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The CYP701B1 of *Physcomitrella patens* is an *ent*-kaurene oxidase that resists inhibition by uniconazole-P

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ABSTRACT

The moss *Physcomitrella patens* produces both *ent*-kaurene and *ent*-kaurenoic acid, which are intermediates of gibberellin biosynthesis in flowering plants. The CYP701 superfamily of cytochrome P450s functions as *ent*-kaurene oxidases in the biosynthesis of *ent*-kaurenoic acid. A candidate gene encoding *ent*-kaurene oxidase in *P. patens*, *CYP701B1*, was cloned and heterologously expressed in yeast to examine enzyme activities in vitro. The recombinant CYP701B1 protein catalyzed the oxidation reaction from *ent*-kaurene to *ent*-kaurenoic acid. CYP701B1 activity was highly resistant to the *ent*-kaurene oxidase inhibitor uniconazole-P (IC_{50} 64 μM), even though the activity of *Arabidopsis ent*-kaurene oxidase (CYP701A3) was sensitive (IC_{50} 0.26 μM).

1. Introduction

Bryophytes have been used as model non-vascular land plants for research on the evolution of growth and development from ancient to vascular flowering plants. The moss *Physcomitrella patens* became the typical example of a primitive land plant since sequencing of its entire genome and the establishment of genetic techniques [1]. The life cycle of *P. patens* is mediated by plant hormones, which have the same structures as those found in flowering plants [2]. Auxins and cytokinins play roles in many developmental processes, including chloronemal to caulonemal differentiation, inhibition of chloronemal branching, and induction of formation of buds and gametophores. Abscisic acid is also found in *P. patens*, but its exact role remains unclear. Unlike the other plant hormones, gibberellins (GAs) have never been detected in mosses. Studies on hormonal regulation in moss showed that GAs and GA-signaling are not involved in moss growth [3]. In 2010, a ground-breaking report described GA-related diterpenes derived from *ent*-kaurene that were involved in growth of *P. patens* [4]. *ent*-Kaurene-deficient mutants of *P. patens* grew abnormally under red light. When *ent*-kaurene and *ent*-kaurenoic acid were individually supplied to mutants, both compounds rescued growth, suggesting that *ent*-kaurene and its metabolites are required for chloronemal differentiation and caulonemal growth. The report also described that no GAs downstream of *ent*-kaurenoic acid, such as GA_{12} and GA_4, were detectable by GC–MS and LC–MS/MS analyses, even though it accumulated *ent*-kaurenoic acid (Fig. 1).

Here, we describe the functional analysis of the gene encoding the *ent*-kaurene oxidase in *P. patens*. In flowering plants, the biosynthetic reaction from *ent*-kaurene to *ent*-kaurenoic acid is catalyzed by CYP701A, a member of the cytochrome P450 monooxygenase (P450) superfamily [5]. This enzyme catalyzes the multistep oxidation of the methyl group at C-19 of *ent*-kaurene, which is converted to *ent*-kaurenoic acid *via ent*-kaurenol and *ent*-kaurenal [6]. *P. patens* has 71 genes encoding P450 enzymes [7]. Recently, the CYP78A subfamily of P450s in *P. patens* was reported to be involved in protonemal growth and gametophore formation [8]. An orthologous gene of *CYP701A*, designated as *CYP701B1*, was found in the *P. patens* genome. This gene encodes a candidate *ent*-kaurene oxidase (KO). We anticipated that CYP701B1 may catalyze the oxidation reaction; therefore, we analyzed its function using recombinant CYP701B1 protein produced in a *Pichia* yeast heterologous expression system [9]. The recombinant CYP701B1 protein showed oxidation activity, converting *ent*-kaurene to *ent*-kaurenoic acid. In addition, it was highly resistant to uniconazole-P (UNI), a compound that inhibits the activities of KOs from higher plants, including CYP701A [10].

Abbreviations: GA, gibberellin; KO, *ent*-kaurene oxidase; P450, cytochrome P450 monooxygenase; UNI, uniconazole-P

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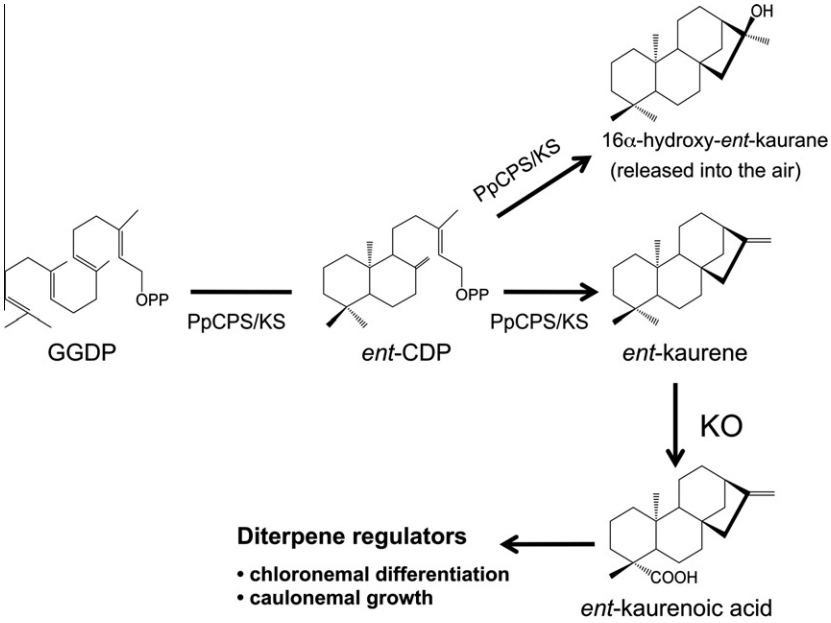

Fig. 1. Biosynthetic pathway of *ent*-kaurenoic acid from geranylgeranyl diphosphate in *P. patens*. These diterpene metabolites were identified from gametophytic tissues [4]. Growth inhibition of PpCPS/KS-knock out mutant was rescued by *ent*-kaurene and *ent*-kaurenoic acid, but not GAs. The fate of *ent*-kaurenoic acid is still unclear.

2. Materials and methods

2.1. Chemicals, plasmids, and genes

The substrate *ent*-kaurene was extracted from leaves of *Cryptomeria japonica* and purified as described previously [4]. Chemicals including antibiotics, PCR, plasmids and *Pichia* host cells used in this work were the same previously reported [11]. The *CYP701B1* gene (clone number: pdp63558) was supplied by the Riken Bio Resource Center (RBRC). The *Arabidopsis thaliana CYP701A3* gene (AtKO, At5g25900) [5] was cloned from the cDNA library prepared from the aerial part of the plant [11]. UNI was purchased from Sumitomo Chemical Co., Ltd. (Osaka, Japan).

2.2. Vector construction

The open reading frame of *CYP701B1* was amplified by PCR (Advantage HF2, Clontech, CA, USA) using the following primers: CYP701B1-fwd, 5′-AGAATTCATTATGGCGAAACATCTGG-3′ (*Eco*RI site underlined); CYP701B1-rev, 5′-GAGGGCCCTGGTAATCTGTGGCTGATTCTAG-3′ (*Apa*I site underlined). The PCR-amplified product was ligated into the pCR2.1vector, and the plasmid was used for transformation of *Escherichia coli*. The DNA sequence was confirmed using an ABI 3130xl Genetic Analyzer (Applied Biosystems), and then the *CYP701B1* fragment digested with *Eco*RI and *Apa*I was ligated into pPICZ-A to construct an expression vector (pPICZA-*CYP701B1*) for *Pichia* yeast. According to our previous report [11], this pPICZA*CYP701B1* was used for transformation of a line of *Pichia* yeast that already contained the *Arabidopsis ATR1* gene. Thus, double transformants resisting two antibiotics (blasticidin S and zeocin) were isolated on YPDS agar plates containing blasticidin S (300 μg ml^{-1}) and zeocin (100 μg ml^{-1}). Transformants harboring a high copy number of genes in the genome were screened on high-concentration zeocin YPD plates (1000 μg ml^{-1}). The *AtKO* ORF fragment was cloned by PCR using the primers 5′-CTACTCGAGATGGCCTTCTTCTCCATGATC-3′ (fwd) and 5′-CTTCTAGAACGCCTTGGATTGATAATAGCC-3′ (rev). The amplified ORF fragment was ligated into the pPICZ-B vector, and then used for transformation of *Pichia* yeast.

2.3. Heterologous production in Pichia and detection of recombinant proteins

The selected transformants were incubated in BMGY medium described in manufacturer's protocol at 30 °C until they reached an OD_{600} value of 2. Cells were collected by centrifugation and resuspended in 100 ml BMMY medium and culture was continued for four days at 30 °C to produce recombinant CYP701B1 protein. Cells harvested after induction culture were suspended in a reaction buffer (50 mM potassium phosphate (pH 7.5), 1 mM EDTA, 20% glycerol, and 1 mM dithiothreitol) and disrupted at 4 °C using acid-washed glass beads (212–300 μm, Sigma–Aldrich, St. Louis, MO, USA). A microsomal fraction was prepared using PEG-4000 and centrifugation (10 000*g*, 1 h, 4 °C). The microsomal fraction was suspended in reaction buffer for use in enzyme assays.

The recombinant protein was detected by Western blotting using an anti-*c-myc* antibody conjugated with alkaline phosphatase (Invitrogen). The staining was performed using 5-bromo-4-chloro-3-indolyl-1-phosphatase/nitroblue tetrazolium reagent (Promega, Madison, WI, USA).

2.4. Enzyme assay

The enzyme solution containing KO-overproducing yeast microsomes was used for the enzyme assay in vitro. The radioisotope labeled *ent*-kaurene was prepared from *RS*-[2-^{14}C]mevalonic acid lactone (Amersham, Little Chalfont, UK, 1.93GBq $mmol^{-1}$) as described previously [12]. The reaction was performed at 30 °C for 2 h after addition of the substrate and NADPH (2 mM). The products were extracted with ethyl acetate and spotted onto a silica gel TLC plate. After development with *n*-hexane-ethyl acetate-acetic acid (75:24:1, v/v), the TLC plate was exposed to an imaging plate (BAS-IP TR-2040, 20 × 40 cm, Fuji Film, Tokyo) and radioactivity was detected using an FLA-7000 image analyzer (Fuji Film). To determine the *ent*-kaurenoic acid by GC–MS, the substrate (*ent*-kaurene, 1 μg) was added to the enzyme solution (100 μl), and the reaction was started by addition of 5 mM NADPH at 30 °C. The reaction was stopped by adding 200 μl 1 N HCl and the products were extracted three times with 1 ml ethyl acetate.

The sample was subjected to GC–MS after derivatization with diazomethane/ether solution. The condition of GC–MS analysis was previously described [9]. To confirm the effect of UNI on enzyme activity of KOs, UNI dissolved in methanol was added to the enzyme solution and incubated as described above.

3. Results and discussion

3.1. GA biosynthesis-related genes in P. patens EST library

The *P. patens* EST library of Riken contains full-length cDNAs expressed in gametophytic tissues that were auxin-treated, cytokinin-treated, or untreated [13]. We found a bifunctional *ent*-kaurene synthase (PpCPS/KS) gene and several pseudogenes in this EST library [14]. In our previous report, we proposed that an orthologous gene of a candidate KO, which belongs to *CYP701A* subfamily of flowering plants, is present as a single copy gene in the *P. patens* genome [4]. We obtained and sequenced the *CYP701A* orthologous gene of *P. patens*, *CYP701B1* (DDBJ accession number: AB618673). This gene is a 1641 bp full-length clone that possibly encodes 547 amino acids. The deduced amino acid sequence shared 42% identity with that of CYP701A3 and 43% with that of CYP701A1.

3.2. CYP701B1 catalyzes oxidation reaction of ent-kaurene to ent-kaurenoic acid

Enzymes within the same P450 subfamily can recognize different substrates and catalyze different reactions, such as the CYP90A, CYP90B, CYP90C, and CYP90D enzymes in brassinosteroid biosynthesis [15]. Hence, the enzymatic function of CYP701B1 of *P. patens* should be confirmed using recombinant protein.

The *Pichia* expression system has been used for production of a wide variety of recombinant proteins. Recombinant KO and CYP88A proteins from lettuce were successfully heterologously expressed and produced using the *Pichia* system [9]. Here, we used the *Pichia* expression system for functional analysis of the *CYP701B1* gene. The *CYP701B1* cDNA was transformed into *P. pastoris* harboring *ATR1* gene. After the second screening of transformants on high-concentration zeocin plates (1000 μg/ml), four transformants were isolated; #4, #9, #12 and #27. To confirm the production of recombinant CYP701B1 protein, a culture of a transformant (strain #12) was induced with MeOH for 72 h, and then a microsomal fraction was prepared by cell disruption and used for immunostaining assays. The production of both CYP701B1 and ATR1 proteins were confirmed by Western blot analysis using an anti-*myc* antibody conjugated with alkaline phosphatase (Fig. 2). The calculated molecular masses of CYP701B1 and ATR1 were 62 and 77 kDa, respectively. Both bands stained with BCIP/NBT were detected at the predicted sizes on the membrane, confirming that both recombinant proteins were produced in the *Pichia* cells. Thus, this microsomal fraction was subsequently used for in vitro enzyme assays.

The function of CYP701B1 protein was analyzed using [^{14}C]*ent*-kaurene. Microsomes containing CYP701B1 and AtKO (CYP701A3), respectively, were incubated in the presence of ATR1 protein, [^{14}C]*ent*-kaurene and NADPH. The radio-chromatogram of the reaction products (Fig. 3) indicated that both AtKO (Fig. 3, lane 2) and CYP701B1 (Fig. 3, lane 3) converted *ent*-kaurene (R_f = 0.79) to *ent*-kaurenoic acid (R_f = 0.52). Two weak spots (R_f = 0.39 and 0.71) may represent *ent*-kaurenol and *ent*-kaurenal, respectively. There were no reaction products from *ent*-kaurene produced by the negative control, ATR1 (Fig. 3, lane 1).

Radioisotope experiments suggested that CYP701B1 and AtKO produced the same products from *ent*-kaurene. To confirm the

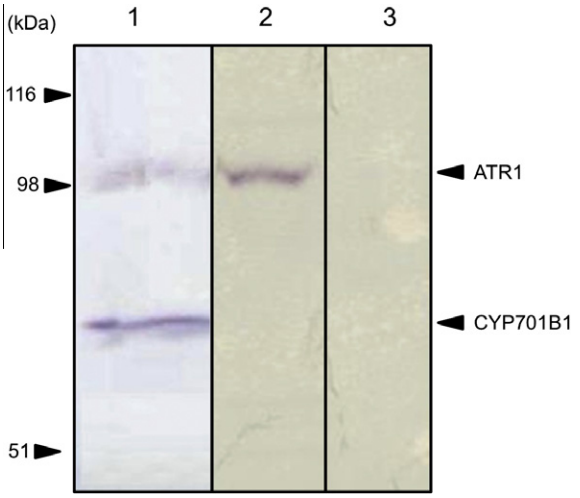

Fig. 2. Detection of recombinant CYP701B1 and ATR1 proteins in microsomal fraction from *Pichia* transformant. Lane 1: Both CYP701B1 (62KDa) and ATR1 (77KDa) proteins produced in transformant #12, Lane 2: ATR1 protein produced in host cell, lane 3: *Pichia* X-33 wild-type.

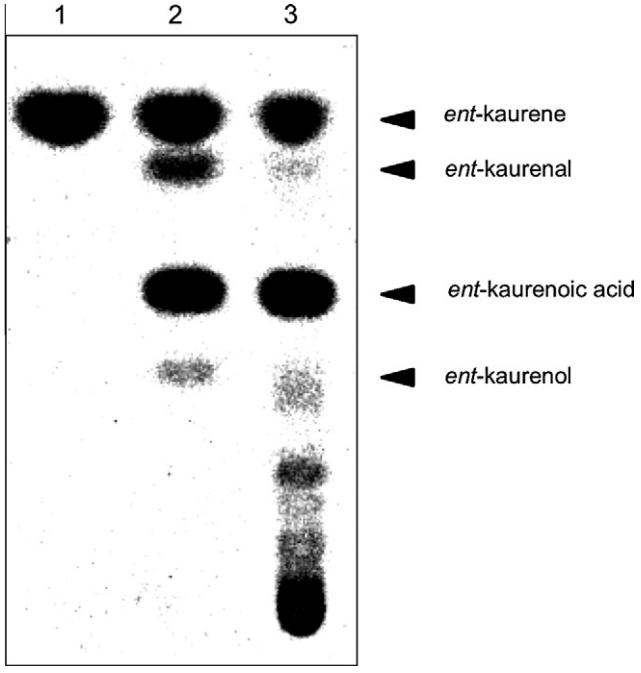

Fig. 3. Radioactivity distribution on TLC plates after incubation of microsomal fractions with [^{14}C]*ent*-kaurene. Lane 1: ATR1 protein, Lane 2: AtKO and ATR1 proteins, Lane 3: CYP701B1 and ATR1 proteins. Arrowheads show R_f values of *ent*-kaurene, *ent*-kaurenol, *ent*-kaurenal, and *ent*-kaurenoic acid.

ent-kaurenoic acid production from *ent*-kaurene by GC–MS, non-labeled *ent*-kaurene was incubated with the microsome containing recombinant CYP701B1 and ATR1 proteins in the presence of NADPH. In the GC–MS analysis of the reaction product, the data of the product produced by CYP701B1 were identical to those of the product obtained by incubation with AtKO and authentic *ent*-kaurenoic acid methyl ester (Fig. 4). In this experiment, the two intermediates *ent*-kaurenol and *ent*-kaurenal were not detected. Microsomal fractions containing ATR1 but not CYP701B1 protein did not convert *ent*-kaurene to *ent*-kaurenoic acid. CYP701B1 proteins produced in other transformants (#4, #9, and #27) also converted *ent*-kaurene to *ent*-kaurenoic acid (data not shown). Hence,

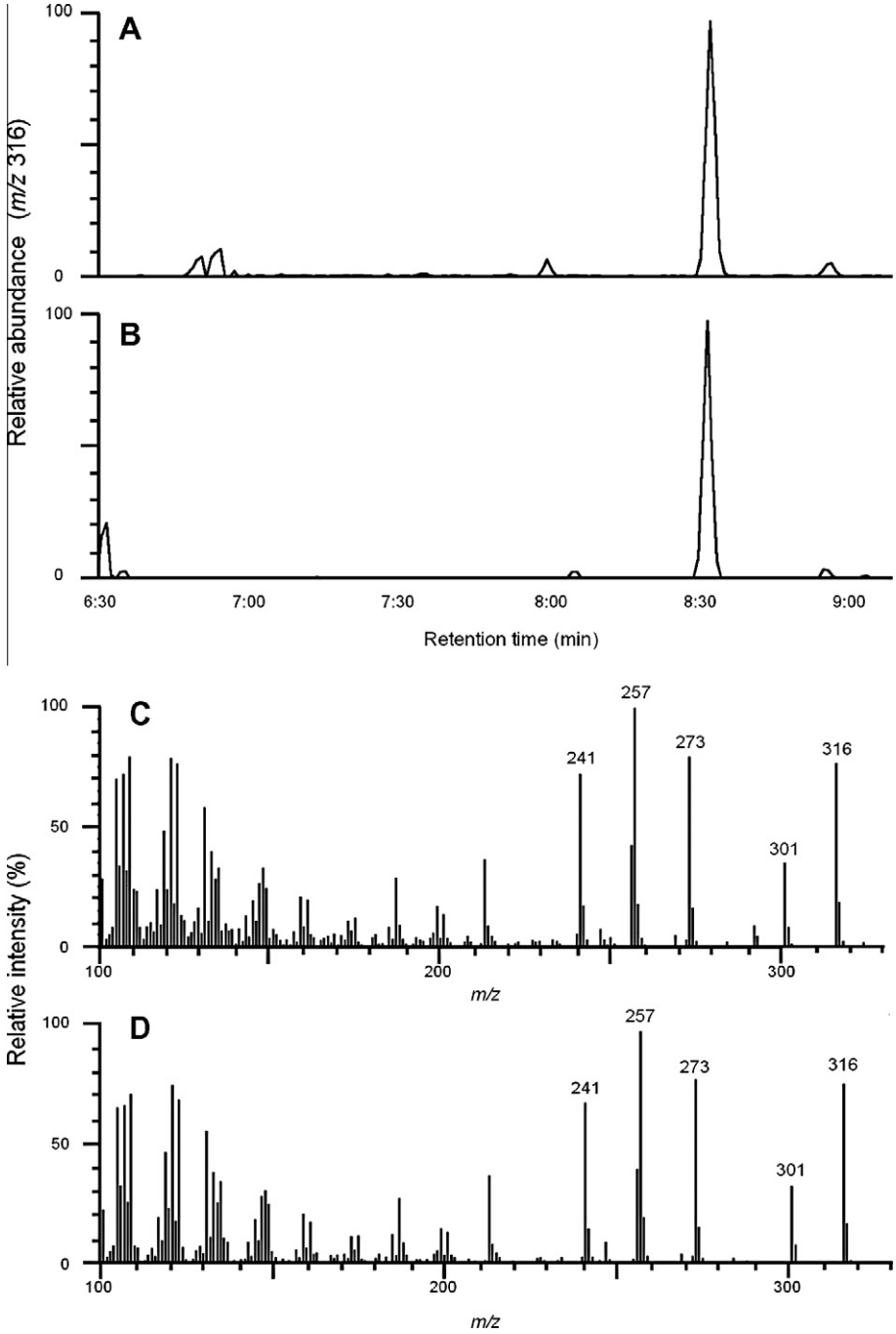

Fig. 4. GC–MS analysis of in vitro assay for *ent*-kaurene oxidase activity in CYP701B1 (PpKO). (A and C) Mass chromatogram scanned by *m/z* 316 and mass spectrum of peak at 8.5 min obtained by incubation with PpKO and *ent*-kaurene. Sample was analyzed as methyl ester. (B and D) Mass chromatogram scanned by *m/z* 316 and mass spectrum of authentic *ent*-kaurenoic acid methyl ester.

CYP701B1 encodes a KO enzyme in *P. patens* (PpKO), and endogenous *ent*-kaurenoic acid is produced via CYP701B1 activity.

3.3. PpKO resists inhibition by UNI

UNI is a well-known triazole-type plant growth retardant [10]. UNI inhibits several P450 enzymes including CYP701A and ABA 8′-hydroxylase (CYP707A), and also inhibits brassinosteroid biosynthesis [16,17]. In *P. patens*, 2 μM UNI inhibited gametophore growth [4]. However, this UNI-induced gametophore growth inhibition was not recovered by exogenous application of *ent*-kaurenoic acid, suggesting that the P450 targeted by UNI in *P. patens* might be not KO. To examine the effects of UNI on PpKO enzyme activity in vitro, we compared UNI inhibitory activity between PpKO and AtKO.

As described above, both AtKO and PpKO catalyzed the oxidation of *ent*-kaurene to produce *ent*-kaurenoic acid. The intermediates, *ent*-kaurenol and *ent*-kaurenal, barely accumulated during these multistep oxidation reactions. From these observations, we estimated the inhibitory activity of UNI as its ability to prevent production of *ent*-kaurenoic acid from *ent*-kaurene. The IC_{50} values of UNI against AtKO and PpKO under 10 μM *ent*-kaurene were 0.26 μM and 64 μM, respectively. It was reported that the IC_{50} value of UNI against CYP707A, *Arabidopsis* ABA 8′-hydroxylase,

was 68 nM under 5 μM ±ABA [16]. The sensitivity of PpKO to UNI showed 200 times lower than that of AtKO. Thus, in *P. patens*, low concentration of UNI may not inhibit KO, but instead may inhibit other P450 enzymes, leading to growth inhibition. Although the full-length amino acid sequence of PpKO shares 44% identity with that of AtKO, the identity of active sites between PpKO and AtKO is significantly low (46%), whereas sequences of active sites of KOs are highly conserved among higher plants (approximately 64%). This may be reason for the tolerance of PpKO to UNI.

In conclusion, CYP701B1 of *P. patens* catalyzes the *ent*-kaurene oxidation reaction that forms *ent*-kaurenoic acid. These metabolites were isolated from *P. patens*. The PpKO enzyme is resistant to UNI, which inhibits KOs of flowering plants. When we are able to obtain specific PpKO inhibitors by in vitro screening using recombinant protein, future research should proceed to characterization of the metabolites, the biosynthetic pathway, and the genes involved in diterpene regulators downstream of *ent*-kaurenoic acid in *P. patens*.

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