Engineering the MEP pathway enhanced ajmalicine biosynthesis

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Abstract

The 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway genes encoding DXR and MECS from *Taxus* species and *STR* from *Catharanthus roseus* were used to genetically modify the ajmalicine biosynthetic pathway in hairy root cultures of *C. roseus*. As expected, the STR-overexpressed root cultures showed twofold higher accumulation of ajmalicine than the control. It was important to discover that overexpression of the single *DXR* or *MECS* gene from the MEP pathway also remarkably enhanced ajmalicine biosynthesis in transgenic hairy root cultures, and this suggested that engineering the MEP pathway by overexpression of *DXR* or *MECS* promoted the metabolic flux into ajmalicine biosynthesis. The transgenic hairy root cultures with co-overexpression of *DXR* and *STR* or *MECS* and *STR* had higher levels of ajmalicine than those with overexpression of a single gene alone such as *DXR*, *MECS*, and *STR*. It could be concluded that transgenic hairy root cultures harboring both *DXR*/*MECS* and *STR* possessed an increased flux in the terpenoid indole alkaloid biosynthetic pathway that enhanced ajmalicine yield, which was more efficient than cultures harboring only one of the three genes.
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Abbreviations: *DMAPP, dimethylallyl pyrophosphate; DW, dry weight; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; FPS, farnesyl diphosphate synthase; G10H, geraniol 10-hydroxylase; H6H, hyoscyamine 6β-hydroxylase; IPP, isopentenyl pyrophosphate; MECS, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; MEP, 2-C-methyl-D-erythritol-4-phosphate; PMT, putrescine-N-methyltransferase; STR, strictosidine synthase; TDC, tryptophan decarboxylase; TIAs, terpenoid indole alkaloids.*

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1. Introduction

The terpenoid indole alkaloids (TIAs) consist of the terpenoid moiety and the indole moiety, which include many pharmaceutical alkaloids such as ajmalicine, vinblastine, and vincristine

with high values. The terpenoid moiety is provided by the plastidial 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway (or namely nonmevalonate pathway) and the indole moiety by the tryptamine pathway [1]. *Catharanthus roseus* is the model plant to unveil biosynthesis of TIAs at the molecular and biochemical levels. Up to now, more than 10 enzymes and corresponding genes have been identified from *C. roseus*, which are involved in the biosynthetic pathway of TIAs (Fig. 1). This makes it possible to engineer TIA biosynthesis at the molecular level.

The reported metabolic engineering of TIA biosynthesis mainly focused on genetic modification of the indole moiety biosynthetic pathway and the strictosidine-after pathway. The overexpression of both anthranilate synthase and tryptophan decarboxylase in hairy root cultures led to an increase in tryptamine and serpentine but did not result in overaccumulation of other major alkaloids of interest such as ajmalicine [2]. Overexpressing both *TDC* and *STR* resulted in an increased level of TIAs, including ajmalicine, but overexpressing *TDC* alone did not produce more ajmalicine [2]. The overexpression of *G10H* and *ORCA3* in the hairy roots of *C. roseus* improved catharanthine production [3]. The plastidial MEP pathway provides the general acyclic 5-carbon isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) for biosynthesis of terpenoids, especially monoterpenoids and diterpenoids [4]. Ajmalicine belongs to the family of monoterpene indole alkaloids, so the MEP pathway also provides its precursors theoretically. The studies of gene expression demonstrated that the MEP pathway might be involved in ajmalicine biosynthesis [5, 6]. Unfortunately, these reports lacked direct evidence such as transgenic results indicating that the MEP pathway participated in ajmalicine biosynthesis. In the present study, the MEP pathway was genetically modified by overexpressing *DXR* and *MECS* in hairy root cultures of *C. roseus* to analyze their effects on ajmalicine biosynthesis, and the effects of overexpressing *STR* combined with *DXR* and *MECS* are also discussed.

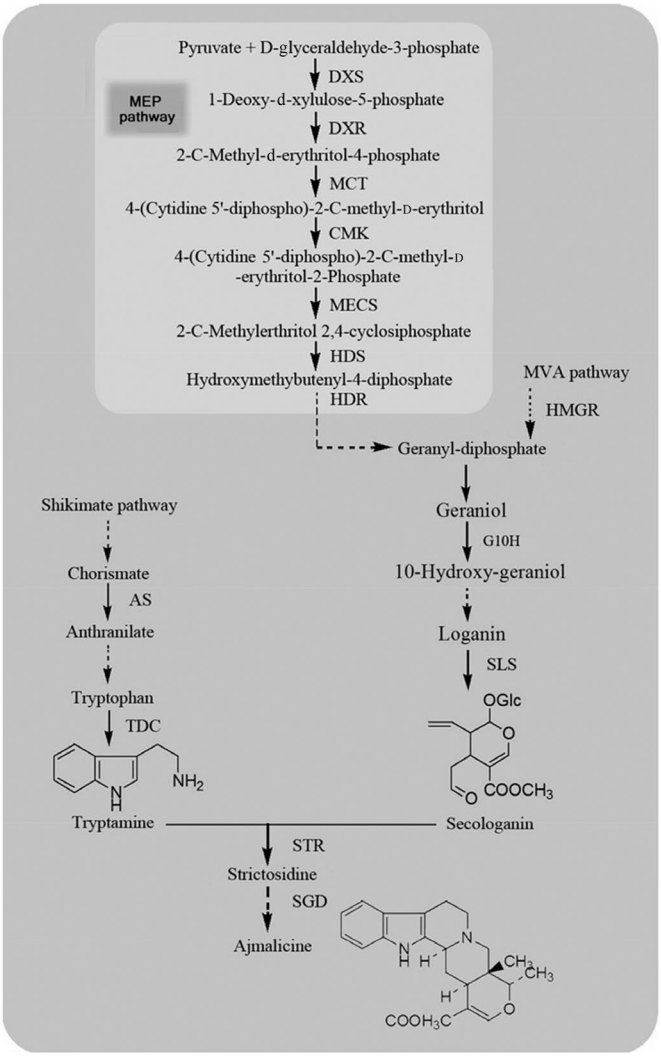

FIG. 1 *The theoretical biosynthetic pathway of ajmalicine. DXS: 1-deoxy-*D*-xylulose 5-phosphate synthase; DXR: 1-deoxy-*D*-xylulose 5-phosphate reductoisomerase; MCT: MEP cytidyltransferase; CMK: 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol kinase; MECS: 2-C-methylerythritol 2,4-cyclodiphosphate synthase; HDS: hydroxymethylbutenyl 4-diphosphate synthase; HDR: hydroxymethylbutenyl 4-diphosphate reductase; HMGR: 3-hydroxy-3-methylglutaryl-CoA reductase; G10H: geraniol 10-hydroxylase; SLS: secologanin synthase; AS: anthranilate synthase; TDC: tryptophan decarboxylase; STR: strictosidine synthase; and SGD: strictosidine-β-glucosidase.*

2. Materials and Methods

2.1. Plant expression vector construction

The *DXR* (accession number: KF710461) and *MECS* (accession number: KC161200) genes were isolated from *Taxus chinensis* and *STR* (accession number: X53602.1) gene from *C. roseus*. The coding sequence of *TcDXR* gene was amplified using primers with the restriction sites of *Bgl*II and *Bst*pI. Both fragments of *TcDXR* and the plasmid p1304$^+$ were digested with *Bgl*II and *Bst*pI. Subsequently, *TcDXR* was cloned into the expression vector p1304$^+$ to obtain the plasmid p1304$^+$-TcDXR (Fig. 2). The p1304$^+$-TcDXR was transformed into the *Agrobacterium tumefaciens* strain C58C1 (pRi4). The expression vector of *TcMECS* (Fig. 2) was constructed using the same method as *TcDXR*. The expression vector of *CrSTR* (Fig. 2) was constructed via inserting *CrSTR* instead of the *gus* gene of p1304$^+$ with *Bam*H I and *Sac* I. The expression vectors harboring *TcDXR* + *CrSTR* (Fig. 2) or *TcMECS* + *CrSTR* (Fig. 2) were constructed by inserting *TcDXR* or *TcMECS,* respectively, instead of the *gfp–gus* fusion gene of p1304$^+$-CrSTR with restriction sites of *Bgl* II and *Bst*p I. The primers used to isolate the genes of interest are listed in Table 1.

2.2. Establishment of hairy root cultures

The seeds of *C. roseus* were germinated into seedlings on MS medium. A disarmed *A. tumefaciens* strain C58C1 [7] harboring

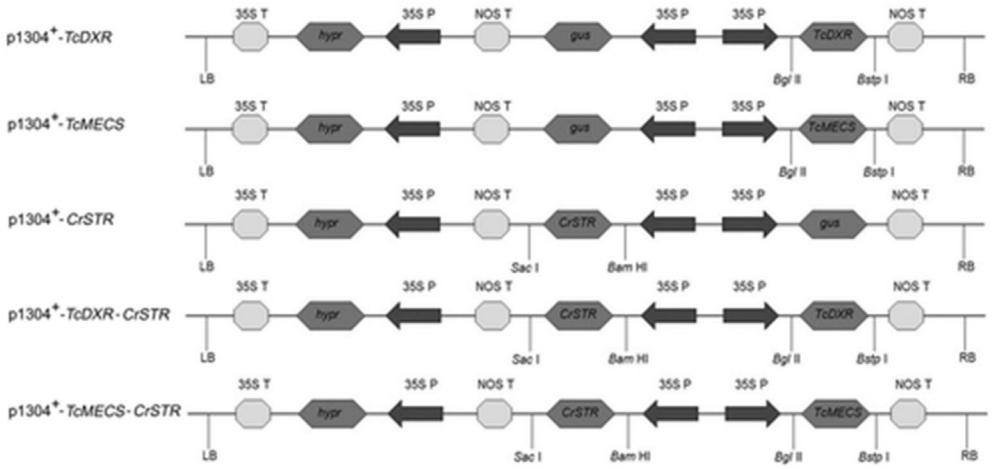

FIG. 2 *Schemes of reconstructed plant expression vectors. LB: left border; 35S T: 35S terminator; hypr: hygromycin; 35S P: 35S promoter; NOS T: NOS terminator; gus: β-glucuronidase; RB: right border.*

with the plasmids of pRiA4 and reconstructed plant expression vectors was restored in our laboratory. The bacteria-free leaves isolated from 5-week-old seedlings of *C. roseus* were used as the initial materials for genetic transformation. The explants were cut into small pieces (about 1.5 cm) followed by preincubation on MS medium in dark for 2 days, and then infected with engineered C58C1. After infection with C58C1, the explants were plated on MS medium for cocultivation in dark for 2 days. After 2 days of coculture, the explants were transferred onto 1/2 MS medium containing 300 mg dm^{-3} cefotaxime to eliminate *A. tumefaciens,* and the transformed hairy roots were selected by 10 mg dm^{-3} hygromycin. Control explants were treated similarly except that they were not cocultivated with *Agrobacterium*. The apical tips of hairy root induced from explants were excised and subcultured on 1/2 MS medium with 300 mg dm^{-3} cefotaxime at 20-day intervals. Rapidly growing root clones with no bacterial contamination were used to establish hairy root lines. About 100 mg fresh roots with 2 cm length were inoculated into 100 dm^3 liquid 1/2 MS medium in 250 dm^3 conical flasks on an orbiter at 120 rpm in dark at 25 °C. Thirty-five days later, the hairy root cultures were harvested for analysis of the gene expression level and ajmalicine content. The genomic PCR analysis was performed to confirm the hairy root lines with target DNA. The control hairy root lines were induced from C58C1 only harboring pRiA4.

2.3. Molecular analysis of hairy root cultures

After 35 days of culture, the hairy root cultures were harvested for future analysis. The root-inducing genes including *rolB* and *rolC* were detected by genomic PCR according to the previous report [8]. The genomic PCR was used to detect the integration of the genes of interest into the genome of *C. roseus*. The primers used are listed in Table 1. Total RNAs were isolated using the RNA plant reagent (Tiangen, People's Republic of China) according to the manufacturer's instructions and stored in −80 °C. cDNA was synthesized using AMV reverse transcriptase (Takara, Shiga, Japan) according to the manufacturer's instructions. Quantitative PCR of each gene was conducted with two primers specific to the coding sequence of four genes, including *TcDXR*, *TcMECS*, *CrSTR*, and *18S*, using SYBR Premix ExTaq (Takara). Amplifications were performed under the following conditions: 94 °C for 2 Min, 40 cycles (denaturation at 94 °C for 15 Sec, annealing at 55 °C for 15 Sec) and followed by extension at 68 °C for 20 Sec. The primers used for quantitative PCR (qPCR) analysis are listed in Table 1. Meanwhile, the housekeeping gene 18S *rRNA* gene was used as the reference gene. There were triplicates in the analysis of each sample.

2.4. Detection of ajmalicine by HPLC

We improved the method of ajmalicine extraction based on the previous report [9]. Dry powder of hairy root cultures was treated with ultrasound with methanol after drying and grinding. The extraction solutions were combined and concentrated under vacuum. The residues were dissolved in water with 5% glacial acetic acid and quickly extracted with ligarine. The alkaloids were quickly extracted three times with chloroform. Then, the chloroform extractions were combined and concentrated on vacuum. The residues were dissolved in 1 mL of the mobile phase and analyzed by HPLC. The detecting wavelength was 268 nm, and injection volume was 20 μL. The temperature of the column was set at 40 °C. Each sample was detected for three times. The mobile phase of HPLC detection was methanol–acetonitrile–ammonium acetate (0.025 M)–triethylamine (15:40:45:0.1, v/v) at a flow rate of 1 mL/Min. There were triplicates in analysis of each sample. The statistical analysis was performed by Duncan's multiple range tests.

3. Results and Discussion

3.1. Establishment of hairy root cultures

The transformed roots could emerge from the infected leaves at the wounded sites 10 days after infection with *Agrobacterium*

TABLE 1 *The primers used to construct plant expressing vectors and detect the genes of interest by qPCR or genomic PCR*

Gene	*Primer sequences (5′-3′)*	*Restriction sites*	T_m (°C)	*Purposes*
TcDXR	Forward: GAAGATCTATGGCTCTGAAAATTGCAC	*Bgl*II	58.0	Vector construction
	Reverse: AGGTAACCTCAAACCATGGCAGG	*Bst*pI	58.0	
TcMECS	Forward: GAAGATCTATGGCAGCCGTGAAAGC	*Bgl*II	55.8	
	Reverse: AGGTAACCCTTCTTATGAAGAAATAATTACAT	*Bst*pI	55.8	
CrSTR	Forward: GCGGATCCATGGCAAACTTTTCTGAATCTAAATCCAT	*Bam*HI	63.0	
	Reverse: TCGAGCTCCTAGCTAGAAACATAAGAATTTCCCTTGT	*Sac*I	63.0	
TcMECS	Forward: CCGTGAAAGCCGCAGTCTC	–	59.0	Gene expression analysis by qPCR
	Reverse: TGACAAGAGGTTGAAGGTGGATG	–	59.0	
CrSTR	Forward: CACTGACTTCGCCTACGCATCTCC	–	61.5	
	Reverse: TGTGGCTAGTTGTGTGGCATACCC	–	61.5	
TcDXR	Forward: GCTGCCCGCTGCTCTATACAAG	–	60.5	
	Reverse: GGCTTTGGACCATCCCATACTTTC	–	60.5	
18S	Forward: ATGATAACTCGACGGATCGC	–	60.5	
	Reverse: CTTGGATGTGGTAGCCGTTT	–	60.5	
f35S-TcMECS	Forward: TTCATTTGGAGAGAACACGGG	–	55.0	Genomic PCR detection
	Reverse: CATGCATAAGTCTGACCGC	–	55.0	
f35S-TcDXR	Forward: TTCATTTGGAGAGAACACGGG	–	55.0	
	Reverse: ATGGTACCACCTGCTCG	–	55.0	
f35S-CrSTR	Forward: TTCATTTGGAGAGAACACGGG	–	55.0	
	Reverse: CTGTTCTCTGATCAACAGTTACTG	–	55.0	

strains. The transformed roots, which had much lateral branching, could grow very rapidly on hormone-free 1/2 MS medium, just like in previous reports on hairy roots of TIA-producing *C. roseus* [10]. According to the typical morphologies of hairy root, it might be concluded that the subcultured roots were the hairy roots genetically transformed by Ri plasmid. The transgenic hairy roots could grow well on half-strength MS medium supplemented with 10 mg L^{-1} hygromycin, but the nontransgenic one could not grow and finally died. The hairy root cultures were confirmed by genomic PCR and used for liquid culture. The cultured hairy roots in half-strength liquid MS medium grew well.

3.2. Molecular detection of hairy root cultures

To confirm that the hairy roots were successfully transformed by the target plasmids, genomic PCR was performed to detect the *rolC* gene, *DXR*, *MECS,* and *STR*. A 626-bp *rolC*-specific PCR product could be amplified from all hairy root lines and the positive control, but could not be amplified from the negative control (Fig. 3). This suggested that the *rol* genes integrated into the genome of *C. roseus*, which led to formation of hairy root [11]. The specific gene fragments of *DXR*, *MECS,* and *STR* could be generated from the corresponding transgenic hairy root lines, which were, respectively, 1255 (*DXR*), 570 (*MECS*), and 500 bp (*STR*). The genomic PCR results suggested that hairy root lines of interest were obtained (Fig. 3). These transgenic lines included hairy root lines with overexpression of a single *DXR* gene, a single *MECS* gene, and a single *STR* gene and overexpression of the combination of *DXR* and *STR* and *MECS* and *STR*.

Real-time qPCR was used to detect the relative gene expression levels in hairy root cultures of *C. roseus* (Fig. 4). In the *DXR*-transformed hairy root lines, the expression of *DXR* could be detected at higher levels but could not be detected in the nontransgenic hairy roots (control). The *MECS*-transformed hairy root lines showed *MECS* expression, but the expression of

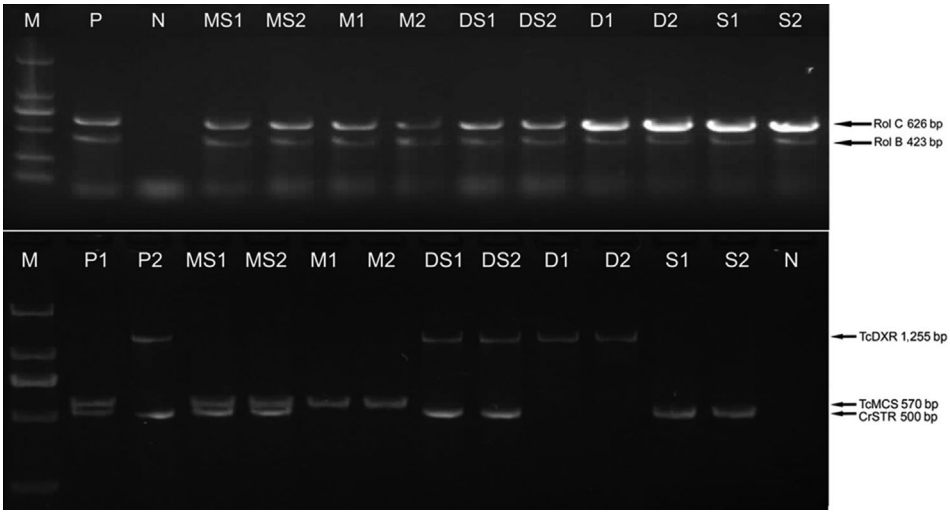

FIG. 3 *Genomic analysis of transgenes in hairy root cultures of C. roseus. M: DNA marker; positive control; P1: positive control of TcMECS and CrSTR genes; P2: positive control of TcDXR and CrSTR genes; DS: transgenic hairy root cultures with overexpressing TcDXR and CrSTR; D: transgenic hairy root cultures with overexpressing TcDXR; MS: transgenic hairy root cultures with overexpressing TcMECS and CrSTR; M: transgenic hairy root cultures with overexpressing TcMECS; S: transgenic hairy root cultures with overexpressing CrSTR; and N: negative control.*

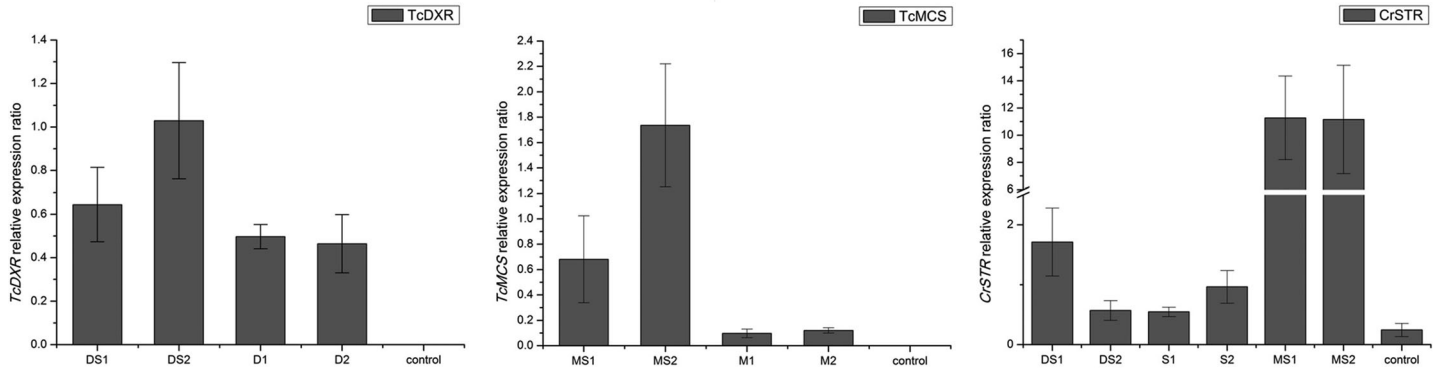

FIG. 4 *Relative gene expression levels in hairy root cultures of C. roseus. DS: hairy root line with overexpressing both TcDXR and CrSTR; D: hairy root line with overexpressing TcDXR; MS: hairy root line with overexpressing both TcMECS and CrSTR; M: hairy root line with overexpressing TcMECS; and S: hairy root line with overexpressing CrSTR.*

MECS could not be detected in the control. In all the hairy root lines, the expression of *STR* could be detected. The hairy root lines with overexpression of *STR* showed a higher expression level than the control. These results demonstrated that in the transgenic hairy root lines, the expression levels of transgenes showed higher levels than nontransgenic hairy root lines. The expression analysis suggested that the ajmalicine biosynthetic pathway was genetically modified by overexpressing the genes of interest, including *DXR*, *MECS,* and *STR*.

3.3. Engineering the MEP pathway by overexpression of DXR/MECS-enhanced ajmalicine biosynthesis

The terpenoid moiety of ajmalicine is theoretically provided by the plastidial MEP pathway, in which the seven functional genes (*DXS*, *DXR*, *MECS*, *CMK*, *MCT*, *HDS*, and *HDR*) were involved [12]. The enzyme of DXR is generally considered to be the committed-step enzyme involved in the MEP pathway and is usually employed to engineer the terpenoid pathways [13]. The overexpression of *DXR* led to a 50% increase in monoterpene

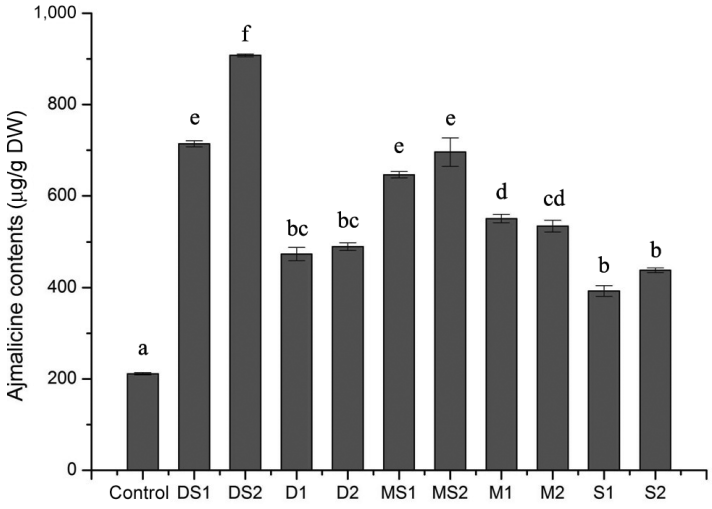

FIG. 5

Ajmalicine content in hairy root cultures of C. roseus. Control: nontransgenic hairy root cultures without target gene overexpression; DS: transgenic hairy root cultures with overexpressing TcDXR and CrSTR; D: transgenic hairy root cultures with overexpressing TcDXR; MS: transgenic hairy root cultures with overexpressing TcMECS and CrSTR; M: transgenic hairy root cultures with overexpressing TcMECS; S: transgenic hairy root cultures with overexpressing CrSTR. The statistical analysis was performed by Duncan's multiple range tests. The different letters indicate significant difference ($P < 0.01$).

essential oils in transgenic peppermint due to breaking of the committed step of the MEP pathway by overexpressing *DXR* [14]. The tobacco transformants with overexpression of *DXR* showed an increase in the content of various isoprenoids such as chlorophyll a, β-carotene, lutein, antheraxanthin, solanesol, and β-sitosterol [15]. These transgenic reports indicated that the DXR reaction was the key step controlling isoprenoid or terpenoid levels in plants. The previous research reported that the gene expression profile of the MEP pathway was consistent with the accumulation of ajmalicine in *C. roseus* [6]. However, the direct evidence of the MEP pathway involved in TIAs is still limited.

In the present study, transgenic hairy root cultures of *C. roseus* with *DXR* overexpression showed a remarkable increase ($P < 0.01$) in ajmalicine biosynthesis (Fig. 5). In the *DXR*-overexpressed hairy root cultures D2, the content of ajmalicine reached 0.49 mg g^{-1} DW, about 2.3-fold compared with control nontransgenic hairy roots (0.21 mg g^{-1} DW). This suggested that overexpression of *DXR* could lead to an increase in ajmalicine biosynthesis because of the breakage of the committed step of the MEP pathway defined by DXR. Our result was consistent with the transgenic studies in peppermint and tobacco of *DXR* overexpression and supported the hypothesis that the MEP pathway was involved in TIA biosynthesis by transgenic data.

Metabolic engineering of the MEP pathway mainly focused on the DXR-catalyzed step. However, according to the studies on gene expression, *MECS* showed the MeJA-induced expression [6] and displayed an identical cell-specific expression pattern with *G10H*, a cytochrome P450 monooxygenase that was the committed-step enzyme involved in TIA biosynthesis [16]. These studies suggested that MECS might play an important role in ajmalicine biosynthesis. In this study, MECS was employed to genetically modify the MEP pathway in plant for the first time. The results showed that overexpression of *MECS* remarkably enhanced biosynthesis of ajmalicine (Fig. 5). The *MECS*-overexpressed hairy root cultures M1 produced ajmalicine at a level of 0.55 mg g^{-1} DW, which was 2.5-fold compared with the control ($P < 0.01$). The transgenic results of *MECS* overexpression in *C. roseus* suggested that it might be another committed-step enzyme involved in the MEP pathway, just like DXR in ajmalicine biosynthesis in *C. roseus*.

3.4. Engineering the ajmalicine pathway by overexpression of STR increased ajmalicine biosynthesis

STR was the committed-step enzyme involved in ajmalicine biosynthesis [17], and this was supported by transgenic results. The overexpression of *STR* in *C. roseus* cell lines improved the level of ajmalicine [18]. Hairy roots of *Weigela styriaca* overexpressing *STR* and *TDC* cDNAs from *C. roseus* were also able to produce tryptamine and ajmalicine at higher levels [19]. However, there is no report on the overexpression of *STR* in hairy root cultures of *C. roseus*. As expected, the overexpression of *STR* promoted biosynthesis of ajmalicine in hairy root cultures of *C. roseus*. The *STR*-overexpressed hairy root cultures S2 accumulated ajmalicine to 0.44 mg g^{-1} DW, which was 2.1-fold compared with the control (Fig. 5). The results in the present study were consistent with those of the previous reports [18, 19].

3.5. Simultaneously engineering the MEP and ajmalicine-specific pathways led to higher levels of ajmalicine biosynthesis

Breaking the committed-step or rate-limiting reaction involved in the pathways of interest is a widely used strategy of metabolic engineering to enhance biosynthesis of the targeted natural products. A general method is overexpression of committed-step enzymes, which has been successfully applied to engineering a number of pathways. Transgenic *Atropa belladonna* with a higher level of scopolamine was developed by overexpressing H6H, the rate-limiting enzyme involved in scopolamine biosynthesis [20]. The overexpression of farnesyl diphosphate synthase (FPS) results in overaccumulation of artemisinin in transgenic *Artemisia annua* [21]. These successful studies were based on a single-gene transformation. The effect of a single-gene transformation might be limited when the pathway of interest has more than one committed step. Thus, co-overexpression of two or more genes has been

applied to genetically modify the pathways of interest, and that usually leads to better results.

Our previous study reported that transgenic hairy root lines expressing both *pmt* and *h6h* produced significantly higher levels of scopolamine compared with the wild-type and transgenic lines harboring a single gene (*pmt* or *h6h*). The best line produced much higher levels of scopolamine, which was over nine times more than that in the wild type and more than twice the amount in the highest scopolamine-producing *h6h* single-gene transgenic line [22]. In the present study, the same results were obtained. Co-overexpression of a single gene (*DXR* or *MECS*) of the MEP pathway and *STR* resulted in remarkably higher levels of ajmalicine production in hairy root cultures of *C. roseus* (Fig. 5). Transgenic hairy root line DS2 expressing both *DXR* and *STR* produced ajmalicine at a level of 0.91 mg g^{-1} DW, which was, respectively, 4.3-, 1.9-, and 2.1-fold ($P < 0.01$) those levels produced by the control nontransgenic hairy root line, *DXR*-overexpressed line D2 (with the highest level of ajmalicine in *DXR*-overexpressed lines), and *STR*-overexpressed line S2 (with the highest level of ajmalicine in *STR*-overexpressed lines) (Fig. 5). Furthermore, transgenic lines overexpressing both *DXR* and *STR* produced the highest levels of ajmalicine compared with all the other lines: transgenic line MS2 overexpressing both *MECS* and *STR* produced ajmalicine by 0.70 mg g^{-1} DW, which was, respectively, 3.3-, 1.3-, and 1.6-fold ($P < 0.01$) the levels of control nontransgenic hairy root line, *DXR*-overexpressed line D2, and *STR*-overexpressed line S2 (Fig. 5). It could be concluded that transgenic hairy root cultures of *C. roseus* harboring both *DXR/MECS* and *STR* possess an increased flux in the TIA biosynthetic pathway that enhances ajmalicine yield, which is more efficient than plants harboring only one of the three genes.

4. Conclusions

Engineering the plastidial MEP pathway enhanced biosynthesis of ajmalicine by overexpression of a single gene, *DXR* or *MECS*, in hairy root cultures of *C. roseus*, and the transgenic results provided direct evidence that the MEP pathway participated in ajmalicine biosynthesis. Furthermore, it was also demonstrated that both DXR and MECS were the key enzymes of ajmalicine biosynthesis. Finally, transgenic line overexpression of both *DXR/MECS* and *STR* produced higher levels of ajmalicine than the lines only overexpressing a single gene, *DXR*, *MECS,* or *STR*. Thus, the push–pull strategy based on expressing both an upstream gene and a downstream gene was a better choice for metabolic engineering of ajmalicine biosynthesis.

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The authors declare no conflict of interest.

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