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RAPID COMMUNICATION

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Antisense and chemical suppression of the nonmevalonate pathway affects *ent*-kaurene biosynthesis in *Arabidopsis*

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Abstract Transgenic plants of *Arabidopsis thaliana* (L.) Heynh. (ecotype Columbia) expressing the antisense *AtMECT* gene, encoding 2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase, were generated to elucidate the physiological role of the nonmevalonate pathway for production of *ent*-kaurene, the latter being the plastidic precursor of gibberellins. In transformed plants pigmentation and accumulation of *ent*-kaurene were reduced compared to wild-type plants. Fosmidomycin, an inhibitor of 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), caused a similar depletion of these compounds in transgenic plants. These observations suggest that both AtMECT and DXR are important in the synthesis of isopentenyl diphosphate and dimethylallyl diphosphate and that *ent*-kaurene is mainly produced through the nonmevalonate pathway in the plastid.

Keywords *Arabidopsis* · *ent*-Kaurene · Fosmidomycin · Gibberellin · Isoprenoids · Nonmevalonate pathway

Abbreviations DMAPP: dimethylallyl diphosphate · DXP: 1-deoxy-D-xylulose 5-phosphate · DXR: DXP reductoisomerase · GA: gibberellin · IPP: isopentenyl diphosphate · MECT: 2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase · MEP: 2-*C*-methyl-D-erythritol 4-phosphate · MS: Murashige and Skoog medium · MVA: mevalonate · WT: wild type

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Introduction

Isoprenoids are an important group of compounds that play a critical role in plant growth and development, and are also involved in the way plants adapt to changes towards environmental stimuli (Gray 1987). Many of the isoprenoids are derived from farnesyl diphosphate (FPP) or geranylgeranyl diphosphate (GGPP) through condensation and cyclization reactions, with these intermediates having common precursors, such as isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP; Cunillera et al. 1997; Okada et al. 2000). Gibberellins (GAs) are diterpene plant hormones that are also derived from GGPP. To initiate GA biosynthesis, GGPP is converted to *ent*-kaurene by two terpene cyclases, GA1 (copalyl diphosphate synthase) and GA2 (*ent*-kaurene synthase). Current evidence suggests that *ent*-kaurene is the plastidic precursor of GA biosynthesis, since these cyclases are localized in the plastids (Sun and Kamiya 1994; Yamaguchi et al. 1998). The GA biosynthetic pathway after *ent*-kaurene production is well documented (Yamaguchi and Kamiya 2000; Helliwell et al. 2001), but the stage before GGPP production remains ambiguous due to the complexity of the pathway in plants.

Plants can synthesize IPP via the mevalonate (MVA) and the nonMVA pathways; the latter was recently discovered in some eubacteria (Rohmer et al. 1993; Rohdich et al. 1999; Herz et al. 2000; Kuzuyama et al. 2000a, b, c; Luttgen et al. 2000; Takagi et al. 2000). Isotope-feeding experiments indicated that the nonMVA pathway is located in the plastid (Schwender et al. 1996; Arigoni et al. 1997; Lichtenthaler et al. 1997), and is involved in pigment biosynthesis in plants. Since the nonMVA pathway is responsible for IPP and DMAPP biosyntheses in the plastid, and *ent*-kaurene is derived from IPP and DMAPP, the regulation of this pathway may affect 'downstream' GA biosynthesis due to alterations in the levels of IPP and DMAPP.

Figure 1 shows the possible sequence of events in the nonMVA pathway. 1-Deoxy-D-xylulose 5-phosphate (DXP) reductoisomerase (DXR) and 2-*C*-methyl-D-erythritol 4-phosphate (MEP) cytidylyltransferase (MECT) catalyze the second and third steps in that DXP is converted to 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol (CDP-ME) via MEP. However, as DXP is a precursor of both thiamine and pyridoxol in *Escherichia coli* (Sprenger et al. 1997), DXR and MECT are considered to be more-specific enzymes for IPP biosynthesis in the nonMVA pathway. In this case, studying the steps downstream from DXP synthesis is essential to understanding the nonMVA pathway in plants.

Although several genes that are involved in the nonMVA pathway in plants have been cloned (Schwender et al. 1999; Estevez et al. 2000; Rohdich et al. 2000b), there are still steps that have not been elucidated in the IPP biosynthetic pathway. Moreover, the physiological role of this pathway in plant hormone biosynthesis has not been studied. Recently, the *MECT* gene homologue (*ispD*) from *Arabidopsis* was cloned and its function analyzed (Rohdich et al. 2000a). Here, we have studied the in vivo function of the *AtMECT* gene by generating a population of transformed *Arabidopsis* plants containing *AtMECT* in antisense orientation, and have compared these plants with *Arabidopsis* plants treated with fosmidomycin, a specific inhibitor of DXR.

Results and discussion

To obtain the *Arabidopsis MECT* gene, a possible cDNA sequence that has high similarity to *E. coli MECT* was amplified by reverse transcription (RT)-PCR using the oligonucleotide primers YacM-S (5′-GCATGCATGGCGATGCTTCAGACGAATCTT GG-3′) and YacM-A (5′-CTGCAGTCATGAGTCCTC GCTCAAGATTCTCTC-3′). The amplified fragment was cloned, sequenced, and used in subsequent experiments. The sequence of the cloned *AtMECT* gene (accession number AB037877) was identical to that of the *ispD* gene (Rohdich et al. 2000a, accession number AF230737). Northern blot analysis confirmed that the expression of *AtMECT* was elevated in aerial parts including leaves, stems and flowers, but not in the root (data not shown). The pattern of *AtMECT* expression was similar to that of *CLA1*, which is involved in chloroplast development, suggesting that *AtMECT* has an important role in isoprenoid biosynthesis in the plastid.

We also investigated the in vivo functions of *AtMECT* using the *AtMECT* antisense line of *Arabidopsis*. The construct carried the *AtMECT* cDNA in antisense orientation, driven by the CaMV 35S promoter on a pBI121 vector (Clontech) and was introduced into *Arabidopsis* by *Agrobacterium*-mediated infiltration (Bechtold et al. 1993). Thirty transgenic plants (T1 generation) were resistant to kanamycin selection. All transformed plants carried the anti-*AtMECT* gene and were deficient in pigment production

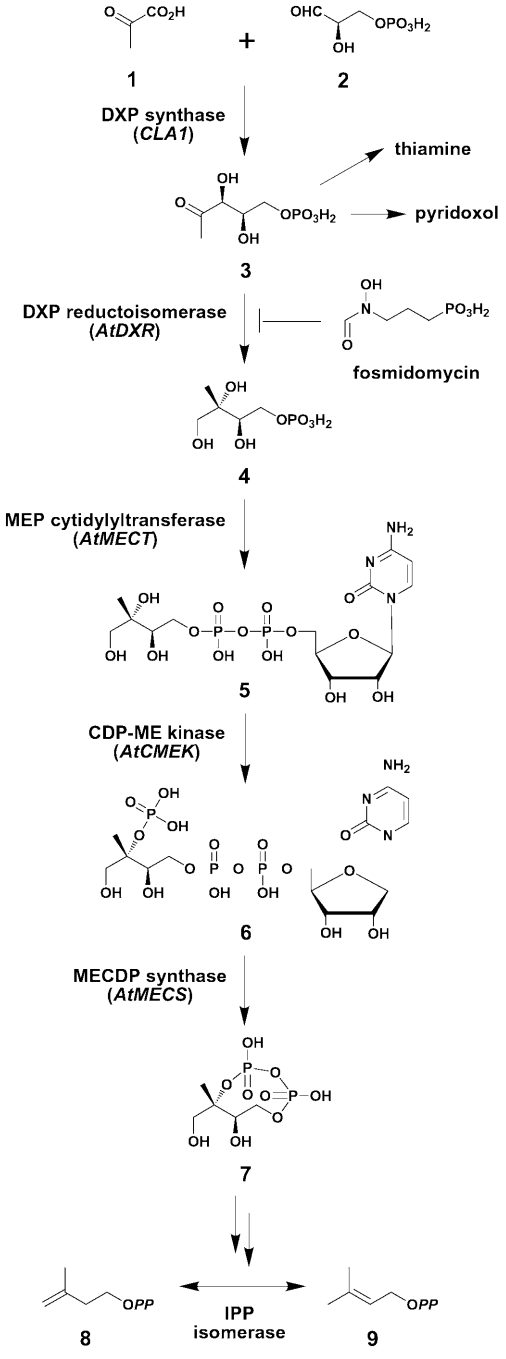

Fig. 1 Biosynthesis of IPP in the nonMVA pathway. *1* Pyruvate, *2* D-glyceraldehyde 3-phosphate, *3* 1-deoxy-D-xylulose 5-phosphate (*DXP*), *4* 2-*C*-methyl-D-erythritol 4-phosphate (*MEP*), *5* 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol (*CDP-ME*), *6* 2-phospho-4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol (*CDP-ME2P*), *7* 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (*MECDP*). The subsequent reaction leading to the formation of IPP (*8*) or DMAPP (*9*) from MECDP remains to be elucidated. Fosmidomycin specifically inhibits the production of MEP. The homologous genes cloned from *Arabidopsis thaliana* are shown in parentheses

compared with wild-type (WT) plants (Fig. 2A–C). About 30% of transformed T2 plants, showed an albino phenotype (lines 3-1, 3-2) and died shortly before anthesis. Plants that exhibited the albino phenotype were

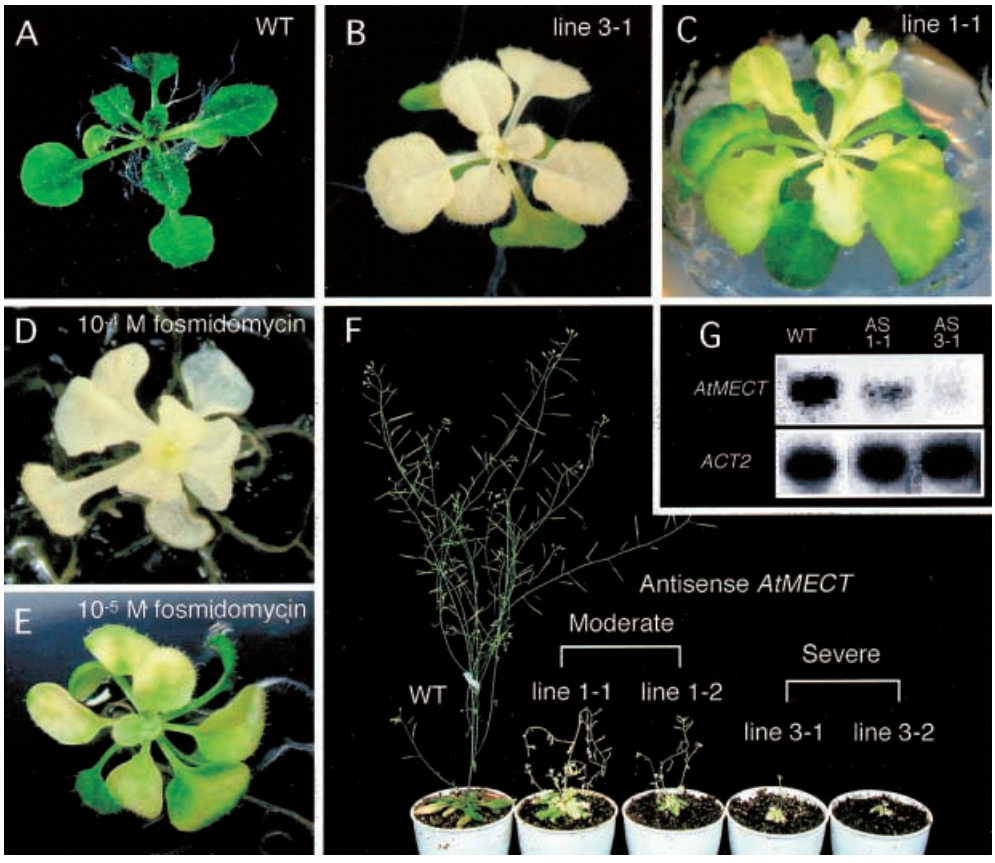

Fig. 2A–G Phenotypes of the antisense *AtMECT*-transformed plants. Plants of *Arabidopsis* ecotype Columbia were grown under continuous light at 22 °C on an MS-based medium (GIBCO-BRL) or on soil. Transformed plants were produced by vacuum infiltration using 28-day-old stock plants. Antisense transformed plants were selected on semi-solidified MS medium supplemented with 100 μg/ml kanamycin, such plants were transferred to soil to obtain seed. Homozygous plants were identified by a 100% segregation of kanamycin-resistant plants in the T3 generation. **A** 3-week-old WT plant. **B** 3-week-old antisense plant (severe line). **C** 3-week-old antisense plant (moderate line). **D** 3-week-old WT plant treated with 10^{-4} M fosmidomycin. **E** 3-week-old plant treated with 10^{-5} M fosmidomycin. **F** 5-week-old plants grown on soil. Four independent lines of the antisense plant were compared with a single WT plant. **G** Expression of the *AtMECT* transcript was examined by quantitative RT-PCR and expression levels were compared using the *ACT2* gene (positive control). Isolated total RNAs used for the RT-PCR were treated with DNaseI (Promega) to eliminate contamination from genomic DNA. First-strand cDNA was synthesized from 1 μg of total RNA using an oligo $d(T)_{12}$ primer by SuperscriptII reverse transcriptase (GIBCO-BRL) at 42 °C for 50 min. Subsequent PCR reactions were performed using the poly-T primed cDNAs as a template along with the primers for *AtMECT* (YacM-S, YacM-A) and *ACT2* (Act-S, 5′-CCTCATGCCATCCTCCGTCTTG-3′; Act-A, 5′-GCAAGAAT GGAACCACCGATC-3′). PCR products were separated on a 1.0% agarose gel. A Southern blot was performed using a radiolabeled probe of *AtMECT*, and then, stripped and re-probed with a radiolabeled *ACT2* cDNA (Sambrook et al. 1989). Results were visualized by a BAS2500 Imaging Analyzer. *WT* Wild-type plant, *AS1-1* moderate antisense plant, *AS3-1* severe antisense plant

collected from heterozygous T2 seeds and used for subsequent experiments as severe phenotypic lines (Fig. 2B, F right). Conversely, moderate lines that showed a mild pale-green phenotype (lines 1-1, 1-2) were more vigorous in growth than albino plants and produced mature seeds. Although the moderate lines were relatively small and looked wilted compared with WT plants, homozygous T3 populations were selected and used for further analysis (Fig. 2C, F left).

The suppression of *AtMECT* mRNA in the antisense plants was confirmed by RT-PCR, followed by Southern hybridization. The expected 900-bp cDNA fragment of *AtMECT* and the 613-bp control fragment of *ACT2*, encoding the actin protein (An et al. 1996), were detected in WT plants (Fig. 2G). Expression of *AtMECT* was poor in the antisense severe line (line 3-1) and slightly more elevated in the moderate line (line 1-1). The *ACT2* transcript was detected in both antisense lines and WT plants. These results indicate that the expression of *AtMECT* mRNA was severely suppressed at the transcript level by the antisense mRNA, and that suppression of *AtMECT* was linked to the phenotype of the antisense plant.

Fosmidomycin, a specific inhibitor of DXR, inhibits the production of MEP in bacteria (Kuzuyama et al. 1998) and reduces pigment production in plants (Zeidler et al. 1998), and was therefore used to confirm the phenotypic results obtained from the transgenic antisense plants. In culture, WT plants treated with 10^{-4} M fosmidomycin had an albino phenotype similar to the severe line expressing the antisense *AtMECT* gene fragment (Fig. 2D). Plants treated with 10^{-5} M fosmidomycin had a pale-green to pale-yellow phenotype,

similar to the moderate line of antisense *AtMECT*, and the growth rate was more vigorous than that of plants treated with 10^{-4} M fosmidomycin (Fig. 2E).

Chlorophylls (Chls) *a* and *b* and total carotenoids were quantified in fosmidomycin-treated WT and *AtMECT* antisense transformed plants (Table 1), according to the method of Lichtenthaler (1987). Antisense plants exhibiting the severe phenotype (line 3-1), showed a reduction in both total Chl (Chl $a+b$) and carotenoid contents to about 7% and 14%, respectively, of the WT level. The moderate antisense transformed plant (line 1-1) showed a reduction in Chl $a+b$ and carotenoids to 38% and 53% of the WT level, respectively. Fosmidomycin-treated WT plants also showed a reduction in these pigments, similar to the severe antisense lines.

Since the early steps of GA biosynthesis occur in the chloroplast (Sun and Kamiya 1994; Yamaguchi et al. 1998), it is possible that a decrease of IPP in the plastid could affect *ent*-kaurene biosynthesis together with pigment production. Endogenous *ent*-kaurene content is regulated at an extremely low level and cannot be detected in WT plants. In a previous study, treating *Arabidopsis* plants with uniconazole, a specific inhibitor of *ent*-kaurene oxidase, resulted in *ent*-kaurene being accumulated to a detectable level (Zeevaart and Gage 1993). Therefore, we measured the accumulation level of *ent*-kaurene in both WT and antisense transformed plants by GC-MS according to the methods of Großelindemann et al. (1991), but in this case, using uniconazole as an inhibitor of *ent*-kaurene oxidase. It was found that treatment with 10^{-4} M fosmidomycin effectively decreased the accumulation of *ent*-kaurene in both the WT and the antisense moderate line 1-1 (Table 2). In the antisense severe line 3-1, the accumulation of *ent*-kaurene was much lower than in line 1-1, indicating that the level of *ent*-kaurene accumulation was related to an increase in severity of the antisense effect of *AtMECT*. Thus, a decrease in plastidial IPP, which is caused by the suppression of the nonMVA pathway, also affected the endogenous level of *ent*-kaurene in the plastid. In this case, the suppression within the nonMVA pathway correlates well with the decreased level of *ent*-kaurene accumulation. This result also supports the idea that the early steps of GA biosynthesis begin in the plastid using IPP from the nonMVA pathway.

From these observations, we expect the GA content in the antisense plant to have decreased due to the reduction in the level of *ent*-kaurene. Our results show that the antisense plant exhibited an albino phenotype and both a slow growth rate and a reduction in plant height compared with WT plants. The application of GA to the antisense plant could not complement its small phenotype (data not shown), probably due to the reduction in photosynthetic activity of the plant. In addition, the application of uniconazole to the antisense plant produced a more severely dwarfed plant than the untreated anti-*AtMECT* transformed plants (data not shown). This result possibly indicates that the endogenous level of active GAs in the uniconazole-treated plants was lower than in the antisense suppressed plant. It has also been reported that the chloroplast membranes possess permeability to IPP during plastid development (Heintze et al. 1990), and that plastids isolated from cell suspensions of Muscat can take up IPP (Soler et al. 1993). Therefore, a very small quantity of IPP, possibly via the cytosolic MVA pathway, would be sufficient to produce the *ent*-kaurene used for biosynthesis of an appropriate level of active GAs. In this case, blocking of the nonMVA pathway is not sufficient for complete suppression of *ent*-kaurene biosynthesis.

In conclusion, we have demonstrated that suppression of the *AtMECT* gene and inhibition of DXR protein in the nonMVA pathway affect *ent*-kaurene biosynthesis and pigment biosynthesis in the chloroplast. Due to a limitation in the number of samples available for GC/MS analysis, we could not determine GA contents, and so our studies focused on the accumulation of *ent*-kaurene. Our results cannot exclude the possibility that incomplete chloroplast development caused by suppression of the nonMVA pathway may itself affect accumulation of *ent*-kaurene. However, as the antisense *AtMECT* plant can grow to some extent on the MS medium with sucrose, *ent*-kaurene is likely to be accumulated in developing plastids of the antisense plant whose level of *ent*-kaurene accumulation is less than that of WT plants. Further studies using isotopic precursors of the MVA and nonMVA pathways will be necessary to

Table 1 Pigment quantification in WT and antisense transformed plants of *Arabidopsis thaliana*. Pigments were extracted from leaves of 15-day-old plants, cultured on semi-solidified (0.8% agar) MS-based medium (Murashige and Skoog 1962), and quantified according to the method by Lichtenthaler (1987). Fosmidomycin was used at 10^{-4} M. Data are means ± SE of three experiments

Samples	Pigment (mg (g FW)$^{-1}$)			
	Chl *a*	Chl *b*	Chl $a+b$	Carotenoids
WT	578 ± 9	230 ± 16	807 ± 11	176 ± 6
WT/fosmidomycin	58 ± 4	29 ± 2	87 + 7	25 ± 1
Antisense line 1-1	219 ± 8	84 ± 3	303 ± 11	94 ± 2
Antisense line 3-1	39 ± 1	17 ± 1	55 ± 2	26 ± 1

Table 2 *ent*-Kaurene contents of WT and antisense transformed *Arabidopsis* plants grown in the presence or absence of fosmidomycin. Seeds were sown on semi-solidified MS-based medium. After 14 days, plants were transferred to MS-based medium supplemented with 10^{-7} M uniconazole. Following 6 days treatment with uniconazole, the plants were harvested (approx. 0.5 g FW) and frozen in liquid nitrogen prior to analysis. *ent*-Kaurene levels were measured according to Großelindemann et al. (1991), except uniconazole was used instead of paclobutrazol. Fosmidomycin was used at 10^{-4} M. Data are means ± SE of three experiments

Samples	*ent*-Kaurene (ng (g FW)$^{-1}$)
WT	510.5 ± 13.5
WT/fosmidomycin	204.6 ± 12.8
Antisense 1-1	468.3 ± 82.3
Antisense line 1-1/fosmidomycin	99.2 ± 9.8
Antisense line 3-1	118.3 ± 10.0

elucidate the contribution of the nonMVA pathway to the biosynthesis of plant hormones.

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