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Note

Platform for "Chemical Metabolic Switching" to Increase Sesquiterpene Content in Plants

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Abstract The biosynthetic pathway of cytosolic isoprenoids bifurcates after farnesyl diphosphate into sesquiterpene and triterpene pathways. "Metabolic switching" has been used to increase sesquiterpene content in plants by suppressing the competitive triterpene pathway using transgenic technology. To develop "metabolic switching" without using transgenic technology, we developed a model system of "chemical metabolic switching" using inhibitors of the competitive pathway. Arabidopsis plants that overexpress the *amorpha-4,11-diene synthase* gene were treated with squalestatin, a squalene synthase inhibitor, or terbinafine, a squalene epoxidase inhibitor. We then analyzed total sterol content as major triterpenes and amorpha-4,11-diene in the plant. Plants treated with squalestatin showed decreased total sterol content and increased amorpha-4,11-diene content. In contrast, plants treated with terbinafine showed decreased total sterol content, but amorpha-4,11-diene accumulation was quite low. These results suggest that inhibition of the enzyme just below the branch point is more effective than inhibition of enzymes far from the branch point for "chemical metabolic switching". In addition, the activity of 3-hydroxy-3-methylglutaryl-CoA reductase, the rate-limiting enzyme of the cytosolic isoprenoid biosynthetic pathway, was upregulated in plants treated with squalestatin, suggesting that feedback regulation of 3-hydroxy-3-methylglutaryl-CoA reductase may contribute to amorpha-4,11-diene production. Here we demonstrated the effectiveness of "chemical metabolic switching" in plants.

Key words: Artemisia, chemical metabolic switching, isoprenoid, mevalonate pathway, squalestatin.

Isoprenoids have extensive structural diversity and over 40,000 compounds are present in nature (Withers and Keasling 2007). Some plants and microorganisms produce species-specific isoprenoids, and some of these isoprenoids are used in various industrial processes because of biological activities that have various purposes in society, such as in medicines, functional foods, fragrances, and pigments.

Isoprenoids are composed of C-5 building blocks, isopentenyl diphosphate (IPP), and the isomer dimethylallyl diphosphate (DMAPP). In higher plants, IPP and DMAPP are produced via two pathways: cytosolic mevalonate (MVA) pathway and plastidic 2-C-methyl-D-erythritol 4-phosphate pathway. Sesquiterpenes and triterpenes (including sterols) are mainly produced via the MVA pathway (Chappell 2002). Farnesyl diphosphate (FPP), a downstream metabolite of the MVA pathway, is a common precursor of sesquiterpene and triterpene biosynthesis. Sesquiterpenes are C-15 isoprenoids that are biosynthesized from one FPP molecule (Figure 1). Some sesquiterpenes are known to have pharmacological activity. There are many reasons why it would be beneficial to establish a standard platform for high accumulation of various sesquiterpenes. For example, artemisinin is a sesquiterpene biosynthesized in *Artemisia annua* and has been used as an antimalarial drug (Klayman et al. 1985). Amorpha-4,11-diene is a key intermediate of artemisinin biosynthesis (Wallaart et al. 2001). For biotechnological artemisinin production, the *amorpha-4,11-diene synthase* (*ADS*) gene was overexpressed in tobacco; however, amorpha-

Abbreviations: ADS, amorpha-4,11-diene synthase; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; IPP, isopentenyl diphosphate; MVA, mevalonate; SQE, squalene epoxidase; SQS, squalene synthase.

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4,11-diene accumulation was limited (Wallaart et al. 2001). This result is thought to be due to the limited supply of FPP. To increase the supply of FPP, three strategies have been considered. First strategy considered is "metabolic switching", which is a strategy that enables a switch from the original metabolic pathway to a specific metabolite through inhibition of metabolism of the common precursor in the original pathway. In the MVA pathway, FPP is the branching point of sesquiterpene and triterpene biosynthesis. If triterpene biosynthesis is inhibited, FPP will be used in sesquiterpene biosynthesis and the product level will increase. In *A. annua*, artemisinin content was significantly increased in plants by silencing squalene synthase (SQS), which biosynthesizes squalene from two molecules of FPP (Zhang et al. 2009). It was recently shown that inhibition of SQS and 5-epi-aristolochene synthase by RNAi methods increased exogenous (+)-valencene accumulation in (+)-valencene synthase overexpressing tobacco (Cankar et al. 2015). In the second strategy, the FPP pool was localized in small compartments, such as the mitochondria and plastids. The third strategy involved upregulation of the MVA pathway. 3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR) is the key enzyme of the MVA pathway (Masferrer et al. 2002; Schaller et al. 1995; Suzuki et al. 2004). The overexpression of truncated HMGR (catalytic domain of HMGR) resulted in increased isopentenyl block accumulation (Manzano et al. 2004). The second and third strategies have been performed in the mitochondria (van Herpen et al. 2010) and plastids (Wu et al. 2006). Although these reports showed success in biotechnological sesquiterpene production, their methods included transgenic or transient transgene expression technology. These technologies cannot always be applied to all plant species. Therefore, we used a chemical inhibitor to allow "chemical metabolic switching" from the triterpene (including sterols) pathway to a specific sesquiterpene pathway because inhibitors are easy to handle and can be applied to any plant species. During sterol biosynthesis, FPP is sequentially metabolized by SQS and squalene epoxidase (SQE) (Figure 1). We used squalestatin (Hartmann et al. 2000) and terbinafine (Wentzinger et al. 2002), which are inhibitors of SQS and SQE, respectively, as inhibitors of the early step of triterpene biosynthesis. In this study, we developed a model system for "metabolic switching" from sterols, the major triterpene in plant, to sesquiterpene using these inhibitors.

To estimate "metabolic switching" of FPP from sterol to sesquiterpene, we generated Arabidopsis plants that exogenously overexpressed the sesquiterpene synthase gene encoding ADS derived from *Artemisia annua* (*35S:ADS* plants; supplemental information) because the *ADS* gene does not exist in the wild-type Arabidopsis genome. Because narrowing of sterol biosynthesis severely affects plant growth and development (Ishiguro et al. 2010; Jin et al. 2012; Rasbery et al. 2007; Suzuki et al. 2004), *35S:ADS* plants were grown without inhibitors on MS agar plates supplemented with 1% sucrose for 1 week, then transferred to liquid MS medium supplemented with 1% sucrose, and cultured for another 1 week with various concentrations of inhibitors. After treatment with inhibitors, the total sterol (sitosterol, campesterol, and stigmasterol) and amorpha-4,11-diene contents were measured using GC-MS/MS (supplemental information). Compared with control treatment, the total sterol content of *35S:ADS* plants treated with 10 and 15 μM squalestatin was reduced to 30% (Figure 2A). Amorpha-4,11-diene was not detected in *35S:ADS* plants grown under a controlled condition (Figure 2B). Treatment with squalestatin increased amorpha-4,11-diene accumulation in *35S:ADS* plants, and *35S:ADS* plants treated with 15 μM squalestatin showed the highest amorpha-4,11-diene accumulation, which was equal to 0.48 $\mu g\,g^{-1}$ FW.

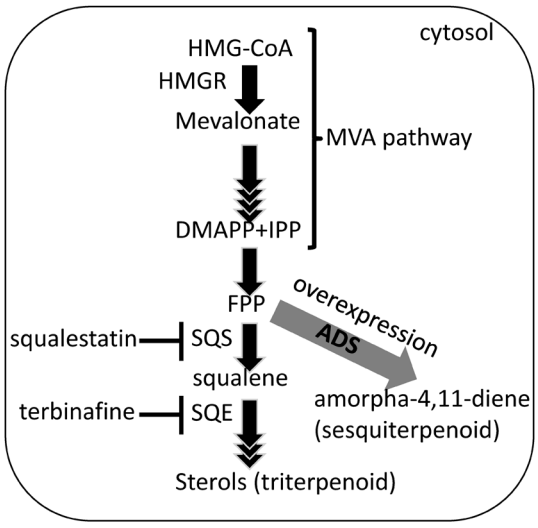

Figure 1. Scheme of "chemical metabolic switching" steps for amorpha-4,11-diene production in Arabidopsis.
HMG-CoA; 3-hydroxy-3-methylglutaryl-CoA, HMGR; HMG-CoA reductase, IPP; isopentenyl diphosphate, DMAPP; dimethylallyl diphosphate, FPP; farnesyl diphosphate, SQS; squalene synthase, SQE; squalene epoxidase.

Next, to investigate the effect of other inhibitors of sterol biosynthesis, we treated *35S:ADS* plants with terbinafine. When *35S:ADS* plants were treated with terbinafine, the total sterol content was reduced to 20%–40% (Figure 3A). However, amorpha-4,11-diene accumulation in plants treated with terbinafine was lower than that of plants treated with squalestatin. *35S:ADS* plants treated with 5 μM terbinafine showed the highest amorpha-4,11-diene accumulation (Figure 3B), which was equal to 0.0037 $\mu g\,g^{-1}$ FW. Squalene accumulation in *35S:ADS* plants increased upon terbinafine treatment in a concentration-dependent manner (Figure 3C).

HMGR activity is reportedly feedback regulated by

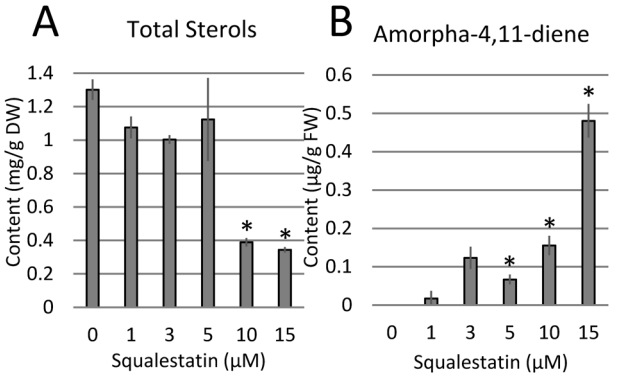

Figure 2. Sterol and amorpha-4,11-diene accumulation in *35S:ADS* Arabidopsis plant treated with squalestatin.

Effect of squalestatin on total sterol (sitosterol, campesterol, and stigmasterol) (A) and amorpha-4,11-diene content (B) in *35S:ADS* plants grown in MS liquid medium supplemented with indicated concentrations of squalestatin. All plants were grown under 16-h light, 8-h dark cycles at 23°C in growth chambers. Squalestatin and terbinafine were dissolved in dimethylsulfoxide and diluted to different final concentrations in the growth medium. The mean±SE for three biological replicates are shown. Asterisk indicates statistical significance relative to control treatment (Student's *t*-test; $*p<0.05$).

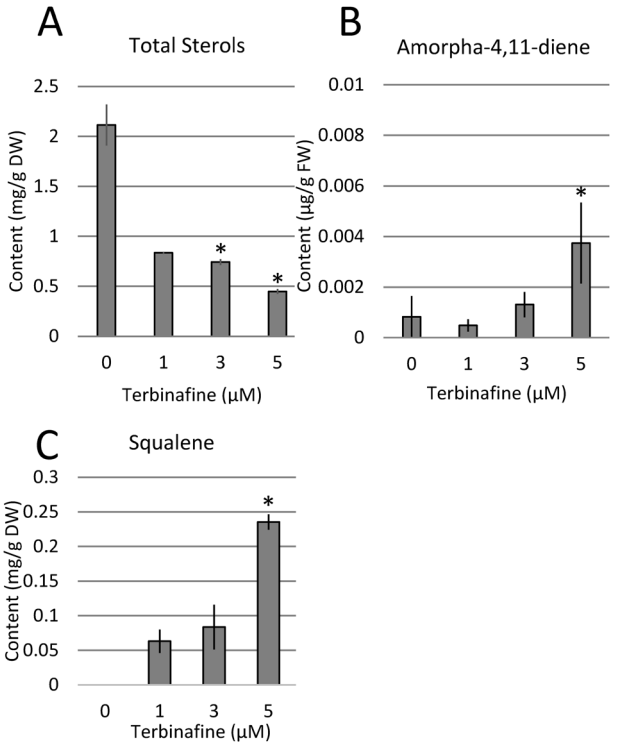

Figure 3. Sterol, amorpha-4,11-diene, and squalene accumulation in *35S:ADS* Arabidopsis plants treated with terbinafine.

Effect of terbinafine on total sterol (sitosterol, campesterol, and stigmasterol) (A), amorpha-4,11-diene (B), and squalene (C) content in *35S:ADS* plants grown in MS liquid medium supplemented with indicated concentrations of terbinafine. The mean±SE for three biological replicates are shown except for the sterol content of *35S:ADS* plants treated with 1 μM. The means of two biological replicates for total sterol contents of 1 μM treatment are shown. Asterisk indicates statistical significance relative to control treatment (Student's *t*-test; $*p<0.05$).

squalestatin and terbinafine treatment in Arabidopsis (Nieto et al. 2009). To determine if HMGR was subjected to feedback regulation in our system, HMGR gene expression and enzyme activity were measured in

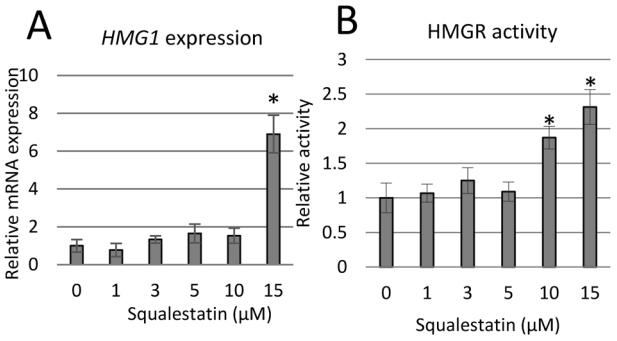

Figure 4. *HMG1* expression and HMGR activity in *35S:ADS* Arabidopsis plants treated with squalestatin.

qRT–PCR analysis of *HMG1* using cDNA synthesized from total RNA extracted from seedlings grown in MS liquid medium supplemented with indicated concentrations of squalestatin (A). Transcript levels are presented as values relative to those of the control treatment, defined as 1, after normalization to *Actin2* expression. HMGR activity using total protein extracted from seedlings grown in MS liquid medium supplemented with indicated concentrations of squalestatin (B). Activity in control treatment plants, 3.7±0.8 pmol min^{-1} mg^{-1} protein, was given a value of 1. Values are the average of three experiments. Asterisk indicates statistical significance relative to control treatment (Student's *t*-test; $*p<0.05$).

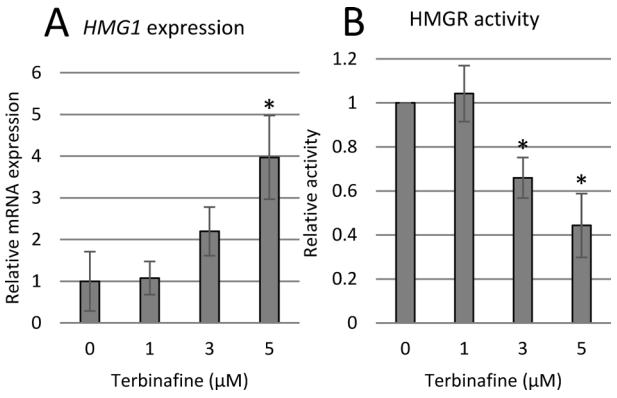

Figure 5. *HMG1* expression and HMGR activity in *35S:ADS* Arabidopsis plants treated with terbinafine.

qRT–PCR analysis of *HMG1* using cDNA synthesized from total RNA extracted from seedlings grown in MS liquid medium supplemented with indicated concentrations of terbinafine (A). The transcript levels are presented as values relative to those of the control treatment, defined as 1, after normalization to *Actin2* expression. HMGR activity using total protein extracted from seedlings grown in MS liquid medium supplemented with indicated concentrations of terbinafine (B). Activity in control treated plants, 25.9±9.1 pmol/min/mg protein, was given a value of 1. Values are the average of three experiments. Asterisk indicates statistical significance relative to control treatment (Student's *t*-test; $*p<0.05$).

35S:ADS plants treated with inhibitors (supplemental information). The expression of *HMG2* was not detected at significant levels. The expression of *HMG1* was increased 7-fold when 15 μM squalestatin was used to treat *35S:ADS* plants (Figure 4A). HMGR activity of *35S:ADS* plants treated with 10 and 15 μM squalestatin was increased 1.9- and 2.3-fold, respectively (Figure 4B). Although *HMG1* mRNA levels were increased 4-fold in *35S:ADS* plants treated with 5 μM terbinafine (Figure 5A), HMGR activity of *35S:ADS* plants treated with 3 and 5 μM terbinafine was reduced to 66% and 44%, respectively (Figure 5B).

Total sterol content was decreased and amorpha-4,11-diene content was increased in *35S:ADS* plants treated with squalestatin, although amorpha-4,11-diene was not detected in untreated *35S:ADS* plants. These results indicated that "chemical metabolic switching" from triterpene to sesquiterpene was effective. However, although sterol contents were decreased in *35S:ADS* plants treated with terbinafine, amorpha-4,11-diene contents were limited under this condition. Squalene which is a substrate for SQE was increased in *35S:ADS* plants treated with terbinafine. These results indicate that inhibition of an enzyme far from the branching point was not effective in "metabolic switching". It can be also considered that terbinafine inhibits ADS activity so that accumulation level of amorphadiene does not increase. Though the possibility cannot be excluded, we consider that the possibility may be quite low, because the structure of ADS is very different from that of SQE.

In tobacco, it was reported that exogenous (+)-valencene contents were also increased using the "metabolic switching" method. Compared with the overexpression of the exogenous sesquiterpene synthase, (+)-valencene synthase alone, the overexpression of (+)-valencene synthase and silencing of the endogenous 5-epi-aristolochene synthase and SQS caused a 2.8-fold increase in the production of (+)-valencene (Cankar et al. 2015). Furthermore, (+)-valencene accumulation was improved to 37-fold by the overexpression of truncated HMGR and FPS. This report demonstrates that both "metabolic switching" and overexpression of upstream components in metabolic pathways is effective, suggesting that sufficient supply of FPP may be critical for biotechnological sesquiterpene synthesis. In our system, HMGR activity was increased in Arabidopsis treated with squalestatin, suggesting that squalestatin treatment resulted in "chemical metabolic switching" and upregulation of upstream components in the MVA pathway.

This "metabolic switching" system may also be effective in endogeneous pathways. In *A. annua*, artemisinin content was significantly increased in plants in which SQS was silenced, with the highest values reaching 31.4 mg g^{-1} dry weight, which is an approximately 3.14-fold increase in the content observed in untransformed control plants (Zhang et al. 2009). Our results reveal the possibility that "chemical metabolic switching" is effective for any plant species in which transformation technology is not established and/or sesquiterpene synthase has not yet been identified.

Expression levels and activity of HMGR were increased in *35S:ADS* plants treated with $>10\,\mu$M squalestatin, suggesting that feedback of *HMG1* gene expression was induced by squalestatin treatment. This result is consistent with previous research demonstrating that HMGR gene expression and activity were induced in tobacco cells treated with squalestatin (Hartmann et al. 2000; Wentzinger et al. 2002).

We found that HMGR activity was decreased in *35S:ADS* plants treated with terbinafine, which conflicts with the results of other studies; HMGR activity was increased in tobacco BY-2 cells and Arabidopsis treated with terbinafine (Nieto et al. 2009; Wentzinger et al. 2002). Differences in growth conditions and terbinafine treatment may affect gene expression and enzyme activity of HMGR.

Although the effectiveness of "metabolic switching" has been demonstrated previously, "chemical metabolic switching" has two advantages. One advantage is that it does not use transgenic technology. "Chemical metabolic switching" is easy to perform and can be examined even in plants in which transgenic technology has not been established. The second advantage is the flexibility of inhibitor treatment. Inhibition of sterol biosynthesis leads to growth inhibition and reduced biomass. Arabidopsis germinated in MS plates containing inhibitors have scarcely grown. To avoid this disadvantage, inhibitors can be used after normal growth. Although growth after inhibitor treatment partially inhibited, we succeeded accumulation of amorpha-4,11-diene. However, the amount of decreased sterol was not consistent with amorpha-4,11-diene accumulation using inhibitor treatments. Understanding the metabolism of excess isoprene units formed because of the decrease in sterol biosynthesis will be important for the improvement of "metabolic switching".

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