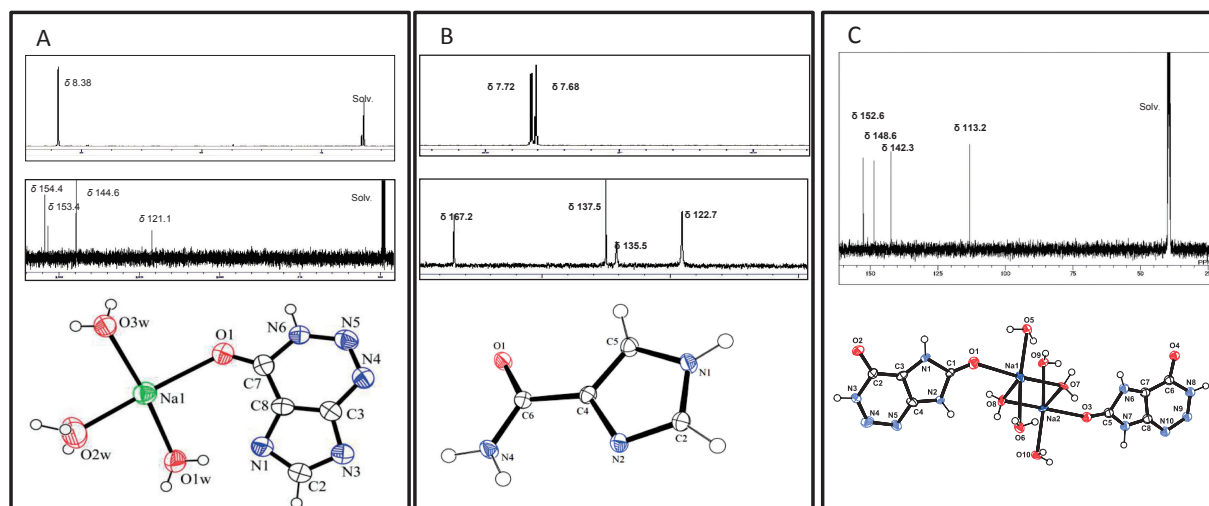


Fig. 3. $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ spectra, and ORTEP drawings of FCs. **A.** AHX; **B.** ICA; **C.** AOH (ORTEP drawing, AOH Na salt hydrate).

(0.1 M NaCl), similar to the previous reports about the GST-transgenic rice. In addition, rice seedlings developed tolerance to high temperature stress (35 °C) by treatment with **1**.⁷⁾ Tonoplast intrinsic proteins (TIPs) are the major components of the vacuolar membranes and the most abundant aquaporins in plants.¹⁷⁾ In addition to the water channel function of the aquaporins, AtTIP2;1 and AtTIP2;3 of Arabidopsis and TaTIP2 of wheat have been demonstrated to transport ammonia/ammonium ion.^{18),19)} In order to reveal the effect of **1** on the absorption of ammonia/ammonium ion into rice, we treated the rice seedlings with **1** and ¹⁵NH₄NO₃, which was the sole nitrogen source. AHX (**1**) at 200 μM increased ¹⁵N absorption from ¹⁵NH₄NO₃ into the plant, especially into the root where *OsTIP2;1* was up-regulated by **1**. However, when NH₄¹⁵NO₃ was used as the sole nitrogen source, ¹⁵N absorption was not affected.⁷⁾

These results indicated that the plant developed tolerance to continuous stress from the environment and increased the absorption of ammonia/ammonium ion by treatment with **1**, resulting in growth promotion.⁷⁾

Further oligo DNA microarray analysis using rice indicated that the up-regulated and down-regulated genes upon treatment with **1** (50 μM) or **3** (50 μM) were very similar to each other; however, treatment with **2** (2 μM) gave opposite results.²⁰⁾ Nevertheless, these three compounds equally provided tolerance to various stresses and increased crop yields. In addition, recent results indicated that the treatment of plants with FCs increased stress tolerance of plants by strengthening their functions, such as transpiration control, elimination of active oxygen, and reduction of cell membrane damage (inhibition of membrane lipid peroxidation) (data not shown).

To elucidate the structure-activity relationship, **1**, **2**, **3**, **4**, hypoxanthine (**6**), and AICA (**7**) were tested with bentgrass. Compounds **1**, **3**, and **7** exhibited growth-promoting activity and **2** inhibited the growth.^{7),9),11)}

In order to investigate the relationship between **1** and plant hormones, tea cells were incubated in the presence of a cytokinin 6-benzylaminopurine (BAP), an auxin naphthaleneacetic acid (NAA), and/or **1**. Both the hormones were necessary for normal cell proliferation. In the presence of both the hormones, **1** did not influence cell proliferation. When **1** was used instead of BAP or NAA, it did not act as a substitute for either hormone. On the other hand, in the absence

of both the hormones, **1** promoted cell proliferation dose-dependently. This result suggests that the effect of **1** is independent of the two hormones and functions like any another hormone.⁷⁾

Recently, we found that FCs also promoted the growth of mycelia of the mushroom-forming fungi (unpublished data).

The proof of endogenous existence of fairy chemicals and their biosynthetic pathways in plants and fungi

2-Azahypoxanthine (**1**) is chemically synthesized from 5-aminoimidazole-4-carboxamide (AICA, **7**) via 4-diazo-4H-imidazole-4-carboxamide (DICA, **8**) (Fig. 5).⁸⁾ Imidazole-4-carboxamide (**2**) is also synthesized from **7**, and **3** is enzymatically converted from **1**.^{8),11)} The interesting points are that the precursor of FCs, **7**, is on purine pathway that is common to animals, plants and microorganisms, but further metabolism of **7** had remained unknown (Fig. 7). From our findings and the facts mentioned above, I hypothesized that the plants themselves produced FCs through a pathway very similar to the chemical synthesis (Fig. 5).

To prove the endogenous existence of **1** and **3** in rice, we synthesized isotope-labeled FCs (Fig. 6) and tried to detect and quantify **1** and **3** in rice cultivated in an aseptic condition by liquid chromatography/tandem mass spectrometry (LC-MS/MS) using [4-¹³C, 2-¹⁵N]AHX (**9**) and [4-¹³C, 2-¹⁵N]AOH (**10**) as internal standards. As a result, **1** and **3** were detected in the plant.¹¹⁾ These results indicated that **1** and **3** are endogenously synthesized in the shoot and root of rice. The amounts of endogenous **1** (457 ± 75 ng/kg fresh weight (F.W.) in shoot; 273 ± 52 ng/kg F.W. in root) and **3** (not detected in shoot; 1289 ± 406 ng/kg F.W. in root) in rice were similar as those of known plant hormones, strigolactones and brassinosteroids.^{11),21)–23)} Furthermore, **1** and/or **3** were detected in various living organisms (Table 1).¹¹⁾ Since **1** was originally obtained from a fungus, there is a possibility that the surrounding microorganisms produced **1** and the compound was taken into plants. Therefore, rice and Arabidopsis were aseptically cultivated, and **1** and **3** were also detected from the extracts of the plants.¹¹⁾ Recently, we developed a method to detect **2** in LC-MS/MS and the endogenous existence of **2** in various plants was also proved (paper submitted).

To verify the existence of our hypothetical biosynthetic route from **7** to **1**, and then **3** in plants (Fig. 4), we performed experiments using rice seed-

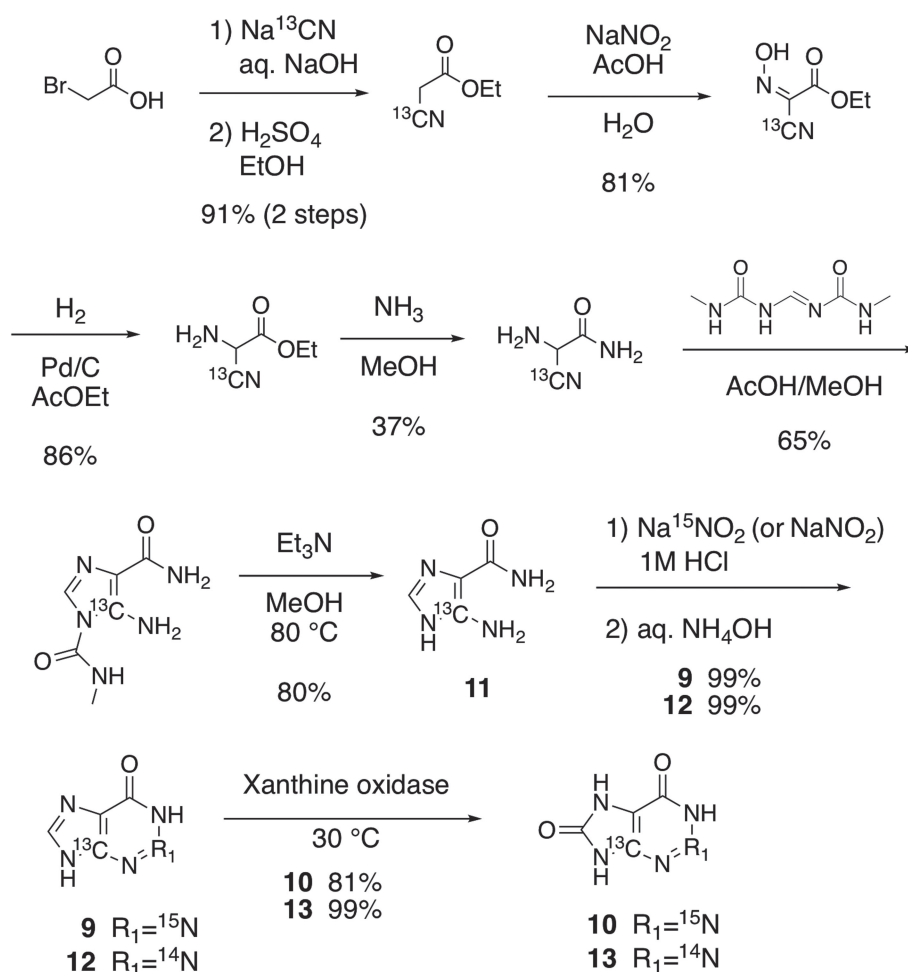


Fig. 6. Chemical synthesis of isotope-labeled FCs.

lings cultivated in liquid medium with [5- ^{13}C]AICA (**11**) and analyzed the incorporation rate of the compound into the seedlings and the amounts of [4- ^{13}C]AHX (**12**) and [4- ^{13}C]AOH (**13**) in the seedlings. The labeled AICA (**11**) was detected in trace level in the medium and **13** was found in the seedlings. The results indicated that **11** in the culture medium was absorbed into rice seedlings and the absorbed **11** was converted into **13** via **12** in rice seedlings.¹¹⁾

Thus, we concluded that **1** and **3** are new metabolites in a novel purine metabolic pathway in plants, at least in rice.¹¹⁾ As mentioned above, many plants, algae, and mushrooms contained **1** and/or **2**, indicating that the pathway is commonly conserved in various living organisms (Table 1). In the fungus *Lepista sordid*, in which **1** was originally discovered, the production of **1** was promoted by adding 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR, **14**) to the culture broth of the fungus.

The expression level of adenine/5-aminoimidazole-4-carboxamide phosphoribosyltransferase (APRT) gene was also stimulated by this addition. The APRT catalyzes the reaction from **14** to **7** and this enzyme activity was found in the fungus. These results suggested that the fungus, and probably other fungi and plants have the pathway from **14** to **1** via **7** (Fig. 7).²⁴⁾

Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) is known as an enzyme that catalyzes a reaction similar to APRT, and catalyzes the reversible interconversion between **6** and IMP (**15**), and guanine and GMP through addition-elimination of the phosphoribose group. Genomic information shows that rice has only one HGPRT gene, and we considered the possibility that this gene converts **1** and **3** to hypothetical ribotides **16** and **17**, respectively. Therefore, we heterologously expressed the enzyme in *Escherichia coli*, and the treatment of **1**

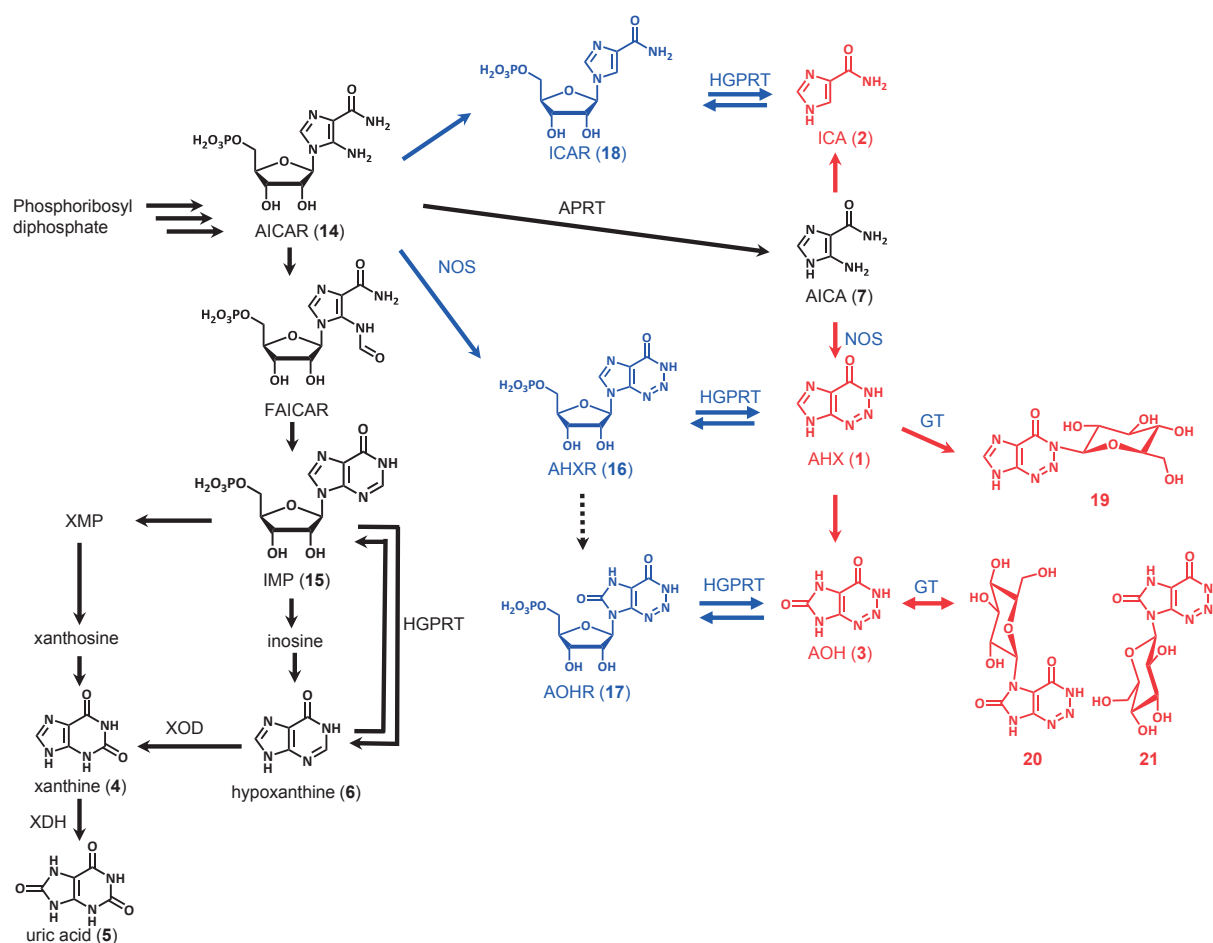


Fig. 7. Purine metabolic pathway in animals, plants, and microorganisms, including the novel route. The route in black was cited from KEGG (Kyoto Encyclopedia of Genes and Genomes). New metabolites and routes are indicated in red (published results). Hypothetic or unpublished routes, metabolites, and enzymes are described in blue.

or **3** with the expressed HGPRT (OsHGPRT) gave products that were thought to be **16** and **17** (unpublished data) (Fig. 7).

Concerning **2**, similar experiments are in progress and preliminary results showed that **2** also was biosynthesized from **7** and its ribotide (**18**) might exist endogenously like in the case of **1** and **3** (data not shown).

It is highly possible that the main origin of nitrogen is nitric oxide (NO) in the biosynthesis of **1** from **7** and **16** from **14**. In the fungus *L. sordida*, incorporation of **7** and a NO generating agent, 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene (NOC5) increased the production of **1**. On the other hand, a nitric oxide synthase (NOS) inhibitor, *N*^G-monomethyl-L-arginine, acetate (L-NMMA), inhibited the production of **1**. In addition, when the fungus was cultured with ¹⁵N-labeled L-arginine that

releases NO catalyzed by NOS, ¹⁵N-labeled **1** was obtained (paper in preparation). Similar experiments are in progress against rice. Various effects of NO on the growth of plants are known, and part of the effects of NO might be due to **1** or **3** incorporating NO in the molecule.

In order to further elucidate the metabolism of **1** and **3**, rice seedlings were cultivated in a solution of **1** and the treated rice produced several metabolites. We purified three metabolites and determined the structures. They were all novel compounds and were glucosides of **1** or **3** (**19**, **20**, and **21**). In the experiment using **3**, **20** and **21** were obtained.²⁵⁾ LC-MS/MS showed that all the glucosides are also endogenous in rice. One of the plant hormone families, cytokinins, has a purine carbon-skeleton like **1** and **3** and is also converted into *N*-glucosides and *O*-glucosides in plants. The glucosides are

Table 1. Detection of AHX and/or AOH by LC-MS/MS

Organism species	AHX	AOH	
Poaceae	rice (Nipponbare; above-ground part)	d	nd
	rice (Nipponbare; root)	d	d
	rice grain (Nipponbare, Koshihikari)	d	d
	wheat grain (Norin No. 61)	d	d
	corn (mille-feuille, Kankanmusume)	d	nd
	bentgrass (above-ground part, root)	d	d
	zoysiagrass (above-ground part)	d	nd
	zoysiagrass (root)	d	d
Liliaceae	asparagus (above-ground part)	d	nd
Eucalyptus	<i>Eucalyptus pellita</i> (above-ground part, root)	d	nd
	<i>Eucalyptus camaldulensis</i> (above-ground part, root)	d	nd
Solanaceae	tomato (House Momotaro, Reika, Endeavour; above-ground part)	d	nd
	tomato (House Momotaro, Reika, Endeavour; root)	d	d
	potato (Danshaku, May Queen)	d	d
Cucurbitaceae	cucumber (above-ground part, root)	d	nd
Theaceae	tea (<i>Camellia sinensis</i> ; above-ground part)	d	d
	tea (<i>Camellia sinensis</i> ; root)	d	nd
Cruciferae	komatsuna (<i>Brassica rapa</i> L. var. <i>perviridis</i>) (above-ground part)	d	nd
	komatsuna (<i>Brassica rapa</i> L. var. <i>perviridis</i>) (root)	d	d
	Japanese radish (<i>Raphanus sativus</i> var. <i>longipinnatus</i>)	d	nd
	<i>Arabidopsis thaliana</i> (above-ground part)	d	nd
	<i>Arabidopsis thaliana</i> (root)	d	d
Araceae	eddo (<i>Colocasia antiquorum</i> , Yamasatoimo)	d	d
Algae	<i>Chlorella vulgaris</i> ; <i>Parachlorella beyerinckii</i>	d	d
	<i>Synechocystis</i> sp. PCC6803	d	nd
Mushroom	<i>Lepista nuda</i>	d	d
	<i>Lepista sordida</i>	d	nd
	<i>Lyophyllum connatum</i>	d	d
	<i>Tricholoma flavovirens</i>	d	nd
	<i>Lyophyllum decastes</i>	d	nd
	<i>Flammulina velutipes</i>	d	nd
	<i>Pholiota adiposa</i>	d	nd
	<i>Hypholoma sublateritium</i>	d	nd
	<i>Lyophyllum shimeji</i>	d	nd
	<i>Cortinarius caperatus</i>	d	nd
<i>Grifola frondosa</i>	d	nd	

d: detected, nd: not detected.

metabolically stable against cytokinin degradation enzymes than the corresponding free bases. In terms of their physiological activity, the glucosides exhibit little or no activity against plants, suggesting that they are inactive forms of the plant hormone. The obtained glucosides, **19**, **20**, and **21**, also showed no activity against rice. Plants may regulate the activity of **1** and **3** by the same mechanism as the hormone.

However, various data indicated that FCs do not act as analogs of cytokinins and are recognized by plants as completely different compounds from cytokinins. Recently, the biosynthetic pathway of **2** in plants was clarified; this compound is also biosynthesized from **7** via **8**, like **1** (Fig. 7) (paper submitted). Other than the proven routes mentioned above, we think that FCs are also synthesized from their ribotides,

16, **17**, and **18** like other metabolites on the purine pathway.

The data mentioned above allowed us to conclude that we discovered new metabolites and a new purine metabolic pathway in plants (Fig. 7).

The possibility of practical use of fairy chemicals in agriculture

Since FCs regulated all the plants tested, regardless of their families, including rice seedlings, I thought that FCs might be able to increase the yield of rice grain, and examined it.

Cultivation experiments need large amounts of FCs. AHX (**1**) and ICA (**2**) are chemically synthesized.^{8),10)} On the other hand, the only method for the synthesis of **3** was the enzymatic conversion of **1** to by XOD.^{11),26)} However, commercially available XOD is not appropriate for the large-scale preparation of **3** because the enzyme is very expensive and the enzymatic reaction should be carried out at a low concentration of **1**. While searching for better methods to synthesize **3**, we happened to notice that the aqueous solution of **1** was accidentally contaminated with microorganisms and HPLC analysis of the solution indicated that **1** in the solution was converted to **3**. Therefore, we tried to isolate the airborne microorganism that produced **1**. As a result, we isolated the bacterium that converted high concentrations of **1** to **3** with high yield and identified the strain as *Burkholderia contaminans*.²⁷⁾

When rice was continuously treated with **1** at 5 μ M or **2** at 2 μ M in greenhouse experiments, the grain yields per plant increased by 25.5% or 26.0%.⁷⁾ This landmark result encouraged us to conduct further studies in the field. **1** or **2** was applied in three stages: when the seedlings rose in nursery boxes (seedling treatment), transplanting, and panicle formation stages. As a result, brown rice yields were increased by all the three treatments. Surprisingly, seedling treatment (for only two-week in nursery boxes; after planting, the rice was cultivated without the compounds) with **1** or **2** increased the yields up to 9.6 and 5.8% of the control, respectively. The treatments did not affect the quality and contents of free fatty acid, amylase, protein, and water of brown rice.²⁸⁾

Field examinations to study the effects of **1** and **2** on wheat were also performed.²⁹⁾ In 2011, only two-week treatment of wheat seedlings with **1** or **2** resulted in the increase of grain yield by 10.2% and 5.6%, respectively. In 2012, germination of seeds was stimulated by soaking in **1** or **2** solution for 36 hours

and the germinated seeds were sown in the field. After sowing, the wheat was cultivated without the compounds. This 36-hour treatment with **1** or **2** increased the grain yield by 20.2% and 9.8%, respectively.

AHX (**1**) also increased yields of potato (pot soil cultivation) by 19%, lettuce (pot culture) by 21%, and asparagus (hydroponic culture) by 100%.⁷⁾

AOH (**3**) also showed effects similar to those of **1** and **2** on rice, wheat, and some other crops (unpublished data).

We have now several years' data examining the effects of FCs on the yields of rice, wheat, and several other crops in greenhouse and/or field experiments. The data indicated that FCs are able to give higher yields without being influenced by stresses from the environment, such as low temperature, high temperature, drought, salt and so on.

Compounds **1** and **2** were positive and **3** was negative in Ames test (an assay of the ability of a chemical compound or mixture to induce mutations in DNA) (data not shown). However, the endogenous existence of FCs in the three major food crops in the world, rice, wheat, and maize, and other crops provide information on the safety of these compounds for the practical use of these compounds in agriculture (Table 1).

Conclusion

Plant hormones are signal molecules produced within the plants, and occur in extremely low concentrations. Plant hormones control all aspects of growth and development, such as embryogenesis, the regulation of organ size, defense against pathogens, stress tolerance, and reproductive development. Unlike animals, each plant cell is capable of producing hormones (https://en.wikipedia.org/wiki/Plant_hormone). Based on our finding mentioned above, we believe that FCs are a new family of plant hormones and are tracing the history of a plant hormone gibberellins. Gibberellins were first isolated as toxins from *Gibberella fujikuroi*, which causes Bakanae disease in rice; one of the symptoms was the abnormal growth of rice internodes. Later, it was proven that gibberellins exist in all plants, and they were recognized as a family of plant hormones.

Now, a company is conducting research to put FCs into practical use in agriculture. In the future, FCs might be contributing to resolve the world food problem.

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Profile

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