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LOVASTATIN INSENSITIVE 1, a Novel Pentatricopeptide Repeat Protein, is a Potential Regulatory Factor of Isoprenoid Biosynthesis in *Arabidopsis*

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Higher plants have two metabolic pathways for isoprenoid biosynthesis: the cytosolic mevalonate (MVA) pathway and the plastidal non-mevalonate (MEP) pathway. Despite the compartmentalization of these two pathways, metabolic flow occurs between them. However, little is known about the mechanisms that regulate the two pathways and the metabolic cross-talk. To identify such regulatory mechanisms, we isolated and characterized the *Arabidopsis* T-DNA insertion mutant *lovastatin insensitive 1* (*loi1*), which is resistant to lovastatin and clomazone, inhibitors of the MVA and MEP pathways, respectively. The accumulation of the major products of these pathways, i.e. sterols and chlorophyll, was less affected by lovastatin and clomazone, respectively, in *loi1* than in the wild type. Furthermore, the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) activity analysis showed higher activity of HMGR in *loi1-1* treated with lovastatin than that in the WT. We consider that the lovastatin-resistant phenotype of *loi1-1* was derived from this post-transcriptional up-regulation of HMGR. The *LOI1* gene encodes a novel pentatricopeptide repeat (PPR) protein. PPR proteins are thought to regulate the expression of genes encoded in organelle genomes by post-transcriptional regulation in mitochondria or plastids. Our results demonstrate that LOI1 is predicted to localize in mitochondria and has the ability to bind single-stranded nucleic acids. Our investigation revealed that the post-transcriptional regulation of mitochondrial RNA may be involved in isoprenoid biosynthesis in both the MVA and MEP pathways.

Keywords: *Arabidopsis* — 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) — Isoprenoid biosynthesis — Lovastatin — Mevalonate (MVA) pathway — Pentatricopeptide repeat (PPR).

Abbreviations: DIG, digoxigenin; DMAPP, dimethylallyl diphosphate; DTT, dithiothreitol; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; HMGS, HMG-CoA synthase; IPP, isopentenyl diphosphate; *loi*, *lovastatin insensitive*; MEP, 2-*C*-methyl-D-erythritol-4-phosphate; MVA, mevalonate; PBS, phosphate-buffered saline; PPR, pentatricopeptide repeat; RT–PCR, reverse transcription–PCR; ssRNA, single-stranded RNA; WT, wild type.

Introduction

Unlike most organisms, plants produce a wide variety of isoprenoids. They are derived from common C5 isoprene units: isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), produced via two pathways, the cytosolic mevalonate (MVA) pathway and the plastidal 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway. Labeling experiments have shown that sesquiterpenes, *cis*-cytokinins, triterpenes, sterols, brassinosteroids, dolichol and mitochondrial ubiquinone side chains mainly comprise isoprene units produced via the MVA pathway (Newman and Chappell 1999, Kasahara et al. 2004), whereas gibberellins, ABA, *trans*-cytokinins, carotenoids, chlorophyll side chains and plastoquinone side chains comprise mainly isoprene units produced via the MEP pathway (Lichtenthaler 1999, Kasahara et al. 2004) (Fig. 1). Despite the compartmentalization of these two pathways, metabolic flow between them has been reported in recent studies (Kasahara et al. 2002, Nagata et al. 2002, Hemmerlin et al. 2003). Therefore, the production of IPP and DMAPP is defined as the activity of the MVA pathway, the MEP pathway, and the metabolic flow between these pathways. However, we have little knowledge of the mechanisms that regulate these complex biosynthetic pathways or their metabolic cross-talk.

Lovastatin, also referred to as mevinolin, is a specific inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), which is thought to be a rate-limiting

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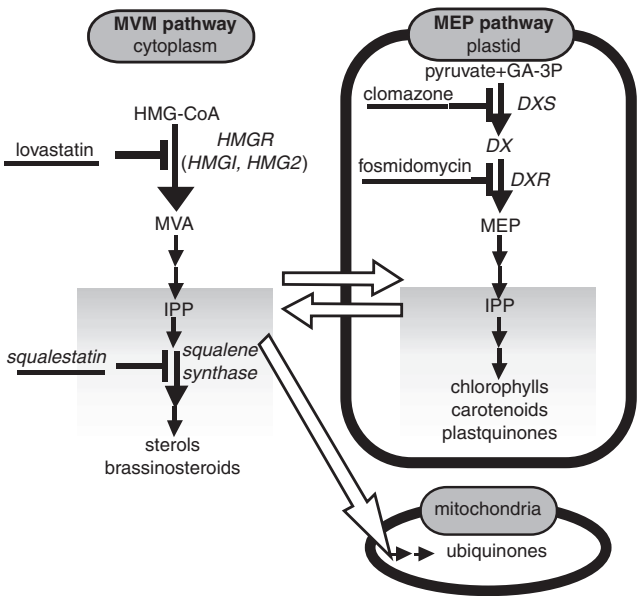

Fig. 1 Isoprenoid biosynthesis in the plant cell. The MEP pathway, localized to plastids, is shown in the box. Genes mentioned in the text are indicated in bold and italics; inhibitors are underlined. White arrows indicate metabolic flow between the cytosol and plastids, and from the cytosol to mitochondria. HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, DX reductase; IPP, isopentenyl diphosphate; MVA, mevalonate; MEP, 2-C-methyl-D-erythritol-4-phosphate.

enzyme of the MVA pathway (Schaller et al. 1995, Masferrer et al. 2002, Suzuki et al. 2004). Lovastatin treatment leads to a reduction in MVA pathway products, such as sterols, and the inhibition of growth (Bach and Lichtenthaler 1983). *rim1*, an allelic mutant of *phyB* that is resistant to mevinolin and fosmidomycin (an inhibitor of the MEP pathway), was screened based on the phenotype of true leaf development with mevinolin treatment (Rodríguez-Concepción et al. 2004). Rodríguez-Concepción et al. (2004) speculated that HMGR is up-regulated and leads to mevinolin resistance in *rim1* because light down-regulates HMGR (Learned 1996), and that enhanced intake from MVA-derived isoprenoid precursors to the plastid causes fosmidomycin resistance. The dramatic phenotype of HMGR inhibitor-treated seedlings or *hmg1* mutation is the severe inhibition of cell elongation. As a result, the root lengths of these seedlings are shortened markedly (Suzuki et al. 2004). We screened lovastatin-resistant mutants using root length as a screening marker to identify new regulatory mechanisms involved in plant isoprenoid biosynthesis.

Here, we report the isolation and characterization of *loi1* (*lovastatin insensitive 1*), which has longer roots than the wild type (WT) under lovastatin treatment. The *loi1* plants showed resistance not only to blockage of the MVA pathway, but also to blockage of the MEP pathway. Because *LOI1* encoded a novel pentatricopeptide repeat (PPR) protein, thought to be a regulator of organelle gene expression, and is localized in the mitochondria, we propose that the novel potential regulatory mechanism of plant isoprenoid biosynthesis is associated with the mitochondria.

Results

Isolation of lovastatin insensitive 1-1 (loi1-1)

We examined *Arabidopsis* development under various concentrations of lovastatin (a specific inhibitor of HMGR) to determine the most appropriate concentration for screening lovastatin-resistant mutants. Blocking the MVA pathway by treatment with mevinolin (lovastatin) results in the inhibition of root elongation, contraction of the cotyledons and arrested development of true leaves in *Arabidopsis* (Rodríguez-Concepción et al. 2004). We observed similar phenotypes in seedlings grown with 300 nM lovastatin. In particular, root elongation was markedly inhibited (data not shown). Therefore, we used root length as a marker of sensitivity to lovastatin at 300 nM.

We screened approximately 3000 of the RIKEN GSC activation tagging T-DNA lines (Nakazawa et al. 2003) for lovastatin-resistant mutants. After germination on MS plates containing 300 nM lovastatin for 14 d, seedlings that had longer roots than those of the WT were transferred to soil and allowed to develop and set seed. The individual that showed the least sensitivity to lovastatin was designated as *lovastatin insensitive 1-1* (*loi1-1*; Fig. 2A). The lovastatin sensitivity of F_1 plants generated by backcrossing *loi1-1* with the WT was comparable with that of the WT, suggesting that *loi1-1* was a recessive mutant.

Lower lovastatin sensitivity in the loi1-1 mutant than in the WT

Root elongation (defined as the root length of 2-week-old seedlings grown with various concentrations of the inhibitor relative to those grown without the inhibitor) in WT plants grown with 300 nM lovastatin was strongly reduced. In contrast, the root elongation of treated *loi1-1* plants was less affected (Figs. 2A, 3). Root elongation in *loi1-1* was reduced by concentrations of lovastatin >1 μM (Fig. 3).

Furthermore, we observed lovastatin-induced root growth arrest under a microscope. In the treated WT plants, lateral root growth was arrested, and tumor-like abnormal shapes appeared. In contrast, the roots of treated *loi1-1* seedlings showed normal development (Fig. 2B). We observed longitudinal sections of the roots of *loi1-1* and the WT. Although the root tip cell size and shape of the

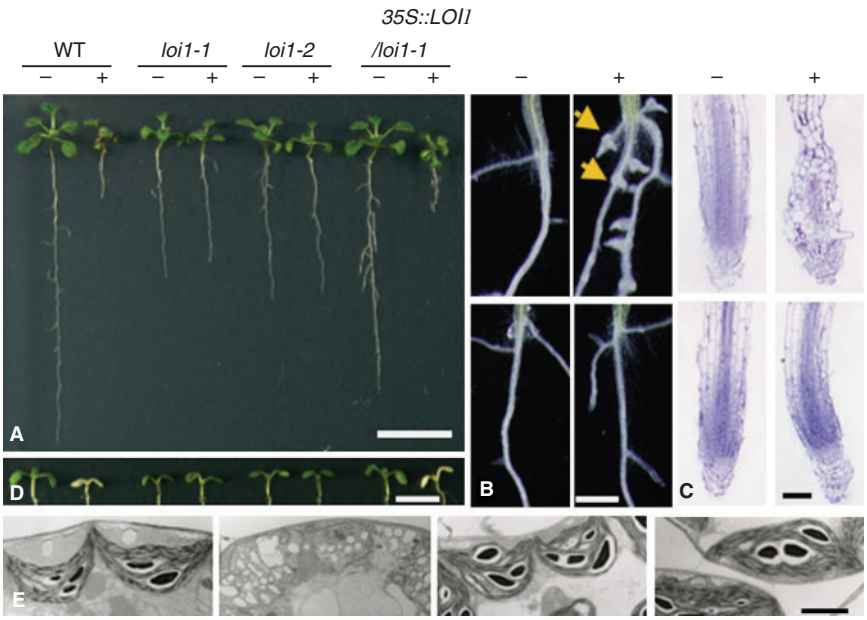

Fig. 2 Characteristic phenotypes of the *loi1* mutant. Seedlings grown on MS solid medium supplemented with or without inhibitor under long-day conditions. (A) Two-week-old seedlings of the wild type (WT), *loi1-1*, *loi1-2* and 35S::*LOI1* in *loi1-1* (left to right) grown with (+) or without (−) 300 nM lovastatin. Bar = 1 cm. (B) Roots of 2-week-old WT (top) and *loi1-1* (bottom) seedlings grown with (+) or without (−) 300 nM lovastatin. Arrows indicate the tumor-like abnormal shape. Bar = 1 mm. (C) Longitudinal sections of the root tip of 2-week-old WT (top) and *loi1-1* (bottom) seedlings grown with (+) or without (−) 300 nM lovastatin. Bar = 100 μM. (D) One-week-old WT, *loi1-1*, *loi1-2* and 35S::*LOI1* in *loi1-1* (left to right) seedlings grown with (+) or without (−) 300 nM clomazone. Bar = 5 mm. (E) Electron microscopy analysis of cotyledons of 1-week-old WT, WT with 300 nM clomazone, *loi1-1*, and *loi1-1* with 300 nM clomazone (left to right). Bar = 2.7 μm.

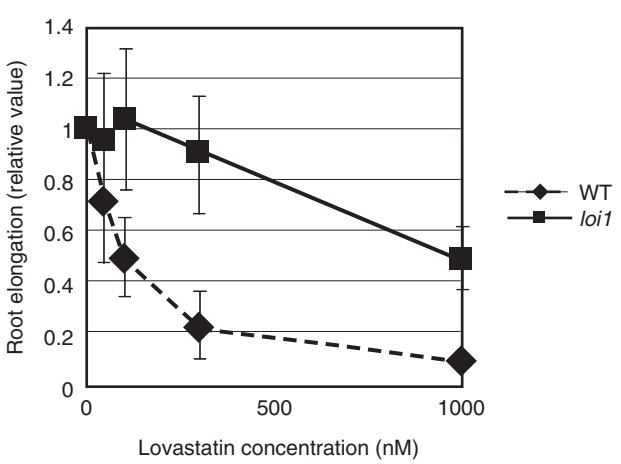

Fig. 3 Effect of lovastatin treatment on root elongation in the WT and *loi1-1*. Relative root elongation in WT (black rhombuses) and *loi1-1* (black squares) seedlings grown on MS solid medium supplemented with the indicated concentrations of lovastatin. Means and standard deviations were calculated from at least three independent experiments using approximately 20 individuals in each experiment.

treated WT were abnormal, the root tip of treated *loi1-1* did not show any abnormality (Fig. 2C). A tumor-like abnormal shape was also observed in the upper region of treated WT roots, but not in treated *loi1-1* roots (data not shown). We considered that the tumor-like shape may be the arrested growth of lateral roots.

The *loi1-1* mutant required treatment with higher concentrations of lovastatin than the WT for the other lovastatin-sensitive phenotypes to appear, i.e. contracted cotyledons and arrested development of true leaves (Fig. 2A). These results indicate that *loi1-1* was more resistant to lovastatin than the WT.

Lower sensitivity to lovastatin in loi1-1 than in the WT in terms of the total amount of sterols

Because sterol (major product of the MVA pathway)-defective mutants showed abnormalities in root elongation and formation, there may be a threshold level of sterols required for root elongation and formation (Diener et al. 2000, Carland et al. 2002, Schaller 2004, Suzuki et al. 2004). Thus, the effect of lovastatin treatment on the amount of sterols in *loi1-1* and the WT was examined. Total sterols were extracted from 2-week-old *loi1-1* and WT seedlings grown with various concentrations of lovastatin. Although the amount of sterols in the WT was markedly reduced by lovastatin, that in *loi1-1* was less affected (Fig. 4). There was no difference in sterol composition between *loi1-1* and the WT (Supplementary data).

Lovastatin resistance of loi1-1 seedlings can be explained by the post-transcriptional up-regulation of HMGR

Whereas the *loi1-1* mutant showed lower sensitivity to lovastatin than the WT, no significant differences in squalestatin (an inhibitor of squalene synthase) sensitivity were found between *loi1-1* and WT plants (data not shown). These results suggest that the metabolic flow upstream of squalene synthase may be up-regulated in *loi1-1*, at least in the presence of lovastatin. This up-regulation may result from either the increased activity of the MVA pathway itself or the up-regulation of the metabolic flow of prenyl diphosphates between the plastid and the cytosol. Because lovastatin is an HMGR inhibitor, an increase in HMGR expression could result in resistance to lovastatin (Re et al. 1995, Rodríguez-Concepción et al. 2004). We examined mRNA and activity of HMGR in WT and *loi1-1* plants grown with or without lovastatin. Reverse transcription–PCR (RT–PCR) analysis showed that the expression levels of *HMG1* and *HMG2* genes, which encode HMGR in *Arabidopsis*, were not affected in *loi1-1* plants (Fig. 5A). Although HMGR activity in *loi1-1* plants without lovastatin was comparable with that in the WT without lovastatin, the HMGR activity in *loi1-1* grown with lovastatin was clearly higher than that in the WT grown with lovastatin (Fig. 5B). The observed phenotype of lovastatin resistance may therefore be derived from the enhanced activation of HMGR.

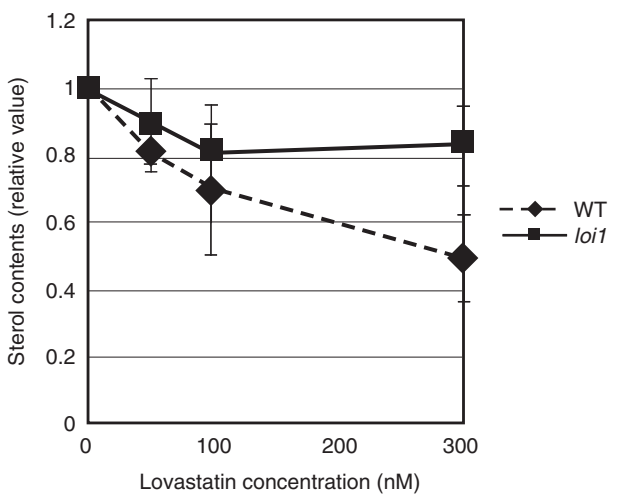

Fig. 4 Effect of lovastatin treatment on sterol contents. Relative sterol contents in WT (black rhombuses) and *loi1-1* (black squares) seedlings grown on MS solid medium supplemented with the indicated concentrations of lovastatin. Means and standard deviations were calculated from at least four independent experiments. The total amounts of sterols in the WT and *loi1-1* grown without lovastatin were 366 ± 131.6 μg 100 mg^{-1} DW and 290 ± 111.9 μg 100 mg^{-1} DW, respectively. Detailed sterol profiles are found in the Supplementary data.

Lower clomazone sensitivity in the loi1-1 mutant than in the WT

To investigate whether the metabolic flow of the MEP pathway was altered in *loi1-1* plants, as a first approach we fed clomazone to *loi1-1* plants. Clomazone is thought to be a specific inhibitor of 1-deoxy-D-xylulose 5-phosphate synthase (DXS) (Mueller et al. 2000), which is the first committed step of the MEP pathway (Dubey et al. 2003). Clomazone treatment leads to a reduction in the MEP pathway products such as chlorophyll side chains and carotenoids, and causes an albino phenotype (Zeidler et al. 2000). A 1-week-old WT seedling grown with 300 nM clomazone showed an albino phenotype and a deficiency of normal thylakoid stacking in the chloroplasts (Fig. 2D, E). In contrast, *loi1-1* was able to produce green leaves and normal chloroplasts in the presence of 300 nM clomazone (Fig. 2D, E). In fact, clomazone treatment resulted in a more marked decrease in total chlorophyll mass in the WT than in *loi1-1* (Fig. 6). These results suggest the potential activation of the MEP pathway in *loi1-1*, at least in the presence of clomazone. Because clomazone inhibits DXS, an increase in the DXS level could result in resistance to

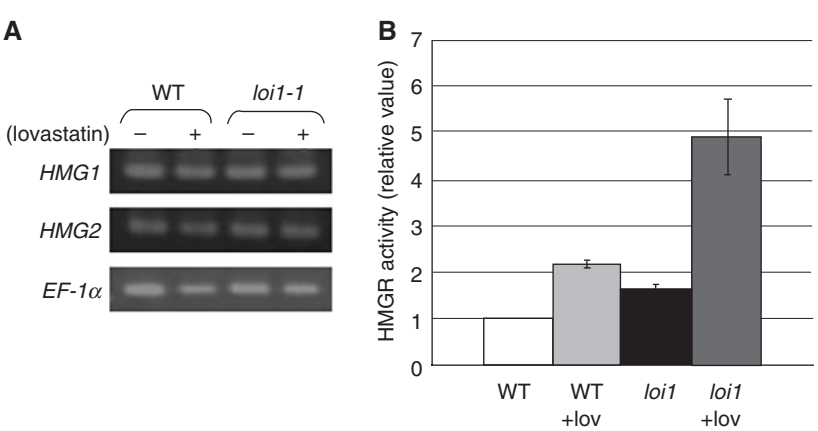

Fig. 5 Expression of HMGR. (A) RT–PCR analysis of *HMG1* and *HMG2* using cDNA made from total RNA extracted from 2-week-old seedlings grown on MS solid medium supplemented with (+) or without (–) 300 nM lovastatin. (B) Relative HMGR activity in the WT, WT grown with lovastatin, *loi1-1*, and *loi1-1* grown with lovastatin (left to right). Activity in the WT was given a value of 1. ER protein fractions were extracted from 12-day-old seedlings grown on MS solid medium supplemented with or without 300 nM lovastatin.

clomazone. We examined mRNA and protein levels of DXS in the WT and *loi1-1* treated or not with clomazone. RT–PCR analysis showed that the expression levels of *DXS1*, *DXS2* and *DXS3* genes, which encode DXS in *Arabidopsis*, were not increased in *loi1-1* (Fig. 7A). An immunoblot analysis with anti-DXS antibody showed nearly equal amounts of DXS in the WT and *loi1-1* treated with clomazone (Fig. 7B).

Identification of the LOI1 gene At4g14850

The *loi1-1* mutant was screened using T-DNA activation tagging lines (Nakazawa et al. 2003). The T-DNA marker (hygromycin resistance) and lovastatin resistance were co-segregated in the F_2 population.

We investigated the location of the T-DNA insertion in *loi1-1* using plasmid rescue. Two different genomic fragments were obtained as the left border flanking sequence of the T-DNA. Both of these were almost identical to the sequence of the first exon of the *At4g14850* gene, and the direction of these fragments was opposite. These results suggest that the T-DNA inverted repeat was inserted in the *At4g14850* gene (Fig. 8A).

We examined the phenotype of another allele, i.e. *loi1-2* (T-DNA insertion in the first exon of the *At4g14850* gene,

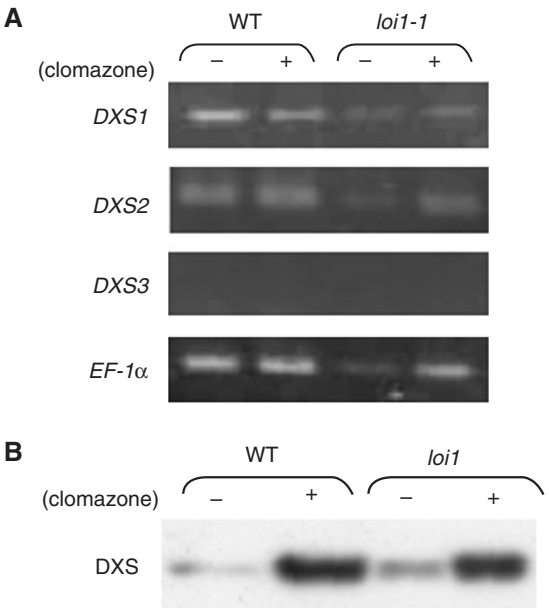

Fig. 7 Expression of DXS. (A) RT–PCR analysis of *DXS1*, *DXS2* and *DXS3* using cDNA made from total RNA extracted from 1-week-old seedlings grown on MS solid medium supplemented with (+) or without (–) 300 nM clomazone. (B) Immunoblotting analysis of DXS using total protein fractions extracted from 1-week-old seedlings grown on MS solid medium supplemented with (+) or without (–) 300 nM clomazone.

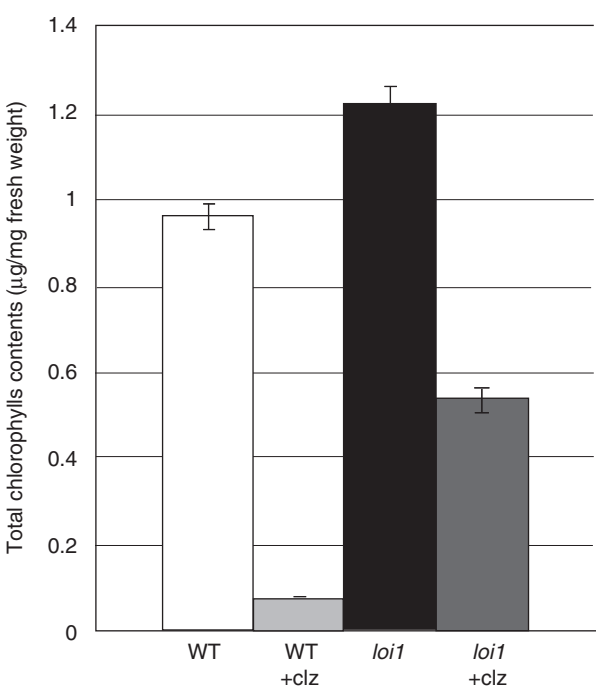

Fig. 6 Effect of clomazone treatment on chlorophyll content. Chlorophyll content of the WT, WT grown with clomazone, *loi1-1*, and *loi1-1* grown with clomazone (left to right). Chlorophyll was extracted from 1-week-old seedlings grown on MS solid medium supplemented with or without 300 nM clomazone under long-day conditions.

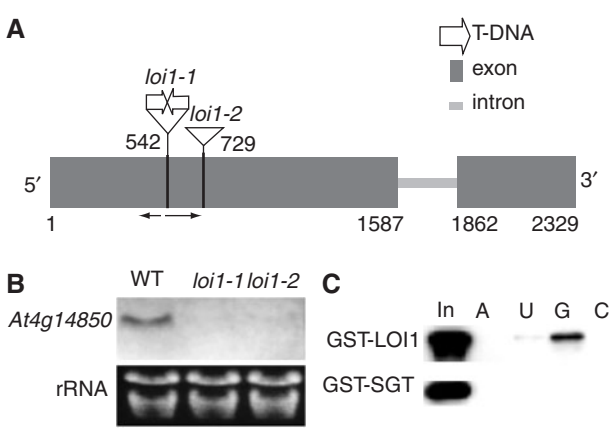

Fig. 8 Structure and expression of the *LOI1* gene and RNA binding ability of LOI1 protein. (A) Structure of the *LOI1* gene. The inserted T-DNA for each allele is flagged. Numbers are assigned to the initiation codon. (B) RNA gel blot analysis of the *At4g14850* gene; rRNA stained with ethidium bromide is shown as a loading control. (C) RNA binding assay of GST–LOI1, and GST–StSGT as the negative control. GST–LOI1 expressed in *E. coli* and purified protein were incubated with the indicated Sepharose- or agarose-bound RNA homopolymers: poly(A), poly(U), poly(G) and poly(C). Bound proteins were resolved by SDS–PAGE and analyzed by immunoblotting with anti-GST antibody. The lane total represents approximately 10% of the input material used in each binding reaction.

purchased from the SALK Institute, La Jolla, CA, USA), grown with 300 nM lovastatin or 300 nM clomazone. The sensitivity of *loi1-2* to these inhibitors was comparable with that of *loi1-1* (Fig. 2A, D).

To investigate *At4g14850* gene expression, total RNA was prepared from seedlings of the WT, *loi1-1* and *loi1-2*, and RNA gel blot analysis was performed. The *At4g14850* transcript was detected in the WT, but not in *loi1-1* or *loi1-2* (Fig. 8B). F_1 plants generated from crossing *loi1-1* and *loi1-2* showed the *loi1* phenotype (data not shown). Furthermore, we performed a complementation test of the *At4g14850* gene. The lovastatin and clomazone sensitivity of *loi1-1* seedlings that overexpressed *At4g14850* cDNA was comparable with that of the WT, indicating that the *At4g14850* gene can complement the *loi1* phenotype (Fig. 2A, D). These results demonstrated that the *LOI1* gene was *At4g14850*. Organ-specific LOI1 gene expression analysis by RT–PCR demonstrated that the *LOI1* gene was expressed ubiquitously (data not shown).

The LOI1 gene encodes a putative protein that has a pentatricopeptide repeat motif

LOI1 encodes a putative protein with 684 amino acids. LOI1 was predicted to be a member of the PPR family. Some PPR proteins are predicted to be targeted to mitochondria or chloroplasts and probably to bind to organelle RNA, taking part in the regulation of organelle gene expression (Small and Peeters 2000).

We investigated whether the LOI1 protein has the ability to bind single-stranded RNA (ssRNA). Glutathione *S*-transferase (GST)-fused LOI1 protein expressed in *Escherichia coli* was extracted and purified. First, we confirmed the binding ability of purified recombinant GST–LOI1 to ssRNA using North-Western analysis (Suzuki et al. 2000). Next, we examined the preferred sequence of LOI1 in a binding assay using RNA homopolymers. Purified recombinant GST–LOI1 and GST were incubated with agarose- or Sepharose-bound RNA homopolymers. After washing with binding buffer, bound proteins were resolved by SDS–PAGE and analyzed by immunoblotting with anti-GST antibody. The LOI1 protein bound preferentially to poly(G) homoribopolymers (Fig. 8C), suggesting that the target of LOI1 may be a G-rich sequence.

To examine the cellular localization of LOI1, we performed a transient expression assay in onion epidermal cells using the LOI1 protein fused to green fluorescent protein (GFP) recombinant protein driven by the 35S promoter. Because GFP fluorescence was rarely detected when the full length of LOI1 was used, we tried using an N-terminal LOI1 fragment (Met1– Pro39) and detected fluorescence (Fig. 9). This demonstrated that the GFP fusion proteins are targeted to the mitochondria, according to the co-localization of GFP images with those stained with mitochondrion-specific dye (MitoTracker Orange) in the same cells (Fig. 9). This indicates that LOI1 may localize to the mitochondria.

Discussion

loi1 is a novel mutant showing resistance to the blockage of the MVA and MEP pathway

Prenyl diphosphate is a component unit of a variety of isoprenoids. Because plants have two prenyl diphosphate biosynthetic pathways, the MVA and MEP pathways, the original regulatory mechanism of prenyl diphosphate biosynthesis may occur in plants. To identify regulators of the MVA pathway, *Arabidopsis* activation tagging lines were screened for mutants that displayed lower sensitivity to lovastatin compared with the WT. We succeeded in

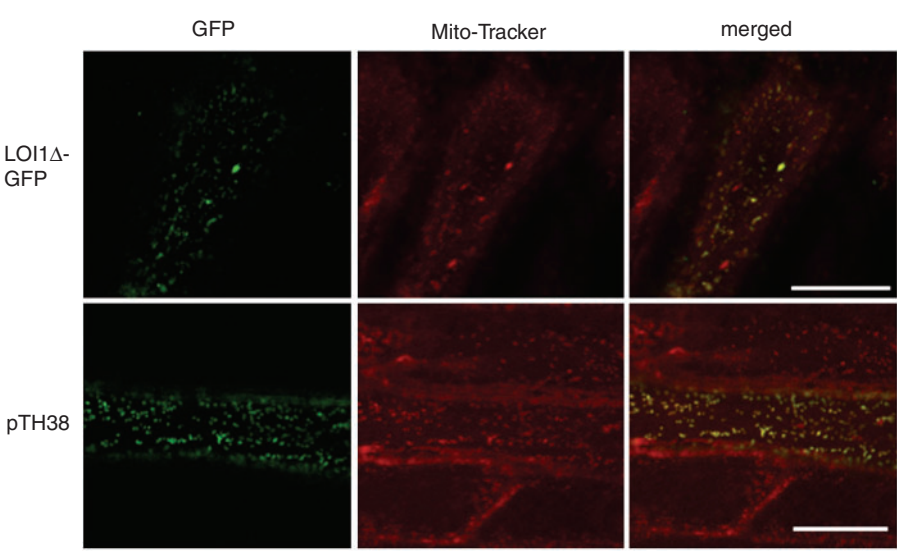

Fig. 9 Localization of LOI1. LOI1Δ–GFP, N-terminus of LOI1 fused to GFP (top); *pTH38*, mitochondrial transit signal fused to GFP (bottom). LOI1Δ–GFP and pTH38 were transiently expressed in epidermal cells of onion transformed by particle bombardment. Green fluorescence shows GFP, red fluorescence shows mitochondria stained by MitoTracker Orange, yellow fluorescence shows images with the two types of fluorescence merged (left to right). Bar = 50 μm.

isolating one mutant, *loi1-1*, that required higher concentrations of lovastatin than the WT for inhibition of root elongation and formation, and arrested development of true leaves (Fig. 2A, B). The cell size, shape and pattern in WT roots treated with lovastatin were similar to those in roots of mutants such as *smt1* and *hyd1*, which are defective in sterol production, the major MVA-derived products of biosynthesis (Souter et al. 2002, Willemsen et al. 2003). In contrast, the cell size, shape and pattern in *loi1-1* roots treated with lovastatin were normal (Fig. 2C). In addition, *loi1-1* showed lower sensitivity to lovastatin than did the WT in its sterol contents (Fig. 4). Furthermore, the HMGR activity analysis showed higher activity of HMGR in *loi1-1* treated with lovastatin than in the WT (Fig. 5B). We consider that the lovastatin-resistant phenotype of *loi1-1* was derived from this post-transcriptional up-regulation of HMGR.

Recently, Rodríguez-Concepción and co-workers reported resistant mutants against inhibitors for the MVA/MEP pathway, i.e. *rim1* (Rodríguez-Concepción et al. 2004) and *rif10* (Sauret-Güeto et al. 2006). The *rim1* mutant is resistant to mevinolin (i.e. lovastatin) and fosmidomycin [an inhibitor of 1-deoxy-D-xylulose 5-phosphate reductase (DX reductase); moreover, it is allelic to *phyB*, and its resistance is caused by the transcriptional and post-transcriptional up-regulation of HMGR (Rodríguez-Concepción et al. 2004). On the other hand, expression of the *HMG1* and *HMG2* genes was not affected in *loi1-1* (Fig. 5A). Thus, the mechanism of lovastatin resistance in *loi1* differs from that of mevinolin resistance in *rim1*.

The *loi1-1* seedlings were also resistant to clomazone. While this suggests potential activation of the MEP pathway in *loi1-1* in the presence of clomazone (Fig. 2D), immunoblot analysis showed the nearly equal accumulation of DXS in the WT and *loi1-1* grown with or without clomazone (Fig. 7B). Although *rim1* was reported to be fosmidomycin resistant, the transcriptional and post-transcriptional up-regulation of DX reductase was not observed in this mutant (Rodríguez-Concepción et al. 2004). Enhanced import of MVA-derived prenyl diphosphates into the plastid was proposed to account for the fosmidomycin-resistant phenotype of *rim1* (Rodríguez-Concepción et al. 2004). However, because *loi1-1 cla1-1* shows an albino phenotype (data not shown), the clomazone-resistant phenotype of *loi1* may not be caused by metabolic flow from the MVA pathway to the MEP pathway. Therefore, the mechanism of clomazone resistance in *loi1* also differs from that of fosmidomycin resistance in *rim1*.

The *rif10* mutant is fosmidomycin resistant. RIF10 is a polyribonucleotide phosphorylase localized in plastids. It has been demonstrated that plastid RNA processing is involved in plastid isoprenoid biosynthesis (Sauret-Güeto et al. 2006). *loi1* did not show fosmidomycin resistance (unpublished data), indicating that *loi1* shows clomazone resistance by a mechanism that differs from that of fosmidomycin resistance in *rif10*.

LOI1 encodes a novel PPR protein that may be localized in mitochondria

Many genes (~450) that are characterized by the presence of tandem arrays of a PPR repeat have been discovered by systematic screening of predicted *Arabidopsis* proteins (Aubourg et al. 2000, Small and Peeters 2000, Lurin et al. 2004). Some plastid PPR-defective mutants have been isolated and analyzed, including *crp1*, which is involved in the processing and translation of the *pet* gene in maize (Fisk et al. 1999), as well as *hcf152* (Meierhoff et al. 2003, Nakamura et al. 2003) and *crr4* (Kotera et al. 2005) in *Arabidopsis*. Some mitochondrial PPR genes have been isolated and characterized, such as the nuclear gene that restores cytoplasmic male sterility mutants, which was associated with aberrant mitochondrial RNA (Brown et al. 2003, Komori et al. 2004). These studies indicated that PPR proteins take part in the post-transcriptional regulation of organelle gene expression. Most PPR proteins have been identified in eukaryotic genomes. In particular, *Arabidopsis* and rice have several hundred PPR genes in their genomes, whereas many fewer PPR genes have been identified from genomes of species other than higher plants (Lurin et al. 2004). LOI1 is expected to be involved in plant-specific regulation of isoprenoid biosynthesis in organelles. The LOI1 protein has the ability to bind ssRNA, especially G-rich sequences (Fig. 8C), consistent with previous findings for PPR (Lurin et al. 2004). LOI1 may be localized in mitochondria (Fig. 9). Higher plant mitochondrial genomes mostly comprise genes encoding subunits of respiratory chain complexes, rRNA and tRNA (Giegé and Brennicke 1999, Handa 2003). If the expression of these genes is impaired, it is likely that respiratory electron transport is affected in *loi1*. Respiratory electron transport in mitochondria is thought to play an important role in protecting chloroplasts against photo-inhibition and in energy metabolism in plant cells (Raghavendra and Padmasree 2003). Given that the side chains of ubiquinone, which act as electron carriers in the mitochondrial respiratory chain, are derived from cytosolic IPP, the up-regulation of the MVA or MEP pathway might arise from any abnormality in these functions of respiratory electron transport in *loi1*.

The biological significance of these findings remains to be elucidated in future experiments, possibly through the identification of physiological RNA targets.

Although the MVA and MEP pathways are considered to be highly regulated, there is little knowledge of the regulatory mechanisms. Here, we reported a novel potential regulatory locus using analyses of the mutant *loi1*,

which shows resistance to inhibitors of the MVA and MEP pathways. Abnormalities in the post-transcriptional regulation of mitochondrial RNA may affect the isoprenoid biosynthetic pathway in *loi1*, probably via the regulation of HMGR activity. Identification of the target RNA of LOI1 should lead to a greater understanding of the plant-specific regulatory mechanisms of isoprenoid biosynthetic pathways.

Materials and Methods

Plant growth conditions

The *loi1* mutant originated from the RIKEN GSC activation tagged lines (Nakazawa et al. 2003) in the Col-0 background. Seeds were surface sterilized and germinated on 1× MS (Invitrogen, Carlsbad, CA, USA) plates containing 3% sucrose and 1.2% agar (Ina Food Industry, Nagano, Japan). After stratification for at least 2 d at 4°C, plates were incubated at 23°C under long-day conditions (16 h under fluorescent white light, 8 h in the dark). Lovastatin (Calbiochem, Darmstadt, Germany), clomazone and squalestatin were dissolved in dimethylsulfoxide and diluted to different final concentrations in the growth medium.

Analysis of the inhibitor-induced phenotypes

Seedlings grown on solid MS medium supplemented with lovastatin for 2 weeks were photographed and the root length was measured. Longitudinal sections of the roots of 2-week-old seedlings were prepared and examined under a light microscope as described by Suzuki et al. (2004). Cotyledons of 1-week-old seedlings were prepared and examined for electron microscopy as described by Nakanishi et al. (2005).

Extraction and quantification of isoprenoids

Sterols were extracted from freeze-dried plant tissues (2-week-old seedlings, 10 mg) and quantified as described by Suzuki et al. (2004). Chlorophylls were extracted from freeze-dried plant tissues (1-week-old seedlings, about 50–100 mg) and quantified by measuring the absorbance at A_{646} and A_{663} as described by Harborne (1998).

Identification of the loi1 mutation

Plasmid rescue was performed to determine the T-DNA flanking genomic sequences of *loi1-1* as described by Ichikawa et al. (2003). The genomic DNA was digested with *Bam*HI and *Sac*I. Plasmids containing the genomic DNA were obtained from self-ligation. Plasmids obtained from the *Bam*HI-digested genomic DNA were sequenced using the LB4 primer 5′-TAGATATCGAT CGTGAAGTTTCT-3′, whereas those obtained from the *Sac*I-digested genomic DNA were sequenced using the RBI primer 5′-ATCTAGATCCGAAACTATCAGTG-3′.

Construction of the 35S promoter::LOI1 fusion gene and transformation of Arabidopsis

The WT LOI1 cDNA fragment amplified using PCR with the forward primer 5′-CACCATGAGCCTCCTCTCCGCGGA-3′ and the reverse primer 5′-CTCGAGCCAATAATCCTTACAA GAACATATC-3′ was cloned into pENTR/D-TOPO (Invitrogen). The resultant entry clone was integrated into the binary vector pBCR-79 (Seki et al. unpublished data) using the GATEWAY system. The resultant construct was transferred into *Agrobacterium tumefaciens* strain GV3101, followed by introduction into *loi1-1* homozygous plants using the floral dip method (Clough and Bent 1998).

Analysis of gene expression

For RNA gel blot analysis, 15 μg of total RNA from 2-week-old seedlings was separated on agarose–formaldehyde gels and blotted to nylon membranes as described by Chomczynski (1992). To obtain gene-specific probes, the full-length LOI1 cDNA was cloned into pCRII-TOPO plasmid (Invitrogen). The resultant plasmid was digested with *Bam*HI, and antisense digoxigenin (DIG)-labeled single-stranded LOI1 RNA probe was synthesized by T7 RNA polymerase (Roche, Basel, Switzerland) and DIG RNA Labeling Mix (Roche). Pre-hybridization, hybridization, wash conditions and detection were as described by Suzuki et al. (2004). RT–PCR analysis was performed using the specific set of primers for each amplification reaction as follows: 5′-CAAAGCTATGACGAAAAGAATTGGAAAGG-3′ and 5′-CACTGTAAGTGCGTCTGTCTTTGACCA-3′ for *DXS2*; 5′-CTCTTAGTAGGCTGCAATCTAATCGTGGA-3′ and 5′-GTCATCAGCTCTCTCTGCGTAAGGATAT-3′ for *DXS3*. The primers for *HMG1*, *HMG2*, *DXS1* and elongation factor were described by Suzuki et al. (2004).

Protein gel blot analysis

Fresh seedlings were homogenized in the extraction buffer [250 mM sucrose, 250 mM tricine, 25 mM $Na_2S_2O_5$, 2 mM EDTA, 100 mM ascorbic acid, 2.5% PVPP, 5 mM dithiothreitol (DTT) and RNase inhibitor; pH 8.0]. The homogenate was centrifuged at 200×*g* for 15 min at 4°C. The supernatant was used as the total protein fraction. The protein concentration was determined using Bradford reagent (Bio-Rad, Hercules, CA, USA), with bovine serum albumin as a standard. A 13 μg aliquot of the total protein was separated by SDS–PAGE and transferred onto nitrocellulose (Immobilon-P; Millipore Corporation, Millipore, Billerica, MA, USA). Immunodetection was performed using a 1: 1,000 dilution of the polyclonal anti-DXS antibodies (Estévez et al. 2000). Detection was performed by the ECL system (Amersham Biosciences, Piscataway, NJ, USA) using an anti-mouse immunoglobulin conjugated to horseradish peroxidase as a secondary antibody [anti-mouse IgG (whole molecule) peroxidase conjugate; Sigma-Ardrich, St Louis, MO, USA].

HMGR enzyme assay

The endoplasmic reticulum protein fraction was prepared and partially modified as described by Chappell et al. (1995). Total protein was extracted with the extraction buffer (see protein gel blot analysis). After centrifugation at 100,000×*g* for 60 min at 4°C, the pellet was washed with suspension buffer (250 mM sucrose, 100 mM tricine, 2 mM EDTA and 50 mM NaCl; pH 7.5) and suspended in suspension buffer containing 1% Triton X-100 and 10 mM DTT. The suspension was rotated for 30 min at 4°C, supplemented with 16% glycerol, and then incubated at 37°C for 20 min. HMGR activity was measured as described by Chappell et al. (1995).

RNA binding assay of the recombinant LOI1 protein

The sequence coding for LOI1 was cloned into pENTR/D-TOPO. The resultant plasmid was integrated into GATEWAY-modified pGEX-5X-1, pBCR-127 (Seki et al. unpublished data). The recombination reaction product was introduced into *E. coli* strain BL21 (Novagen, Madison, WI, USA). Cells were harvested, resuspended in phosphate-buffered saline (PBS), disrupted using

an ultrasonic cell disrupter and centrifuged. The purification of GST–LOI1 and GST–soluble proteins was performed using glutathione–Sepharose 4B (Amersham Pharmacia, UK). The binding assay was performed as described by Mili and Pinol-Roma (2003). The purified GST–LOI1 and GST–StSGT (Kohara et al. 2005), which was used as a negative control, were incubated with the Sepharose-bound RNA homopolymer poly(A) and the agarose-bound RNA homopolymers poly(U), poly(G) and poly(C), respectively, in 50 µl of binding buffer (10 mM Tris–HCl pH 8.0, 100 mM NaCl, 2.5 mM $MgCl_2$, 0.5% Triton X-100, 10% glycerol, 0.2 mM EDTA, 1 mM DTT and RNase inhibitor). The binding reaction mixtures were incubated for 20 min at 4°C, and the reaction products were washed three times with the binding buffer on ice. Bound proteins were separated by SDS–PAGE and detected by immunoblotting with anti-GST antibody (Nacalai Tesuque, Kyoto, Japan). An ECL+ system was used for detection in the immunoblot analysis (Amersham Biosciences).

Transient expression assay of LOI1–GFP

A DNA fragment containing the first 39 amino acid residues of the LOI1 protein and five residues of the glycine linker was amplified by PCR using 5′-TCTAGAGGATCCATGAGCC TCCTCTCCGC-3′ and 5′-ACCACCACCACCACCTGGTGAA TCGAGGGTTTTGAC-3′. The fragment was connected with the 35S promoter and GFP. The resultant fragment was cloned into the pENTR1A vector (Invitrogen). The resultant entry clone was integrated into the binary vector pBCR-112 (Seki et al. unpublished data) using the GATEWAY system.

The constructs were transiently transformed into onion epidermal cells (Scott et al. 1999) on agar plates by a helium-driven accelerator (PDS/1000; Bio-Rad). Bombardment parameters were as follows: 1,100 p.s.i. bombardment pressure, 1.0 µm gold particles, a distance of 6 cm from the macrocarrier to the samples, and a decompression vacuum of 28 inches Hg. After culture for 1 d, the bombarded epidermal cells were treated with 500 nM of the mitochondrion-selective dye MitoTracker Orange CMTMRos (Invitrogen) in PBS for 10 min. After staining, the onion epidermis was washed with PBS three times. GFP expression in general and co-localization of GFP fusion proteins to mitochondria were viewed using a confocal scanning microscope system (FV500; Olympus, Tokyo, Japan) with 488 nm laser light for fluorescence excitation of GFP and 543 nm laser light for excitation of MitoTracker Orange.

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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