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Rapid Paper

Emission of *ent*-Kaurene, a Diterpenoid Hydrocarbon Precursor for Gibberellins, into the Headspace from Plants

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***ent*-Kaurene is a tetracyclic hydrocarbon precursor for gibberellins (GAs) in plants and fungi. To address whether fungal GA biosynthesis enzymes function in plants, we generated transgenic *Arabidopsis* plants overexpressing *ent*-kaurene synthase (GfCPS/KS) from a GA-producing fungus *Gibberella fujikuroi*. GfCPS/KS catalyzes a two-step reaction corresponding to *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS) activities in plants. When GfCPS/KS was overexpressed and targeted to plastids, a range of GA-deficient phenotypes of the *ga1-3* and *ga2-1* mutants (defective in CPS and KS, respectively) were restored to wild type. Unexpectedly, the transgenic lines overproducing GfCPS/KS emitted the GA precursor *ent*-kaurene into the headspace besides its accumulation in the plant body. When co-cultivated with the *ent*-kaurene overproducers in a closed environment, the airborne *ent*-kaurene was able to fully complement the dwarf phenotype of *ga1-3* and *ga2-1* mutants, but not that of the *ga3-1* mutant (defective in *ent*-kaurene oxidase). These results suggest that *ent*-kaurene may be efficiently metabolized into bioactive GAs in *Arabidopsis* when supplied as a volatile. We also provide evidence that *ent*-kaurene is released in the headspace of wild-type *Chamaecyparis obtusa* and *Cryptomeria japonica* plants, suggesting the occurrence of this hydrocarbon GA precursor as a volatile in nature.**

Keywords: *Arabidopsis thaliana* — Diterpene — *ent*-Kaurene — *Gibberella fujikuroi* — Gibberellin.

Abbreviations: CDP, *ent*-copalyl diphosphate; CPS, *ent*-copalyl diphosphate synthase; GC-MS, gas chromatography–mass spectrometry; GA, gibberellin; GFP, green fluorescent protein; GGDP, geranylgeranyl diphosphate; Kan, kanamycin; KO, *ent*-kaurene oxidase; KS, *ent*-kaurene synthase; MEP, methylerythritol phosphate; ODS, octadecylsilane; PDMS, polydimethylsiloxane; QRT–PCR, quantitative reverse transcription–PCR; SPME, solid-phase microextraction; TP, transit peptide.

Introduction

Gibberellins (GAs) are a class of diterpenoid phytohormones essential for many processes of plant growth and development, such as seed germination, stem elongation, leaf expansion and flower development. The growth-promoting activity of GA in plants was initially recognized by observing the 'Bakanae disease' in rice (foolish rice), in which exogenous bioactive GA produced by the pathogenic fungus *Gibberella fujikuroi* causes hyper-elongation of shoots (for review, see Phinney 1983). The roles of endogenous GAs in plant development have been elucidated using a series of mutants that are defective in an enzyme in the GA biosynthesis pathway. For example, severe GA-deficient mutants of *Arabidopsis*, such as *ga1-3*, *ga2-1* and *ga3-1*, are non-germinating, male-sterile and extremely dwarfed (Koornneef and van der Veen 1980).

Recent studies have identified almost all of the genes for GA biosynthesis in *Arabidopsis* and the fungus *G. fujikuroi* (for review, see Hedden et al. 2002). These studies have revealed that there are apparent differences in the enzymes in the plant and fungal GA biosynthesis pathways. For example, in plants geranylgeranyl diphosphate (GGDP), a common precursor of diterpenes, is converted to the tetracyclic hydrocarbon *ent*-kaurene by two separate diterpene cyclases, *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS) (Fig. 1). In contrast, fungal *ent*-kaurene synthases from *G. fujikuroi* and *Phaeosphaeria* sp. L487 are bifunctional terpene cyclases with both CPS and KS activities (Kawaide et al. 1997, Toyomasu et al. 2000). We refer to the bifunctional enzyme from *G. fujikuroi* as GfCPS/KS. As predicted from the bifunctionality of the fun-

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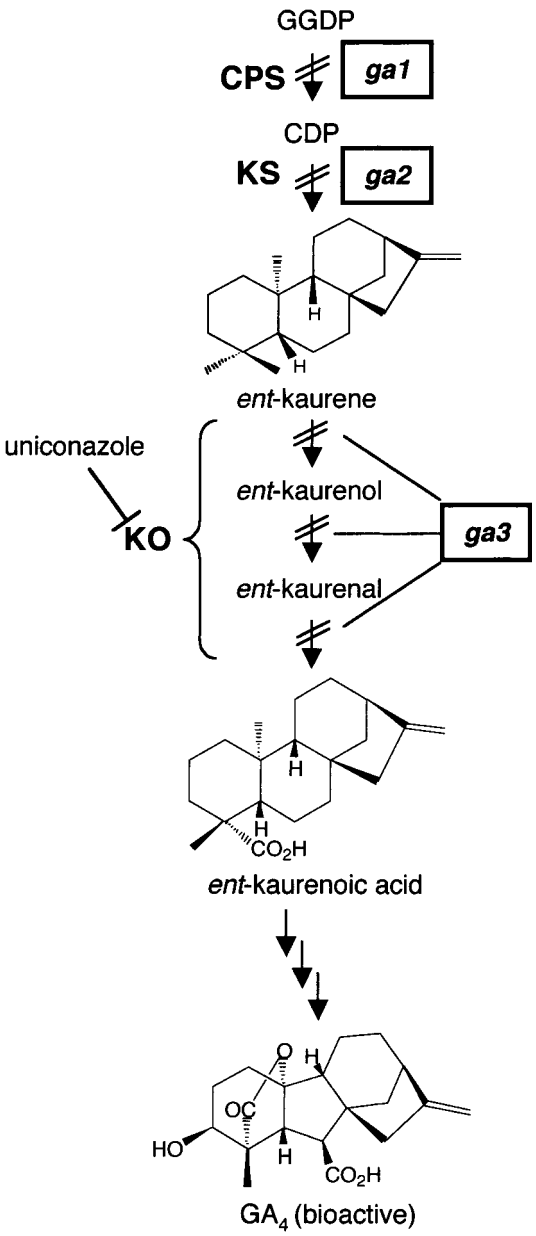

Fig. 1 The major GA biosynthesis pathway in *Arabidopsis*. Enzyme names are given in boldface. Names of mutant alleles are shown in boxes. GA_4 is the major bioactive GA in *Arabidopsis*. GGDP, geranylgeranyl diphosphate; CDP, *ent*-copalyl diphosphate; CPS, CDP synthase; KS, *ent*-kaurene synthase; KO, *ent*-kaurene oxidase. The T bar indicates inhibition of KO activity by uniconazole.

gal enzymes, the overall sequence similarities between CPS and KS from plants and the fungal enzymes are relatively low. Sequence diversities between plant and fungal enzymes are also evident in later GA biosynthesis steps (Hedden et al. 2002), suggesting that the GA biosynthesis pathways of plants and fungi evolved independently.

Recent completion of the genome sequence analysis of *Neurospora crassa* (Galagan et al. 2003) has revealed that this fungus possesses a large number of putative genes for terpenoid secondary metabolism, as do plants. Part of the diversity of fungal terpenoids was realized earlier because of the production of several classes of terpenoids, including such biologically active compounds as fusicoccin from *Fusicoccum amygdali* (Ballio et al. 1968) and aphidicolin from *Phoma betae* (Dalziel et al. 1973). Therefore, functional expression of fungal enzymes in plants may provide new possibilities for useful terpenoid productions through metabolic engineering. Unlike fungal terpenoids, which are produced through the cytosolic mevalonate pathway, the majority of plant diterpenes including GAs, are synthesized through the methylerithritol phosphate (MEP) pathway in plastids (Kasahara et al. 2002, Okada et al. 2002). Thus, a possible challenge in functional expression of fungal diterpene cyclases in plants would be the necessity of the enzyme to be targeted to appropriate subcellular locations.

As an initial step in addressing these questions, we set out to generate transgenic *Arabidopsis* plants overexpressing GfCPS/KS. Our results show that GfCPS/KS is successfully targeted to, and functions in, plastids when produced as a fusion protein with the transit peptide of *Arabidopsis* CPS (AtCPS). Gas chromatography–mass spectrometry (GC-MS) analysis demonstrates that transgenic lines contain extremely high levels of *ent*-kaurene. Unexpectedly, our results also show that *ent*-kau-

Fig. 2 Overexpression of *GfCPS/KS* cDNA results in accumulation of *ent*-kaurene in *Arabidopsis*. (A) Outline of the construct pBIC-TPGK. P35S, cauliflower mosaic virus 35S promoter. TP, a cDNA fragment encoding the putative transit peptide of *Arabidopsis* CPS. GK, full-length cDNA encoding GfCPS/KS; GFP, green fluorescence protein; Tnos, nopaline synthase terminator; Kan^r, a gene cassette to confer kanamycin resistance. (B) Thirty-day-old *ga1-3* mutant (left), *ga1-3* mutant transformed with pBIC-TPGK (center), and wild type (right). Seeds were treated with GA_4 to allow germination of *ga1-3*. Plants were then grown on soil without exogenous GA. (C) Relative *GfCPS/KS* transcript levels in 2-week-old rosette plants. N.D., Not detected. (D) Western blot analysis showing GfCPS/KS-GFP protein using an anti-GFP antibody (upper panel). Total proteins (20 μg per lane) from 2-week-old rosette plants were used. The arrowhead indicates the predicted position of the GfCPS/KS-GFP protein. Shown in the lower panel is Coomassie brilliant blue staining of an SDS–polyacrylamide gel containing the same set of protein samples, to indicate approximately equal loading. Molecular weights are shown on the right. 35S-GFP, transgenic *Arabidopsis* plant overexpressing *GFP* gene alone. (E) Levels of endogenous *ent*-kaurene in 2-week-old rosette plants. To inhibit *ent*-kaurene metabolism, plants were treated with uniconazole (0.1 μM) for 5 d prior to measurements. The values are represented as the mean ± SE of three independent samples. N.D., Not detected. (F) Subcellular localization of GfCPS/KS-GFP protein in plastids. Shown are confocal images of cortical cells of 10-day-old *Arabidopsis* hypocotyls. Left and middle panels indicate the image of GFP fluorescence (green) and chlorophyll auto-fluorescence (red), respectively. Right panels show the merged image, in which both GFP and chlorophyll fluorescence have been overlapped. Bar = 100 μm. 35S-GFP, transgenic plant overexpressing *GFP* alone as a control.

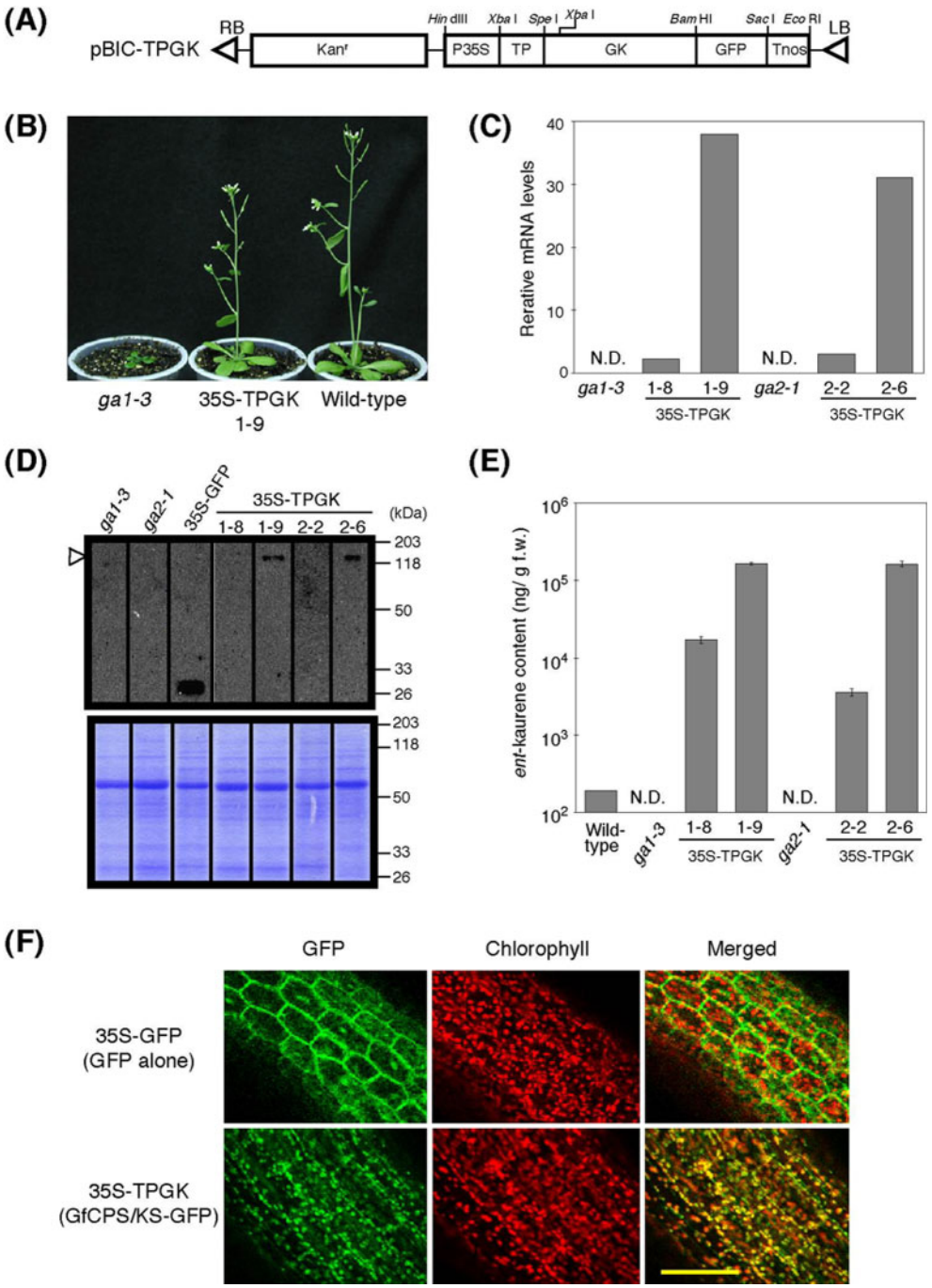

Fig. 2

rene is emitted into the headspace, and that this diterpene hydrocarbon as a volatile is able to rescue the *ent*-kaurene-deficient *ga1-3* and *ga2-1* mutant phenotypes. We show that *ent*-kaurene emission also occurs at a detectable level without overexpressing *ent*-kaurene synthases if its metabolism is blocked by the *ga3-1* mutation. Our results also suggest that *ent*-kaurene, supplied in the headspace, can be taken up and metabolized by plants to modulate their growth and development.

Results

Generation of transgenic lines overexpressing GfCPS/KS

The plant enzymes responsible for *ent*-kaurene biosynthesis possess an N-terminal transit-peptide (TP) that targets proteins to plastids. In contrast, the fungal enzyme GfCPS/KS does not have an apparent TP-like sequence (Toyomasu et al. 2000). To target GfCPS/KS into plastids and to monitor its subcellular location in *Arabidopsis*, we designed a plasmid con-

struct named pBIC-TPGK that would allow the production of GfCPS/KS in plants as a fusion protein with both the TP from AtCPS and green fluorescent protein (GFP) under the control of the 35S promoter of cauliflower mosaic virus (Fig. 2A). It has previously been shown that AtCPS is targeted into isolated plastids in vitro, and that the mature AtCPS protein (after processing of the TP) is approximately 10 kDa smaller than the precursor (Sun and Kamiya 1994). Smith et al. (1998) generated N-terminal deletions of recombinant CPS from pumpkin, and found that the removal of the N-terminal 99 amino acids resulted in loss of CPS activity. Based on these observations, the N-terminal 85 amino acid sequence of *Arabidopsis* CPS was used as a potential TP.

To examine whether GfCPS/KS is functional in *Arabidopsis*, we introduced pBIC-TPGK into the *ga1-3* and *ga2-1* mutants via *Agrobacterium*-mediated transformation. The *ga1-3* and *ga2-1* mutants are defective in CPS and KS, respectively (Sun and Kamiya 1994, Yamaguchi et al. 1998), and exhibit severe GA-deficient phenotypes. Therefore, it was anticipated that the introduction of pBIC-TPGK would result in complementation of both *ga1-3* and *ga2-1* mutations if GfCPS/KS is functional in *Arabidopsis*. Because both *ga1-3* and *ga2-1* mutants are non-germinating, male-sterile dwarfs, we selected T_1 transgenic lines in the presence of GA_4, the major bioactive GA in this species. We first selected transgenic lines in both mutant backgrounds that segregated approximately 3 : 1 for kanamycin (Kan)- resistant versus -sensitive (n = 40–100). We found that Kan-resistant plants grew normally and set fertile seeds without GA application, like wild-type plants (Fig. 2B), indicating that the construct was able to rescue the phenotypic defects of both mutants at these developmental stages. To examine the germination phenotype seeds of T_2 lines with 3 : 1 segregation for Kan resistance were imbibed in the absence of exogenous GA. Approximately 75% of the seeds germinated without GA treatment, indicating that the construct complements the failure of germination of both *ga1-3* and *ga2-1* mutants.

Molecular characterization of GfCPS/KS in the transgenic lines

To verify the expression of *GfCPS/KS* and the production of *ent*-kaurene, we established 10 and six independent lines homozygous for the transgene in *ga1-3* (35S-TPGK1) and *ga2-1* (35S-TPGK2) mutant backgrounds, respectively. Two representative lines from each mutant background were characterized in detail at the molecular level. Quantitative reverse transcription (QRT)–PCR analysis showed that *GfCPS/KS* mRNA accumulated in the transgenic lines at varying levels, whereas this transcript was absent in the parental mutant plants (Fig. 2C). Anti-GFP antisera were used to detect the GfCPS/KS fusion protein in total cell extracts from the transgenic lines. In lines that accumulated *GfCPS/KS* transcript at relatively high levels (1–9 and 2–6), accumulation of the fusion protein was also evident (Fig. 2D), while this protein was not detectable in transgenic lines 1–8 and 2–2, both of which contained *GfCPS/KS* mRNA at relatively low levels.

It has previously been shown that overproduction of native CPS (AtCPS) in *Arabidopsis* results in accumulation of *ent*-kaurene at extremely high levels (Fleet et al. 2003). Therefore, if GfCPS/KS is fully functional in the *Arabidopsis* transgenic lines, they might produce a larger amount of *ent*-kaurene in comparison with wild-type plants. To examine this possibility, we measured *ent*-kaurene contents in *GfCPS/KS* overexpressors by GC-MS using an isotope-labeled internal standard (Fig. 2E). To assess the ability of *ent*-kaurene synthesis directly, plants were cultivated in the presence of uniconazole, which inhibits the metabolism of this hydrocarbon (Fig. 1). As shown in Fig. 2E, all the transgenic lines accumulated *ent*-kaurene at much higher levels than do wild-type plants. Consistent with the mRNA and protein levels (Fig. 2C, D), lines 1–9 and 2–6 produced much larger amounts of *ent*-kaurene relative to the weak lines (1–8 and 2–2) in respective mutant backgrounds. These results confirm that GfCPS/KS is functional in *Arabidopsis*.

To determine the subcellular localization of GfCPS/KS-GFP, we observed GFP fluorescence using a confocal laser-scanning microscope. Fig. 2F shows that GFP alone is distributed predominantly to the cytosol and nuclei in cortical cells of the hypocotyl. In contrast, the major GFP fluorescence from GfCPS/KS-GFP coincided with the location of autofluorescence of chlorophyll, indicating that GfCPS/KS was targeted to plastids. From these results, we can conclude that GfCPS/KS was functionally produced in *Arabidopsis* plastids.

Release of ent-kaurene into the headspace of Arabidopsis plants

As described above, we identified multiple transgenic lines that segregate approximately 3 : 1 for the transgene in the T_2 generation. In order to ensure that the dwarf (GA-deficient) and normal vegetative phenotypes also segregate at approximately 3 : 1, T_2 transgenic seeds were stratified in the presence of GA_4, washed with water and then grown on agar media in the absence of GA_4. Such temporal GA treatment allows GA-deficient mutant seeds to germinate, but they grow into severe dwarfs with dark green leaves if GA is not further supplied exogenously (Koornneef and van der Veen 1980). When the T_2 transgenic lines (in both the *ga1-3* and *ga2-1* backgrounds) were grown in this manner, we found that all individuals of every independent line resembled wild-type plants (30–50 plants per Petri dish), which was inconsistent with the segregation of Kan resistance. However, when T_2 individuals were grown in a Petri dish in an isolated manner from other plants (one plant per Petri dish), they segregated at approximately 3 : 1 for normal versus dwarf phenotypes. These results suggested that the *ga1-3* and *ga2-1* dwarf phenotype was rescued due to the presence of transgenic lines overexpressing GfCPS/KS in the same Petri dish.

As transgenic lines overexpressing *GfCPS/KS* produced a large amount of *ent*-kaurene (Fig. 2E), we speculated that this hydrocarbon intermediate might affect the growth of plants

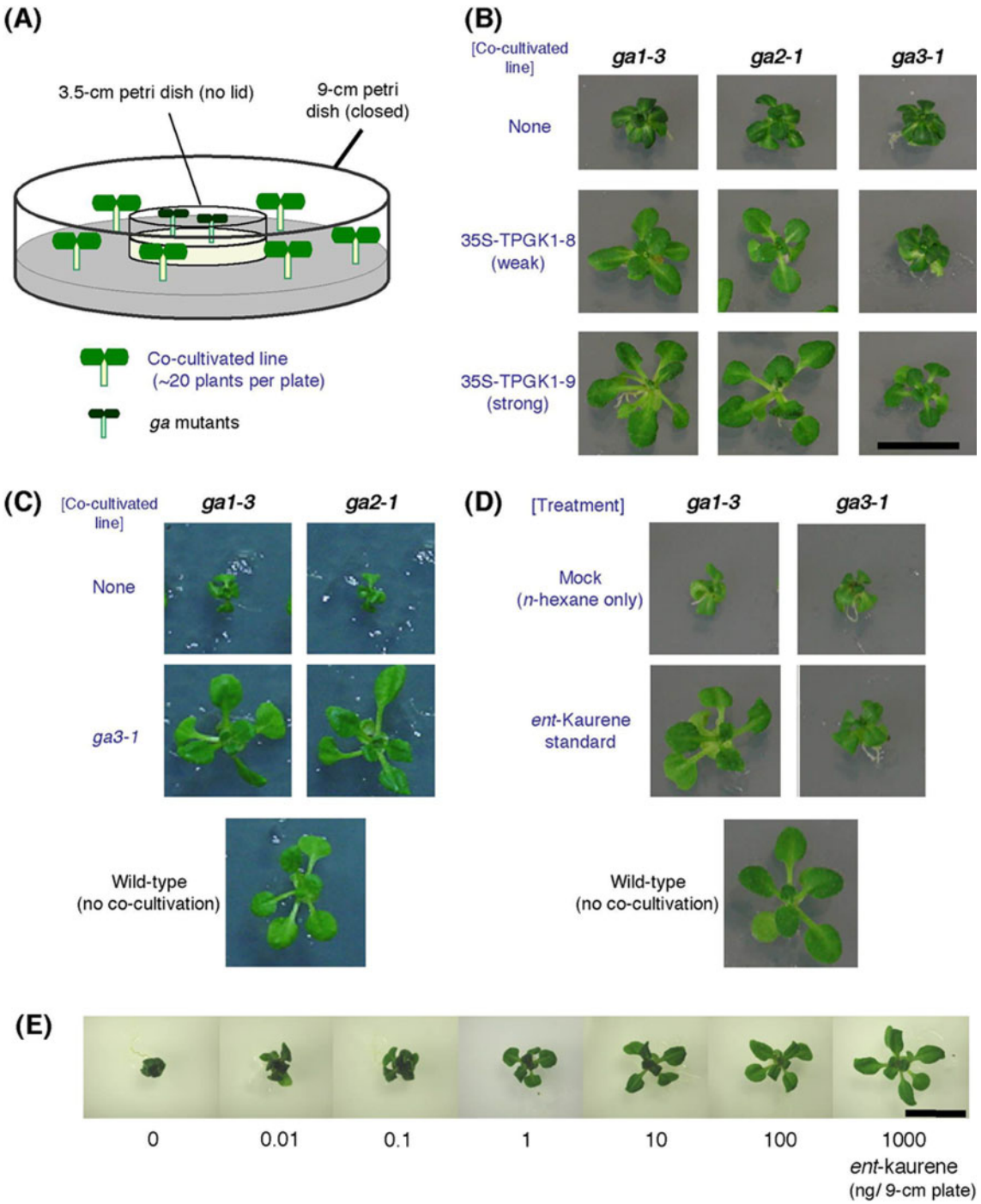

Fig. 3 Co-cultivation experiments. (A) Schematic drawing of co-incubation. An open 3.5 cm Petri dish (containing MS agar medium) was placed on another MS agar medium in a 9 cm Petri dish. During incubation, the lid of only the larger Petri dish was closed, so that two sets of plants do not share the media, but they share the headspace. (B) Phenotypes of GA-deficient mutants co-cultivated with 35S-TPGK transgenic lines for 22 d. (C) Phenotypes of *ga1-3* and *ga2-1* mutants co-incubated with or without *ga3-1* for 18 d. (D) Phenotypes of 16-day-old *ga1-3* and *ga3-1* mutants in the presence of a filter paper containing *ent*-kaurene (1 mg) in the 3.5 cm Petri dish. As a control, the filter paper was treated with *n*-hexane only (mock). (E) Response of *ga1-3* seedlings to headspace *ent*-kaurene at different doses. Twelve-day-old *ga1-3* seedlings were co-incubated with a filter paper containing *ent*-kaurene for 9 d. Bar in (B) and (E) =1 cm.

Table 1 Identification of *ent*-kaurene in the headspace of the 35S-TPGK 1–9 line by full-scan GC-MS

Sample	Retention time on GC (min)	Characteristic ion at m/z (% of relative intensity of base peak)
35S-TPGK 1–9 line	10.45	272 (M^+, 48), 257 (100), 229 (65), 213 (47), 201 (15), 187 (27), 175 (25), 161 (25)
Authentic *ent*-kaurene	10.44	272 (M^+, 52), 257 (100), 229 (65), 213 (44), 201 (15), 187 (25), 175 (24), 161 (24)

nearby. *ent*-Kaurene is a tetracyclic diterpene hydrocarbon, which exists as a solid and sublimes at room temperature. In addition, some diterpene hydrocarbons are found as minor components in essential oil prepared by steam distillation (Ishikura et al. 1984). It was possible therefore, that *ent*-kaurene was released as a volatile from the transgenic lines and affected the growth of *ent*-kaurene-deficient *ga1-3* and *ga2-1* mutants. To examine this possibility we conducted a series of co-cultivation experiments in which a pair of mutant or transgenic lines share the headspace, but are grown on separate agar media (Fig. 3A). As shown in Fig. 3B, when the *ga1-3* or *ga2-1* mutant was co-cultivated with *GfCPS/KS* overexpressor lines, the dwarf phenotype was restored nearly to wild type. Full recovery of the dwarf phenotype was not observed for the *ga3-1* mutant (see Discussion), which is also a GA-deficient dwarf, but is defective in the oxidation of *ent*-kaurene in the GA biosynthesis pathway (Fig. 1; Helliwell et al. 1998). Co-cultivation of transgenic lines overexpressing *AtCPS* alone or both *AtCPS* and *AtKS* (Fleet et al. 2003) exhibited the same effect on the growth of these GA-deficient mutants (data not shown). These results suggested that the major compound affecting the growth of *ga1-3* and *ga2-1* mutants was *ent*-kaurene in the co-cultivation experiments. Besides up-regulation of the synthesis of *ent*-kaurene, down-regulation of its catabolism would also cause accumulation of this hydrocarbon in plants. As shown in Fig. 3C, co-cultivation of *ga3-1* mutant plants was also effective at rescuing the dwarf phenotype of the *ga1-3* and *ga2-1* mutants. To test whether *ent*-kaurene provided as a volatile is able to rescue the *ga1-3* dwarf phenotype, *ent*-kaurene was dissolved in *n*-hexane, spread onto a filter paper. After *n*-hexane evaporated, the filter paper containing *ent*-kaurene was placed in the Petri dish, and incubated with the *ga1-3* or *ga3-1* mutant (Fig. 3D). This *ent*-kaurene treatment was effective at complementing the *ga1-3* mutant phenotype, but the *ga3-1* mutant remained severely dwarfed even at the highest dose. The growth promotion of *ga1-3* mutant seedlings was already evident in the presence of 10 pg *ent*-kaurene in the Petri dish (Fig. 3E).

To examine whether *ent*-kaurene exists in the headspace, volatile compounds from 12-day-old 35S-TPGK plants (line 1–9) grown on Murashige–Skoog (MS) agar media were collected by the solid-phase microextraction (SPME) method and analyzed qualitatively on GC-MS. As shown in Table 1, *ent*-kaurene was identified in the headspace of the 35S-TPGK transgenic plants by full-scan GC-MS. The peak of *ent*-kaurene was absent when the headspace of MS agar media without plants or with wild-type plants was analyzed (Fig. 4). Consistent with its effect on co-cultivated *ga1-3* mutant plants (Fig. 3C), *ent*-kaurene was identified in the headspace of the *ga3-1* mutant and an *AtCPS* overexpressor (data not shown). From these results we can conclude that overexpression of a fungal enzyme GfCPS/KS in *Arabidopsis* results in accumulation of *ent*-kaurene both in the plant body and in the headspace. Our results also show that *ent*-kaurene emission into the headspace is also detectable without engineered up-regulation of *ent*-kaurene synthesis if its metabolism is blocked by the *ga3-1* mutation. Airborne *ent*-kaurene was able to rescue the dwarf phenotype of the *ga1-3* and *ga2-1* mutants.

ent-Kaurene in the headspace of different plant species

In order to assess whether *ent*-kaurene is emitted into the air in nature, it is necessary to determine whether this diterpene hydrocaron exists in the headspace of wild-type (non-engineered) plants. As shown in Fig. 4, *ent*-kaurene was not detectable in the headspace of wild-type *Arabidopsis* plants using the SPME method. In an attempt to analyze volatile *ent*-kaurene at a higher sensitivity, we incubated soil-grown plants in a closed glass jar, and the volatiles released in the headspace were collected at the outlet using an octadecylsilane (ODS) cartridge (Fig. 5A). Quantitative analysis of ODS-trapped *ent*-kaurene from 35S-TPGK transgenic plants (line 1–9, 2 weeks

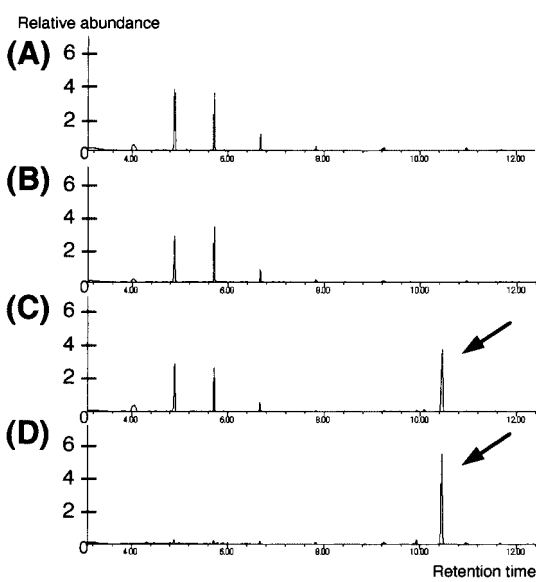

Fig. 4 GC profiles of volatile compounds in the headspace using the SPME method. Arrows indicate the peak of *ent*-kaurene. (A) MS agar media without plants. (B) Wild type and (C) 35S-TPGK line (1–9) on MS agar media. (D) *ent*-kaurene standard.

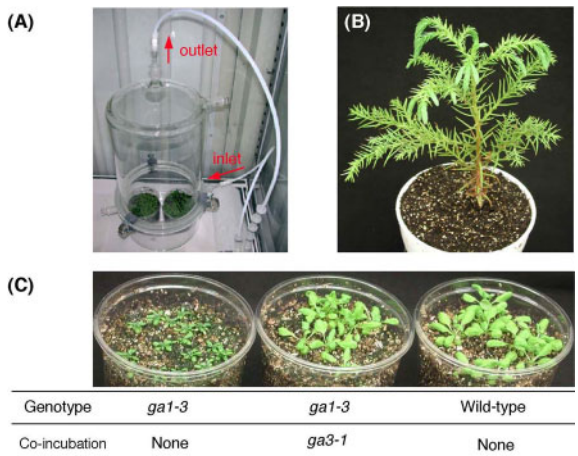

Fig. 5 Analysis of headspace volatiles from soil-grown plants. (A) The ODS-trapping method. Plants are grown in a glass jar placed in a growth chamber. The air through the glass jar was circulated using a pump and the headspace volatiles were collected at the outlet using an ODS cartridge. (B) An approximately 1-year-old Japanese cedar plant used for headspace analysis. The pot diameter is 9 cm. (C) Four-week-old *ga1-3* mutant plants co-incubated with or without 30 *ga3-1* mutant plants (same age) in a closed 300 liter growth chamber. As a reference, 4-week-old wild-type plants under the same growth conditions is also shown.

old) demonstrated that *ent*-kaurene is emitted into the headspace at 60 ng h^{-1} per plant. However, *ent*-kaurene was also not detectable from the headspace of wild-type *Arabidopsis* plants by the ODS-trapping method.

To investigate whether any wild-type plants emit *ent*-kaurene at a detectable level, headspace volatiles from seedlings of tomato (*Lycopersicon esculentum* cv. Moneymaker), lettuce (*Lactuca sativa* cv. Grand rapids), pea (*Pisum sativum* cv. Alaska), pumpkin (*Cucurbita maxima*), Japanese cedar (*Cryptomeria japonica*) and Japanese cypress (*Chamaecyparis obtusa*) were analyzed on GC-MS. We included *C. japonica* in this study because this species has been shown to produce an exceptionally large amount of *ent*-kaurene and its derivatives (Hegnauer 1962). GC-MS analysis showed that *ent*-kaurene was released in the headspace of approximately 1-year-old *Cryptomeria japonica* (380 ng h^{-1} per plant, Fig. 5B) and *Chamaecyparis obtusa* (0.47 ng h^{-1} per plant), but not in the headspace of tomato, lettuce, pea or pumpkin seedlings (2–3 weeks old) under the current growth conditions. These results suggest that a GA intermediate *ent*-kaurene is released into the headspace of some plant species in nature.

Effect of volatile ent-kaurene on seed germination and flower development

As described above, we showed that *ent*-kaurene as a volatile is able to rescue the dwarf phenotype of the GA-deficient *ga1-3* and *ga2-1* mutants at the seedling stage. In addition to being dwarfed, severe GA-biosynthesis mutants are defective in seed germination, bolting and developing fertile flower organs (Koornneef and van der Veen 1980). We examined the effect of volatile *ent*-kaurene on these phenotypic defects in *ga1-3* and *ga2-1* mutants. In the presence of 1 mg *ent*-kaurene in a 9 cm Petri dish, *ga1-3* mutant seeds still failed to germinate (data not shown), indicating that volatile *ent*-kaurene is not effective in rescuing the germination phenotype. To examine the effect of headspace *ent*-kaurene at later developmental stages, dwarfed *ga1-3* mutant plants were grown on soil and co-cultivated with approximately 30 *ga3-1* plants in a closed 300 liter growth cabinet. As shown in Fig. 5C, the *ga1-3* mutant grew normally like wild-type plants; they bolted, produced fertile flowers and set seeds without exogenous GA treatment. In contrast, the *ga3-1* mutant remained severely dwarfed in the same growth chamber, consistent with the block of *ent*-kaurene metabolism in this mutant. Taken together, our results indicate that the GA-deficient *ga1-3* and *ga2-1* mutant phenotypes can be complemented by the presence of volatile *ent*-kaurene, with the exception of their non-germinating phenotype.

Discussion

This study has shown that GfCPS/KS, a diterpene cyclase of fungal origin, functions in *Arabidopsis* when it is targeted to plastids. In plants, the MEP pathway in plastids is thought to provide the majority of diterpenoid precursors. We therefore speculate that an efficient supply of the substrate GGDP to GfCPS/KS in this organelle allowed the production of *ent*-kaurene at a high level. Previous work has indicated that GGDP synthases are also present in other subcellular compartments, such as the endoplasmic reticulum and mitochondria (Okada et al. 2000). Given the success in overproduction of *ent*-kaurene in the plastid, it would now be interesting to test whether the production of this hydrocarbon is possible from other GGDP sources by targeting GfCPS/KS to different subcellular compartments and examine whether such mis-targeted production of *ent*-kaurene affects its conversion to GAs.

Our results have shown that *ent*-kaurene is released into the headspace of *Arabidopsis* plants when *ent*-kaurene accumulates to a high level as a result of its production being increased or its metabolism being blocked. The volatility of mono- and sesquiterpene classes has been well established; some are known to act in the headspace of plants as molecules for pollinator attraction and defense (for review, see Pichersky and Gershenzon 2002). However, our knowledge is limited on the occurrence and roles of diterpenes in the headspace of plants. For example, a recent comprehensive study on headspace volatiles of *Arabidopsis* identified a number of mono- and sesquiterpene compounds, but the presence of diterpenoids was not evident (Aharoni et al. 2003). Although diterpene hydrocarbons often exist in essential oil (extracted with steam distillation) as minor components from many plant species, there are only a few reports on the detection of diterpene-like compounds as headspace volatiles of living organisms (Fravel et al. 2002, Lewis et al. 2003). Our study provided evidence for the occur-

rence of *ent*-kaurene in the headspace of both engineered *Arabidopsis* plants and wild-type *C. obtusa* and *C. japonica* plants. From the latter two tree species, other diterpene hydrocarbon-like compounds were also detected as headspace volatiles (data not shown). These observations suggest that diterpene hydrocarbons are released from living plants into the air in nature, and imply a potential role of this class of natural products in communication between organisms.

Application of *ent*-kaurene as a solution (2.5 μg per plant) has previously been shown to induce stem elongation of adult *ga1-2* and *ga2-1* mutants (Helliwell et al. 1998). Our data indicated that *ent*-kaurene supplied as a volatile is able to rescue dwarf and male-sterile phenotypes of the *ga1-3* and *ga2-1* mutants efficiently. In our experiment using dwarfed *ga1-3* seedlings on agar media, growth response to headspace *ent*-kaurene was visible at a dose of 10 pg in a 9 cm Petri dish (Fig. 3E). In contrast, the *ga3-1* mutant (defective in *ent*-kaurene metabolism) (Fig. 3D) did not respond to *ent*-kaurene supplied as a volatile. These observations illustrate the requirement of *ent*-kaurene oxidase (KO) activity (*ent*-kaurene metabolism) for the growth response to headspace *ent*-kaurene, and suggest that it might be converted to bioactive GAs in the *ga1-3* and *ga2-1* mutants. This hypothesis is supported by the fact that a mutant with a block in the final step in the production of bioactive GAs also did not respond to *ent*-kaurene supplied as a volatile (data not shown). The hypothetical uptake and metabolism of headspace *ent*-kaurene by plants will need to be demonstrated directly by feeding isotope-labeled *ent*-kaurene as a headspace volatile. Nevertheless, the ability of volatile *ent*-kaurene to modulate the growth of *ga1-3* and *ga2-1* mutant suggests a potential role for this GA precursor as a volatile signal involved in interplant communication. To consider the physiological and ecological significance of this hypothesis, there are several questions to be addressed as discussed below.

We were unable to detect *ent*-kaurene in the headspace of wild-type (non-engineered) seedlings of *Arabidopsis*, lettuce, tomato, pea or pumpkin. Therefore, *ent*-kaurene emission into the headspace may be restricted to plant species with elevated *ent*-kaurene synthesis (for secondary metabolites) or reduced *ent*-kaurene metabolism. Alternatively, the release of *ent*-kaurene into the headspace might be a common event among plant species under a specialized condition if elevated synthesis or reduced metabolism of *ent*-kaurene (e.g. by up-regulation of CPS or down-regulation of KO) occurs in response to an unidentified signal. It is interesting to note that the block of *ent*-kaurene oxidation (by the *ga3-1* mutation) resulted in the release of *ent*-kaurene into the headspace. Previous work has illustrated that expression of the *AtCPS* gene is restricted to specific cell types, such as shoot apices and the vascular tissues (Silverstone et al. 1997). It has also been shown that AtCPS, but not AtKS, limits *ent*-kaurene synthesis in *Arabidopsis* (Fleet et al. 2003). In addition, the *AtCPS* gene does not appear to be regulated by the feedback inhibition mechanism via GA activity, unlike later GA biosynthesis genes (Silverstone et al. 1997). Therefore, it is reasonable to speculate that the status of *ent*-kaurene synthesis is not substantially affected in the *ga3-1* mutant. These observations suggest that *ent*-kaurene released into the headspace by the *ga3-1* mutant originates from the plastids of limited cell types expressing the *AtCPS* gene. Our previous work has indicated that the *AtCPS* and *AtKO* genes are expressed mainly in distinct cell layers in germinating *Arabidopsis* embryos, implying a necessity of the intermediate *ent*-kaurene to move across different cell types (Yamaguchi et al. 2001). Our current results support the idea that *ent*-kaurene can move at least locally in *Arabidopsis*, as release into the air necessitates movement from the predicted sites of its synthesis to peripheral tissue layers.

The *ent*-kaurene-deficient *ga1-3* and *ga2-1* mutants responded drastically to exogenous *ent*-kaurene in the headspace (Fig. 3D). However, wild-type *Arabidopsis* plants did not change their morphology in response to headspace *ent*-kaurene (data not shown). In transgenic *Arabidopsis* lines overexpressing *AtCPS*, *ent*-kaurene accumulated at extremely high levels without treatment with a KO inhibitor (up to 1,000-fold relative to wild type), while the content of active GAs remained unchanged (Fleet et al. 2003). In the present study, the 35S-TPGK transgenic plants did not exhibit an obvious GA-overdose phenotype (Fig. 2B), suggesting that active GA levels did not increase significantly in these transgenic lines. These results indicate that exogenous application of *ent*-kaurene on its own would not greatly modulate plant growth and development, due to the ability of later steps in the GA biosynthesis pathway to maintain homeostasis of bioactive GA levels. Thus, to evaluate the potential role of volatile *ent*-kaurene in controlling plant growth as a possible mechanism of interplant communication, further studies will be necessary to investigate whether the amount of *ent*-kaurene is limiting the level of bioactive GAs in any other environmental condition or in different plant species. Our results also suggest that the release of *ent*-kaurene into the headspace might contribute to reducing the flux through the GA biosynthesis pathway in the *AtCPS* overexpressor (Fleet et al. 2003), assisting the establishment of homeostasis of bioactive GA levels.

The *ga3-1* mutation is likely to be null (Helliwell et al. 1998), and the mutant did not respond to exogenous *ent*-kaurene (Fig. 3D). However, the *ga3-1* dwarf phenotype was slightly recovered when co-cultivated with 35S-TPGK transgenic lines (Fig. 3B) or a strong *AtCPS* overexpressor (data not shown). This observation suggests that *ent*-kaurenoic acid and/or later GA intermediate(s) might be responsible for the slight recovery of the *ga3-1* dwarf phenotype in the co-cultivation experiment. However, such compounds are unlikely to be volatile. In the *AtCPS* overexpressor, *ent*-kaurenoic acid, the final product of KO, also accumulated at a high level (up to ~1,000-fold relative to wild type) in plants (Fleet et al. 2003). Helliwell et al. (1998) reported that the *ga3-1* mutant responded slightly to exogenous *ent*-kaurenol. We therefore speculate that, if a small fraction of *ent*-kaurenal (which appears to be more

volatile than *ent*-kaurenol) was present in the headspace of *ent*-kaurene overproducers, it might be oxidized to form *ent*-kaurenoic acid by an unknown mechanism.

ent-Kaurene-deficient mutants and chemical inhibitors of KO (such as uniconazole and paclobutrazol) have been widely used to study GA physiology in a variety of plant species. Our findings illustrate that care should be taken in experiments using these mutants and inhibitors. For example, our results predict that induction of dwarfism by uniconazole treatment will result in the release of an appreciable amount of *ent*-kaurene into the headspace in many plant species, which would affect the growth of any neighbouring plants in which *ent*-kaurene limits the content of bioactive GAs

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Landsberg *erecta* (L*er*) was used as a wild-type plant in this study. The *ga1-3* seeds were originally obtained from M. Koornneef (Wageningen University, Wageningen, The Netherlands). The *ga2-1* and *ga3-1* seeds were obtained from the *Arabidopsis* Biological Resource Center (Ohio State University, Columbus, OH, U.S.A.). To assist germination, GA-deficient *ga1-3*, *ga2-1* and *ga3-1* mutant seeds were imbibed in a 50 μM GA_4 solution at 4°C for 2 d, washed thoroughly with water and then placed on MS agar medium or on soil. All plants were grown under continuous white light at 22°C in a 300 liter growth chamber (Biotron LH-300-RDSS; NK systems, Osaka, Japan). To rescue flower development and seed set, GA-deficient mutants were sprayed with 2 μM GA_4 solution as required. For co-cultivation experiments, a small Petri dish (3.5 cm) containing an MS agar medium was placed on MS medium in a 9 cm Petri dish, so that two sets of plants share the headspace, but not the media. The 9 cm Petri dish was sealed with Micropore surgical tape (3M Health Care, St. Paul, MO, U.S.A.) during incubation. To feed *ent*-kaurene as a volatile, authentic *ent*-kaurene was dissolved in *n*-hexane, and the solution was spread onto a sterilized 3MM filter paper (Whatman, Maidstone, U.K.). The filter paper was then placed in an empty 3.5 cm Petri dish (to avoid direct contact with the agar medium and plants), and co-incubated with test plants. *ent*-Kaurene standard was purified from Japanese cedar leaves (Yasue et al. 1976).

Plasmid construct

Double-stranded cDNA from 1-month-old *Arabidopsis* plants was used as a template to amplify a cDNA fragment encoding the putative transit peptide of AtCPS by PCR with a forward primer 5′-GGATCCATGTCTCTTCAGTATCAT-3′ and a reverse primer 5′-ACTAGTTATCGTAATTTCCCCGTC-3′ (the forward and reverse primers contain *Bam*HI and *Spe*I sites, respectively). The amplified cDNA fragment was cloned into pCRII vector (Invitrogen, Carlsbad, CA, U.S.A.), and the resulting plasmid was named pTP. Double-stranded cDNA from *G. fujikuroi* mycelia (Toyomasu et al. 2000) was used as a template to amplify the coding region of *GfCPS/KS* by PCR with a forward primer 5′-ACTAGTATGCCTGGCAAAATC-3′ (containing a *Spe*I site) and a reverse primer 5′-CCCGGGTCATCACTTCATGCTGCTTGA-3′ (containing an *Sma*I site). The amplified cDNA fragment was cloned into pCRII vector (pGfCPS/KS). Using the restriction enzyme sites introduced by PCR, a chimeric cDNA fragment encoding TP-GfCPS/KS was cloned into pBluescript SK- vector (pTP-GfCPS/KS; Stratagene, La Jolla, CA, U.S.A.). To generate a chimeric construct containing the cauliflower mosaic virus 35S promoter fused to TP-GfCPS/KS-GFP in the binary vector pBIC121 (Okada et al. 2000), new restriction enzyme sites (*Xba*I and *Bam*HI at 5′ and 3′ ends, respectively) were introduced into the ligated cDNA fragment (encoding TP-GfCPS/KS) by PCR using a forward primer 5′-TCTAGAATGTCTCTTCAGTATCATGTT-3′ and a reverse primer 5′-GGATCCCTTCATGCTGCTTGAAAGGTC-3′. The PCR product was cloned into pCRII vector (pTP-GfCPS/KS2). There is an *Xba*I site in the TP-GfCPS/KS cDNA fragment. To excise the full-length TP-GfCPS/KS as a 5′-*Xba*I–*Bam*HI-3′ DNA fragment, pTP-GfCPS/KS2 was cut with *Bam*HI, and then partially digested with *Xba*I. The resulting cDNA fragment was cloned into the *Xba*I and *Bam*HI sites of pBIC121 to generate pBIC-TPGK (Fig. 2A).

Plant transformation

The plasmid construct was transferred into *Agrobacterium tumefaciens* (GV3101) by electroporation. Transformation of the *ga1-3* and *ga2-1* mutant plants was carried out using the floral dip method (Clough and Bent 1998). Transformants were selected on MS agar medium containing 50 μg μl^{-1} Kan. To examine whether Kan resistance segregates at approximately 3 : 1 in the T_2 generation, we used 40–100 plants for each line. The approximate 3 : 1 segregation for Kan resistance of selected lines was confirmed in the T_3 generation using ~200 individuals from T_2 parents heterozygous for the transgene.

QRT–PCR

QRT–PCR was carried out according to Ogawa et al. (2003) using *GFP*-specific primers (5′-CACAACGTCTATATCATGGCCG-3′ and 5′-ATGTTGTGGCGGATCTTGAAG-3′) and a Taqman probe (5′-CAAGCAGAAGAACGGCATCAAGGTGA-3′). The mean value of triplicate analysis was normalized using an 18S rRNA as the internal standard control.

Western blot analysis

Total proteins were extracted according to Silverstone et al. (2001) from 2-week-old rosette plants grown on agar media containing 1 mM GA_4. Samples were separated by 10% SDS–PAGE and analyzed on immunoblots (Sambrook et al. 1989) using a 1,000-fold dilution of anti-GFP polyclonal antibodies from rabbit (MBL, Nagoya, Japan) and a 5,000-fold dilution of peroxidase-conjugated goat anti-rabbit IgG (Pierce, Rockford, IL, U.S.A.). The signals were detected by enhanced chemiluminescence using the Super-signal west pico kit (Pierce).

Observation of GFP fluorescence

To observe GFP and chlorophyll fluorescence, we used a Leica TCS-NT confocal laser scanning microscope (Leica, Mannheim, Germany). Excitation and emission wavelengths for GFP were 488 nm and 500–520 nm, respectively. Chlorophyll auto-fluorescence was detected using an excitation wavelength of 568 nm and an emission wavelength of 580–630 nm.

Analysis of endogenous ent-kaurene and headspace volatiles

Quantitative analysis of endogenous *ent*-kaurene was conducted as reported previously using deuterium-labeled *ent*-kaurene as an internal standard (Kasahara et al. 2002). To analyze *ent*-kaurene in the headspace of agar-grown plants, the SPME method was employed using a 100 μm polydimethylsiloxane (PDMS)-coated silica fiber (Sigma Aldrich Supellco Co., St. Louis, MO, U.S.A.). The PDMS fiber was exposed to the headspace of test plants and to that of a filter paper containing 1 mg *ent*-kaurene (as a standard) for 3 min and 40 s, respectively, at room temperature. Volatiles absorbed on the PDMS fiber were analyzed on full-scan GC-MS. To examine the occurrence of *ent*-kaurene in the headspace of soil-grown plants, the plant pot was

enclosed in a glass jar equipped with inlet and outlet. A vacuum pump was used to draw air through the glass jar at approximately 6 liter min^{-1}. The volatiles released from the plants were collected at the outlet using an ODS cartridge (Sep-Pak Plus PS-Air; Waters Co., Milford, MA, U.S.A.). After incubation, compounds trapped on the ODS cartridge were eluted with methanol, and then analyzed on GC-MS.

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