*Plant Physiology*, February 1999, Vol. 119, pp. 507–510, www.plantphysiol.org © 1999 American Society of Plant Physiologists

# Arabidopsis *ent*-Kaurene Oxidase Catalyzes Three Steps of Gibberellin Biosynthesis

**Chris A. Helliwell*, Andrew Poole, W. James Peacock, and Elizabeth S. Dennis**

Commonwealth Scientific and Industrial Research Organization, Plant Industry, G.P.O. Box 1600, Canberra, ACT 2601, Australia

**The Arabidopsis *GA3* cDNA was expressed in yeast (*Saccharomyces cerevisiae*) and the ability of the transformed yeast cells to metabolize *ent*-kaurene was tested. We show by full-scan gas chromatography-mass spectrometry that the transformed cells produce *ent*-kaurenoic acid, and demonstrate that the single enzyme GA3 (*ent*-kaurene oxidase) catalyzes the three steps of gibberellin biosynthesis from *ent*-kaurene to *ent*-kaurenoic acid.**

GAs are an important group of plant growth regulators with roles in a number of plant growth and developmental processes (Hooley, 1994). Considerable progress has been made in isolating and characterizing the genes encoding enzymes of GA biosynthesis (Hedden and Kamiya, 1997), in particular the enzymes that synthesize *ent*-kaurene and the dioxygenases that catalyze the late steps of GA biosynthesis. The intermediate steps that oxidize *ent*-kaurene to $GA_{12}$ are catalyzed by a number of Cyt P450 monooxygenases. Two Cyt P450 genes implicated in GA biosynthesis have been isolated. The maize *Dwarf3* gene encodes a member of the CYP88 family of Cyt P450 functions (Winkler and Helentjaris, 1995), but although the *dwarf3* mutant responds to GA, the point of the lesion in GA biosynthesis is unknown. The Arabidopsis *GA3* gene encodes a Cyt P450 protein, which is a member of the CYP701 family (Helliwell et al., 1998). The *ga3* mutant accumulates *ent*-kaurene and shows a growth response to *ent*-kaurenoic acid but not *ent*-kaurene, and only a slight response to *ent*-kaurenol (Helliwell et al., 1998). These data are consistent with *GA3*-encoding *ent*-kaurene oxidase, which has been proposed to catalyze the three-step oxidation of *ent*-kaurene to *ent*-kaurenoic acid (Fig. 1). A direct demonstration of this activity has not been made.

The pea *lh-2* mutant is also blocked in *ent*-kaurene oxidation. Using extracts from embryos, Swain et al. (1997) demonstrated that the mutant was not able to oxidize the radiolabeled intermediates *ent*-kaurene, *ent*-kaurenol, or *ent*-kaurenal, but was able to oxidize *ent*-kaurenoic acid. Extracts from wild-type plants could metabolize all four substrates. These data suggest that a single enzyme catalyzes these three reactions, although the mutation could be in a gene encoding a regulatory protein affecting all three steps. Other data also support the proposal that the oxidation of *ent*-kaurene to *ent*-kaurenoic acid is catalyzed by a single enzyme. Coolbaugh et al. (1978) showed that in wild cucumber (*Marah macrocarpus*) the inhibition of each of the three steps from *ent*-kaurene to *ent*-kaurenoic acid by ancymidol had the same kinetics, whereas inhibition of oxidation of *ent*-kaurenoic acid to *ent*-7$\alpha$-hydroxykaurenoic acid was greater, suggesting that it is catalyzed by a different enzyme.

In this paper we describe the expression of the Arabidopsis *GA3* cDNA in yeast (*Saccharomyces cerevisiae*) and show that the GA3 protein does catalyze the three-step oxidation of *ent*-kaurene to *ent*-kaurenoic acid.

## MATERIALS AND METHODS

### Expression in Yeast

The entire *GA3* cDNA was amplified by PCR and inserted between the glyceraldehyde 3-phosphate dehydrogenase promoter and glyceraldehyde 3-phosphate dehydrogenase terminator in the pYE22 multiple cloning site vector, a modification of the pYE2211 vector (Ashikari et al., 1989) in which a polylinker containing *Eco*RI, *Sal*I, *Kpn*I, *Not*I, and *Bam*HI restriction sites was inserted in place of the glyceraldehyde 3-phosphate dehydrogenase open reading frame. Plasmids were prepared from single colonies after the transformation of *Escherichia coli* and the cDNA insert was sequenced to confirm that the fragment would encode the precise protein encoded by the *GA3* cDNA. The sequenced plasmid was then used to transform the yeast (*Saccharomyces cerevisiae*) strain G1315 by a lithium chloride method (Cullin and Pompon, 1988). The transformation mixture was plated on a minimal medium consisting of 0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) Glc and 2% (w/v) agar to select for transformants. Yeast RNA was extracted by the hot acidic-phenol method (Ausubel et al., 1993). Yeast microsomes were prepared using an enzymatic digestion method (Pompon et al., 1996).

### Enzyme Assays

Single colonies of transformed yeast and untransformed controls were used to inoculate 50-mL cultures in a yeast peptone dextrose medium containing 1% (w/v) Bacto yeast

* Corresponding author; e-mail chrish@pican.pi.csiro.au; fax 61–2–6246–5000.

Abbreviations: KRI, Kovat's retention index; TMS, trimethylsilyl.



Downloaded from on October 27, 2020 - Published by www.plantphysiol.org
Copyright © 1999 American Society of Plant Biologists. All rights reserved.

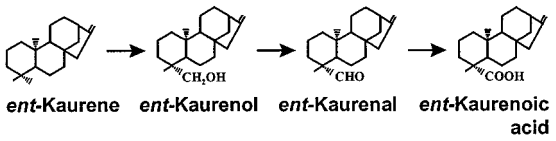


**Figure 1.** The intermediates of the GA biosynthetic pathway from *ent*-kaurene to *ent*-kaurenoic acid.

extract, 2% (w/v) Bacto (Difco, Detroit, MI) peptone, and 2% (w/v) Glc. After growing overnight, 0.5 mL of each culture was removed; the yeast was pelleted and resuspended in 0.5 mL of a reaction mixture containing 100 mM Tris-HCl pH 7.5, 0.5 mM NADPH, and 0.5 mM FAD, according to the method of Hazebroek et al. (1993). The substrates added were 25 $\mu$g of *ent*-kaurene, 5 $\mu$g of *ent*-kaurenol, or 20 $\mu$g of [17,17-$^2H_2$]-*ent*-kaurenal. The substrates were dissolved in 100% methanol before they were added to the reaction mixture; the final methanol concentration in the reaction mixture was 5%. The reactions were incubated for 1 h at 30°C, with shaking at 150 rpm. At the end of the incubation the reaction mixture was extracted, once with 0.5 mL of hexane and twice with 0.5 mL of ethyl acetate. The organic fractions were then pooled and dried using a Speed-Vac (Savant Instruments, Farmingdale, NY) before derivatization for GC-MS. Assays with yeast microsomal fractions were carried out as described above with 100 $\mu$g of microsomal protein replacing the yeast cells.

### Analysis of *ent*-Kaurene Metabolites by GC-MS

For analysis by GC-MS some metabolites of *ent*-kaurene require methylation or trimethylsilylation. Dried samples were dissolved in 50 $\mu$L of methanol and methylated with excess diazomethane (about 200 $\mu$L), after which the samples were redried. Trimethylsilylation was carried out by the addition of 5 $\mu$L each of pyridine and *N,O*-bis(TMS)trifluoroacetamide plus 1% trimethylchlorosilane (Alltech Associates, Deerfield, IL).

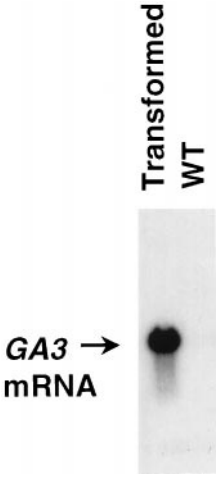


**Figure 2.** RNA gel blot of total RNA from nontransformed yeast and yeast transformed with the *GA3* expression construct probed with a *GA3*-specific probe. WT, Wild type.

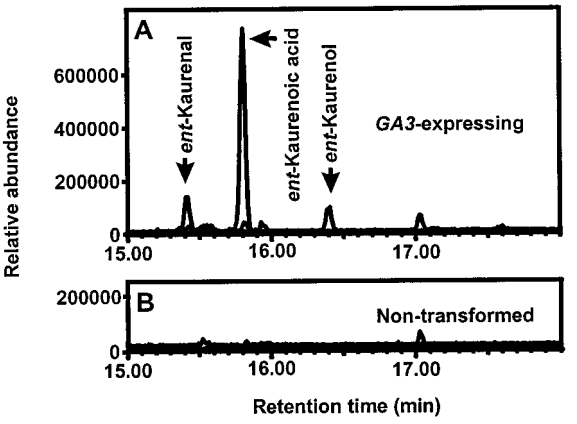


**Figure 3.** Relative abundances of the base peak ions (as in Table I) of *ent*-kaurenal (286), *ent*-kaurenoic acid (316), and *ent*-kaurenol (270) between 15 and 18 min of retention time on the HP-1 column for extracts of *GA3*-expressing (A) and nontransformed (B) yeast after feeding of *ent*-kaurene.

Samples were injected onto a BPX-5 column (25-m × 0.22-mm i.d. [SGE, Austin, TX]) with a 0.25-$\mu$m-thick 5% phenyl (equivalent) polysilphenylene-siloxane stationary phase and analyzed in a full-scan mode. A second injection of the samples was made onto a HP-1 column (25-m × 0.2-mm i.d., [Hewlett-Packard]) with a 0.33-$\mu$m dimethyl polysiloxane stationary phase. Both columns were nonpolar. The HP-1 column achieved better separation of the *ent*-kaurenoic acid and the *ent*-kaurenol in particular and also further confirmed the identities of all metabolites. GC conditions were as described by Green et al. (1997). We co-injected all of the sample (1 $\mu$L) with a series of hydrocarbons derived from Parafilm (Gaskin et al., 1971) for KRI determination, and used authentic standards (from L.N. Mander, Australian National University, Canberra, and J.D. Metzger, Ohio State University, Columbus) for a comparison. We also compared the full scans with a PC-based spectral library (Gaskin and MacMillan, 1991).

## RESULTS

Yeast colonies were picked after a transformation with the *GA3* cDNA construct. We then performed RNA gel-blot analysis to identify the transformed yeast cell line with the highest expression of the *GA3* mRNA (Fig. 2) and used this cell line for the subsequent analysis of GA3 enzyme activity.

### Yeast Expressing GA3 Are Able to Metabolize *ent*-Kaurene to *ent*-Kaurenoic Acid

Yeast cells expressing the *GA3* mRNA and control cells of untransformed yeast were incubated with *ent*-kaurene. Immediately after the incubation the cells were extracted and prepared for GC-MS. Authentic *ent*-kaurene, *ent*-kaurenol, *ent*-kaurenal, and *ent*-kaurenoic acid, derivatized where appropriate, were also injected to generate KRIs and ion spectra for these compounds.

Ion current peaks in the extracts from *GA3*-expressing and nontransformed yeast were initially compared with a

Downloaded from on October 27, 2020 - Published by www.plantphysiol.org
Copyright © 1999 American Society of Plant Biologists. All rights reserved.

**Table I.** *Identification of metabolites after feeding ent-kaurene to yeast transformed with the Arabidopsis GA3 clone*

| Putative[a] and Reference Compound[b] | KRI | | Characteristic Ions | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BPX-5 | HP-1 | Relative Abundance of Base Peak[c] | | | | | | | | | |
| | | | | | | | % | | | | | |
| | | | *360 ($M^+$)* | *345* | *270* | *257* | *255* | *241* | *227* | *187* | *175* | *161* |
| *ent*-Kaurenol TMS | 2244 | 2330 | 25 | 7 | 100 | 80 | 21 | 11 | 22 | 10 | 19 | 23 |
| *ent*-Kaurenol TMS std. | 2244 | 2329 | 23 | 7 | 100 | 72 | 24 | 14 | 14 | 11 | 23 | 26 |
| | | | *286($M^+$)* | *271* | *257* | *243* | *225* | *215* | *199* | *187* | *161* | |
| *ent*-Kaurenal | 2231 | 2257 | 100 | 11 | 35 | 61 | 27 | 19 | 34 | 41 | 42 | |
| *ent*-Kaurenal std. | 2233 | 2258 | 100 | 9 | 35 | 62 | 27 | 12 | 35 | 36 | 34 | |
| | | | *316($M^+$)* | *301* | *273* | *257* | *256* | *241* | *213* | *199* | *187* | *159* |
| *ent*-Kaurenoic acid methyl ester | 2242 | 2286 | 100 | 29 | 55 | 92 | 38 | 69 | 29 | 18 | 27 | 22 |
| *ent*-Kaurenoic acid methyl ester std. | 2242 | 2285 | 100 | 26 | 52 | 93 | 37 | 69 | 29 | 19 | 19 | 21 |

[a] Samples were analyzed, following appropriate derivatization, by full-scan GC-MS on both BPX-5 and HP-1 capillary columns. [b] Comparison was with unlabeled standards (std.), and all samples were coinjected with parafilm so that KRI values could be compared. [c] Data are from full scan on an HP-1 column.

library of spectra. *ent*-Kaurene could be identified in the extracts from both the nontransformed and the *GA3*-expressing yeast. In the extracts from *GA3*-expressing yeast, peaks were present that were putatively assigned as *ent*-kaurene, *ent*-kaurenol, *ent*-kaurenal, and *ent*-kaurenoic acid (Fig. 3). None of these peaks was present in the extracts from nontransformed yeast. In experiments in which the yeast cells expressed a different P450 cDNA, pumpkin CYP88A2 (C.A. Helliwell and E.J. Dennis, unpublished results), only *ent*-kaurene could be detected. KRIs were calculated for the putatively identified peaks and compared with those of authentic standards; this calculation was carried out using data from both the BPX-5 and HP-1 columns (Table I). We found that the KRIs for the putative peaks did not differ significantly from those of the authentic compounds. Confirmation of the identity of the putative peaks was provided by a comparison of the relative abundances of characteristic ions of the putative and reference compounds (Table I). The HP-1 column gave the best separation of the compounds, particularly of *ent*-kaurenol and *ent*-kaurenoic acid.

Extending the length of incubation time increased the abundance of the *ent*-kaurenoic acid ions for the *GA3*-expressing yeast (data not shown). *ent*-Kaurenoic acid accumulation was approximately linear over a 2-h incubation. The abundances of the intermediates *ent*-kaurenol and *ent*-kaurenal were approximately 5-fold lower than *ent*-kaurenoic acid and did not vary greatly over the 2-h incubation, presumably because these intermediates were metabolized to *ent*-kaurenoic acid.

In experiments in which microsomes (approximately 0.2 mg of protein) prepared from *GA3*-expressing and nontransformed yeast were assayed, no metabolism of *ent*-kaurene was observed in preparations from nontransformed cells. In the incubations of the microsomes from *GA3*-expressing cells, *ent*-kaurenol was detected but not *ent*-kaurenal or *ent*-kaurenoic acid.

### Yeast Expressing GA3 Metabolize *ent*-Kaurenol and *ent*-Kaurenal

To confirm that the *GA3*-expressing yeast cells were catalyzing all three steps of the GA biosynthetic pathway from *ent*-kaurene to *ent*-kaurenoic acid, both *GA3*-expressing and nontransformed yeast cells were incubated with *ent*-kaurenol or [17,17-$^2H_2$]-*ent*-kaurenal (Table II). Neither *ent*-Kaurenoic acid nor [17,17-$^2H_2$]-*ent*-kaurenoic acid was detected in extracts from the nontransformed yeast incubated with *ent*-kaurenol or [17,17-$^2H_2$]-*ent*-kaurenal. The extracts from the *GA3*-expressing yeast incubated with *ent*-kaurenol or [17,17-$^2H_2$]-*ent*-kaurenal contained *ent*-kaurenoic acid or [17,17-$^2H_2$]-*ent*-kaurenoic acid, respectively. [17,17-$^2H_2$]-*ent*-Kaurenal was not detected in reactions where it was included as a substrate, which could be due to nonenzymatic oxidation or metabolism by the yeast cells to a product other than *ent*-kaurenoic acid.

## DISCUSSION

We have expressed the Arabidopsis *GA3* gene CYP701A3 in yeast and shown that the transformed cells were able to

**Table II.** *Summary of intermediates detected after a 1-h incubation of yeast expressing the GA3 cDNA with ent-kaurene, ent-kaurenol, and ent-kaurenal*

| Feed | Intermediates Detected by GC-MS | | | |
|---|---|---|---|---|
| | *ent*-Kaurene | *ent*-Kaurenol | *ent*-Kaurenal | *ent*-Kaurenoic acid |
| *ent*-Kaurene | Yes | Yes | Yes | Yes |
| *ent*-Kaurenol | n.d.[a] | Yes | n.d. | Yes |
| *ent*-Kaurenal | n.d. | n.d. | n.d. | Yes |

[a] n.d., Not detected.

Downloaded from on October 27, 2020 - Published by www.plantphysiol.org
Copyright © 1999 American Society of Plant Biologists. All rights reserved.

carry out the three-stage oxidation of *ent*-kaurene to *ent*-kaurenoic acid. The cells were also able to catalyze the oxidation of the intermediates *ent*-kaurenol and *ent*-kaurenal to *ent*-kaurenoic acid. These data showed that all three oxidation steps were enzymatically catalyzed and not due to spontaneous oxidation. The results confirmed previous evidence that *GA3* encodes *ent*-kaurene oxidase. These earlier studies were based on growth responses to fed intermediates and measurements of *ent*-kaurene accumulation in the *ga3-1* mutant (Helliwell et al., 1998).

Our results demonstrated that yeast was a suitable system for analyzing the function of this GA biosynthetic enzyme. It may now be possible to determine which step of GA biosynthesis is catalyzed by the maize Dwarf3 protein using this expression system. In the case of *ent*-kaurene oxidase, whole-yeast cells were a better system than the purified microsomal fraction of the cells. Whole cells expressing *GA3* consistently gave metabolism of *ent*-kaurene to *ent*-kaurenoic acid, whereas the microsomes carried out the single-step conversion to *ent*-kaurenol only. The ability of the microsomes to metabolize *ent*-kaurenol and *ent*-kaurenal was not tested. A possible explanation for the difference in activity between whole cells and microsome preparations is that *ent*-kaurene oxidase was unstable in the microsome preparations. The *ent*-kaurene oxidase from the fungus *Gibberella fujikuroi* has been reported to lose activity rapidly in assays using crude lysates (Ashman et al., 1990). Another possibility is that in purification of the microsomal fraction, a cofactor essential for the oxidation of *ent*-kaurenol was lost, but the oxidation of *ent*-kaurene to *ent*-kaurenol could still proceed.

Our successful expression of the GA3 protein in a functional form in yeast is the first direct demonstration, to our knowledge, of the activity of a Cyt P450 enzyme of the GA biosynthesis pathway.

## ACKNOWLEDGMENTS

We thank Ying Luo for technical assistance and Professors L.N. Mander and J.D. Metzger for providing GA intermediates.

Received September 15, 1998; accepted November 3, 1998.

## LITERATURE CITED

**Ashikari T, Kiuchi-Goto N, Tanaka Y, Shibano Y, Amachi T, Yoshizumi H** (1989) High expression and efficient secretion of *Rhizopus oryzae* glucose amylase in the yeast *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol **30:** 515–520

**Ashman PJ, Mackenzie A, Bramley PM** (1990) Characterization of *ent*-kaurene oxidase activity from *Gibberella fujikoroi*. Biochim Biophys Acta **1036:** 151–157

**Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K** (1993) Current Protocols in Molecular Biology. John Wiley and Sons, New York, pp 13.12.1–13.12.3

**Coolbaugh RC, Hirano SS, West CA** (1978) Studies on the specificity and site of action of α-cyclopropyl-α-[*p*-methoxyphenyl]-5-pyrimidine methyl alcohol (ancymidol), a plant growth regulator. Plant Physiol **62:** 571–576

**Cullin C, Pompon D** (1988) Synthesis of functional mouse cytochromes P-450 P1 and chimeric P-450 P3–1 in the yeast *Saccharyomyces cerevisiae*. Gene **65:** 203–217

**Gaskin P, MacMillan J** (1991) GC-MS of the Gibberellins and Related Compounds: Methodology and a Library of Spectra. Cantock's Enterprises, Bristol, UK. Data converted to MS Chemstation format by Steve Croker

**Gaskin P, MacMillan J, Firn RD, Pryce RJ** (1971) "Parafilm:" a convenient source of *n*-alkanes for determination of gas chromatographic retention indices. Phytochemistry **10:** 1155–1157

**Green LS, Faergestad EM, Poole A, Chandler PM** (1997) Grain developmental mutants of barley. α-Amylase production during grain maturation and its relation to endogenous gibberellic acid content. Plant Physiol **114:** 203–212

**Hazebroek JP, Metzger JD, Mansager ER** (1993) Thermoinductive regulation of gibberellin metabolism in *Thlaspi arvense* L. II. Cold induction of enzymes in gibberellin biosynthesis. Plant Physiol **102:** 547–552

**Hedden P, Kamiya Y** (1997) Gibberellin biosynthesis: enzymes genes and their regulation. Annu Rev Plant Physiol Plant Mol Biol **48:** 431–460

**Helliwell CA, Sheldon CC, Olive MR, Walker ARW, Zeevaart JAD, Peacock WJ, Dennis ES** (1998) Cloning of the *Arabidopsis ent*-kaurene oxidase gene *GA3*. Proc Natl Acad Sci USA **95:** 9019–9024

**Hooley R** (1994) Gibberellins: perception, transduction and responses. Plant Mol Biol **26:** 1529–1555

**Pompon D, Lauerat B, Bronine A, Urban P** (1996) Yeast expression of animal and plant P450s in optimized redox environments. Methods Enzymol **272:** 51–64

**Swain SM, Reid JB, Kamiya Y** (1997) Gibberellins are required for embryo growth and seed development in pea. Plant J **12:** 1329–1338

**Winkler RG, Helentjaris T** (1995) The maize *Dwarf3* gene encodes a cytochrome P450-mediated early step in gibberellin biosynthesis. Plant Cell **7:** 1307–1317

Downloaded from on October 27, 2020 - Published by www.plantphysiol.org
Copyright © 1999 American Society of Plant Biologists. All rights reserved.