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ORIGINAL ARTICLE

Application of transport engineering to promote catharanthine production in *Catharanthus roseus* hairy roots

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Abstract

Low accumulation levels of valuable plant secondary metabolites lead to high costs for these compounds production. In order to promote accumulation levels of these molecules, many efforts have been carried out during the past decades, such as elicitation, precursor feeding, tissue cultures and overexpression of pathway genes. However, these engineering strategies could only slightly increase the amounts of target metabolites, since biosynthesis pathways of these compounds are very complex and involving several different organelles and cell types. In this work, we used *Catharanthus roseus* hairy roots as research material to investigate the effect of transport engineering on monoterpenoid indole alkaloids (MIAs) production. Results showed that overexpresssion of catharanthine transporter, *CrTPT2*, in *C. roseus* hairy roots could dramatically increase the accumulation level of catharanthine to fivefold higher than that in control hairy roots, while other MIAs accumulation levels are not affected. Since the expression of pathway genes are at similar level, timely removal of catharanthine from where it is synthesized could be critical for promoting catharanthine production, which exemplifies the application of transport engineering to effective manipulation of plant secondary metabolites biosynthesis.

Key message

Overexpression of catharanthine transporter, CrTPT2, in Catharanthus roseus hairy rootsspecifically promotes catharanthine production, which exemplifies an effective manipulationstrategy for plant secondary metabolites biosynthesis.

Keywords Transport engineering · *Catharanthus roseus* · Catharanthine transporter · Monoterpenoid indole alkaloids · Hairy roots

Introduction

Catharanthus roseus is extensively investigated as a model medicinal plant for its diverse monoterpenoid indole alkaloids (MIAs) biosynthesis. Many of these MIAs have been demonstrated ecological and pharmaceutical functions, which are widely used for clinical therapy (De Luca et al. 2014). Due to their complicated chemical structures, it is not feasible to produce these compounds by chemical synthesis and most of MIAs are obtained from plant extracts. Therefore, many investigations on regulating MIAs biosynthesis were carried out for possible promoting MIAs amounts in *C. roseus*, which is the only plant source for some of MIAs, such as catharanthine, vindoline, vinblastine and vincristine (Pan et al. 2016).

In order to efficiently produce these valuable compounds from medicinal plants, many engineering strategies were applied for either promoting these metabolites accumulation levels (such as treating plant materials with elicitors/precursors or transgenic plants construction) or enhancing genetic stability and growth rates of plant materials (such as suspension cell and hairy root cultures). In comparison with plant cultivation, suspension cell and hairy root cultures have shown many advantages for secondary metabolites production, such as fast growing

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rates, saving farmland resources, and efficiently transgenic manipulation (Zhou et al. 2011; Ochoa-Villarreal et al. 2016; Saiman et al. 2018; Isah et al. 2018). However, some of secondary metabolites are usually produced in differentiated cells or distinct developmental stages of plant materials, which causes the biggest challenge for producing these compounds through suspension cell culture. Hairy roots, which are differentiated tissues developed by infecting plants with *Agrobacterium rhizogenes*, generally could produce secondary metabolites at comparable levels to their parent plants, thus making of hairy root culture as an effective strategy for producing these compounds (Hao et al. 2015; Zhou et al. 2016; Cao et al. 2018; Shi et al. 2019).

It's generally acknowledged that genetic engineering is a powerful tool for promoting valuable secondary metabolites production in plants. In comparison with construction of transgenic plants, it's comparatively easily to generate transgenic hairy roots by placing desired gene(s) into plant genome for enhanced target metabolites production (Sun et al. 2018a; Deng et al. 2019; Huang et al. 2019). However, most of these valuable secondary metabolites are toxic to plant and its increased accumulation retards hairy roots growth, which leads to low efficiency for producing these metabolites (Goossens et al. 2003). In order to avoid this limitation, regulated translocation of these compounds from its synthesis site seems to be a feasible solution (Cai et al. 2012; Nour-Eldin and Halkier 2013). In *C. roseus*, although transgenic hairy roots were applied to promote MIAs production for many years, only pathway genes or transcription factors were selected for regulating MIAs biosynthesis (Hughes et al. 2004; Magnotta et al. 2007; Peebles et al. 2011; Sun and Peebles 2015; Sun et al. 2018b), while no report on manipulating the movement of pathway intermediates or final products for regulating the efficiency of MIAs biosynthesis in *C. roseus* transgenic hairy roots was delivered. So far, several specific transporters were identified for their biological functions of regulating the translocation of iridoid glucoside, strictosidine, and catharanthine in *C. roseus* (Yu and De Luca 2013; Larsen et al. 2017; Payne et al. 2017). Functional characterization of these transporter genes greatly widens our understanding of MIAs biosynthesis and supplies effective gene elements for possible regulating MIAs biosynthesis through optimizing translocation of pathway intermediates or final products.

In this work, *CrTPT2*, a catharanthine transporter that was identified previously in our lab (Yu and De Luca 2013), was overexpressed in *C. roseus* hairy roots. Our results clearly showed that overexpression of *CrTPT2* were dramatically affecting the accumulation of catharanthine in transgenic hairy roots, which pointed out an effective transport engineering strategy for promoting valuable plant secondary metabolites production.

Materials and methods

Plant materials

Seeds of *C. roseus* cv. Little Delicata were rinsed with running water for about 2 h and surface-sterilized with 75% (v/v) ethanol for 15 s, and then rinsed three times with sterilized water, followed by soaked in 2% (v/v) sodium hypochlorite solution for 8–10 min. After rinsed three times with sterilized water, seeds were placed on MS solidified medium, and germinated in a growth chamber at 25 °C with a dark environment for 1 week. The germinated seeds were then placed in a growth chamber at 25 °C under 16 h photoperiod with light intensity of 3000 lx. When three leaf pairs appeared, the *C. roseus* seedlings were ready for subsequent experiments.

Vector construction for *CrTPT2* overexpression

For construction of *CrTPT2* overexpression vector, the coding sequence of *CrTPT2* (GenBank KC511771) was PCR amplified to add *Xba*I/*Kpn*I restriction sites at both ends of *CrTPT2* by using pGEM-T easy-*CrTPT2* plasmid (Yu and De Luca 2013) as template and then the PCR product was cloned to pGEM-T easy vector. The *CrTPT2* fragment was then obtained by *Xba*I/*Kpn*I double digestion and mobilized to binary vector pBIGD pre-digested with *Xba*I/*Kpn*I under control of 35S promoter and NOS terminator to produce plasmid pBIGD-*CrTPT2*.

A. rhizogenes transformation and generation of *Catharanthus roseus* hairy roots

Agrobacterium rhizogenes strain C58C1 was used in this study for initializing transgenic hairy roots from *C. roseus* seedlings. *A. rhizogenes* was activated on LB solid medium containing 25 µg/ml of rifampicin and 50 µg/ml of gentamicin at 28 °C for 2 days. Single colonies were selected and grown in 50 ml of LB liquid medium with 180 rpm shaking at 28 °C for overnight until OD = 0.8–1.0. The bacteria were then collected by centrifugation of 4500 rpm for 10 min and ready for plasmids transformation. pBIGD (empty vector control), pBI121(*GUS* overexpression control), and pBIGD-*CrTPT2* were then transformed to cultured *A. rihzogenes* strain C58C1 by electroporation and positive colonies were selected for generating transformed *C. roseus* hairy roots.

The transformed *A. rihzogenes* were grown in 100 ml of LB medium containing 10 mM of MES, 20 µM of Acetosyringone and 50 µg/ml of kanamycin at 28 °C with 180 rpm. The cultured bacteria were collected by centrifugation

with 4500 rpm for 10 min, and resuspended in infection buffer (10 mM of $MgCl_2$, 20 mM of MES, and 200 µM of acetosyringone) and incubated for 3 h at 28 °C with shaking (180 rpm). Leaves from *C. roseus* were wounded and infected with one of each respective *Agrobacterium*/construct strain and then incubated in the dark for 48 h on solid MS medium containing 50 µg/ml of kanamycin at 28 °C. After co-cultivation, *C. roseus* leaves were transferred to fresh plates containing additional 250 µg/ml of cefatoxime to kill the remaining bacteria. The transformed hairy roots could be observed after 6 weeks of cultivation and the formed hairy roots were excised and maintained in an antibiotic free solution of 50 ml of half strength of Gamborg's B5 medium salts (Gamborg et al. 1968) and 2% of sucrose with pH 5.8 in 250 ml of Erlenmeyer flasks. The cultures were grown with shaking of 120 rpm at 25 °C and subcultured with fresh medium every 4–5 weeks.

Total RNA extraction and gene expression analysis

Total RNA was isolated from the hairy roots by TRizol reagent (Invitrogen) according to manufacturer's protocol. The RNA pellets were dissolved in diethylpyrocarbonate (DEPC)-treated water and approximately 0.5 µg of total RNA was used to carry out reverse transcription with M-MLV RTase cDNA Synthesis Kit (TaKaRa). The obtained cDNAs were used as templates to perform qRT-PCR analysis for examining gene expression. The primers used in this work are listed in Supplementary Table 1. The PCR amplification condition for qRT-PCR was as follow: 95 °C for 3 min; 40 cycles of 95 °C for 15 S, 50 °C for 20 S, and 72 °C for 15 S. The relative gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method. Results were normalized to *C. roseus ACTIN3* (Genbank: MG813871) and are shown relative to the level in wild type (WT) hairy roots.

HPLC analysis for MIAs accumulation in *Catharanthus roseus* hairy roots

Wild type and Transformed *C. roseus* hairy roots were harvested at late exponential growth stage (20 days after subculture) and dried in 50 °C oven for 24 h, and then about 1 g of dried samples were ground thoroughly with a mortar and pestle followed by MIAs extraction with methanol at room temperature. The extracts were then evaporated in a speedvac and re-dissolved in 500 µl of methanol. After filtered through 0.22 µm acrodic syringe filter, samples are ready for HPLC analysis. 5 µl of the extract was injected into the Waters HPLC system (Alliance 2690) equipped with an ACCHROM Unitary C18 column (250 mm × 4.6 mm, 5 µm) and HPLC protocol was according to the method developed by Sun et al. (2018a).

Results

Generation of transgenic *Catharanthus roseus* hairy root lines

Young leaves of *C. roseus* were infected with *A. rhizogenes* C58C1 carrying the plasmids pBIGD (empty vector control), pBI121 (*GUS* overexpression control), and pBIGD-*CrTPT2* (*CrTPT2* overexpression). 30 hairy roots from each construct were excised and grew on solid selection medium plates containing 50 mg/l of kanamycin and only 15 pBIGD hairy roots, 13 pBI121 hairy roots and 8 pBIGD-*CrTPT2* roots showed strong growth. These selected hairy root lines with different constructs were then transferred to liquid medium for adaptive growth of hairy roots in liquid environment that was reported to be the most difficult step for establishing hairy root cultures (Bhadra et al. 1993). In liquid medium, only 3 of 15 pBIGD hairy root lines, 4 of 12 pBI121 hairy root lines and 2 of 8 pBIGD-*CrTPT2* hairy root lines could be maintained and other hairy root lines stopped growing after being transferred to liquid medium. After five successive subcultures of these transgenic hairy root lines in liquid medium without adding antibiotics, DNA was extracted for positive transgenic line identification by PCR amplifying *nptII* (kanamycin resistance) gene fragment and ruling out possibility of *A. rhizogenes* contamination by PCR amplifying bacterial chromosomal gene *chvH* (Bosselut et al. 2011). Results clearly showed that all obtained transgenic hairy roots could be detected with the presence of *nptII* gene fragment and no *chvH* gene fragment could be

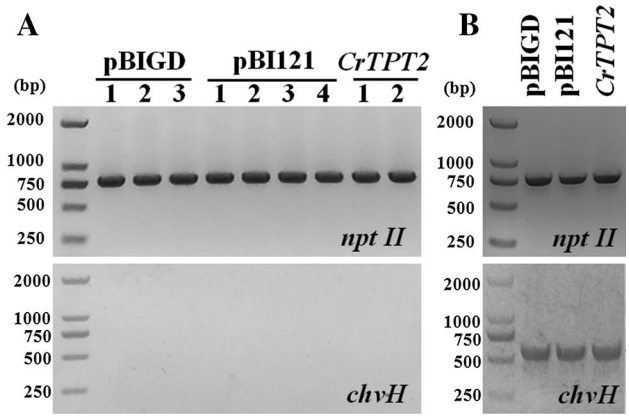

Fig. 1 Identification of positive transformed hairy root lines by PCR amplification. PCR was performed for amplifying *nptII* gene fragments and *A. rhizogenes chvH* gene fragments by using DNA extracted from hairy roots as templates (**a**) or *A. rhizogenes* containing pBIGD, pBI121, or pBIGD-*CrTPT2* as templetes (**b**). pBIGD, empty vector control lines; pBI121, *GUS* overexpression lines; *CrTPT2*, *CrTPT2* overexpression lines

detected, which indicates all hairy roots were successfully transformed through *A. rhizogenes* infection (Fig. 1).

MIAs accumulation in transformed hairy roots

The positive transformed hairy root lines and one WT (wild type) line were then used for examining accumulation levels of MIAs. In *C. roseus*, most of MIAs pathway intermediates or final products and pathway genes were identified recently (Qu et al. 2015, 2018; Stavrinides et al. 2015; Caputi et al. 2018). According to these reports, we selected catharanthine, ajmalicine, tetrahydroalstonine, and tabersonine from different pathway branches (Fig. 2) for investigating the effect of overexpressing *CrTPT2* on MIAs accumulation. In comparison with MIAs accumulation levels in control hairy root lines, overexpression of *CrTPT2* dramatically promoted catharanthine production for about fivefold, while no obvious variations of ajmalicine, tetrahydroalstonine and tabersonine levels between different hairy root lines could be observed (Fig. 3).

Gene expression analysis in transformed hairy roots

HPLC results have clearly shown that overexpression of *CrTPT2* could specifically promote catharanthine production in *C. roseus* hairy roots. In order to figure out whether translocation of catharanthine is the critical and direct cause leading to catharanthine over-accumulation, pathway genes involved in MIAs biosynthesis were selected for examining their expression in response to overexpression of *CrTPT2* in *C. roseus* hairy roots (Fig. 4).

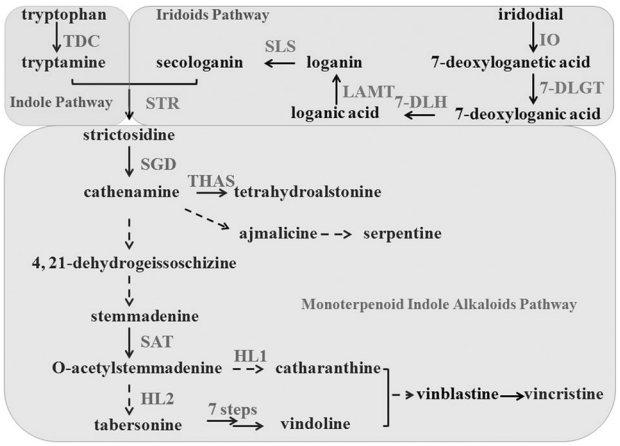

Fig. 2 Monoterpenoid indole alkaloids (MIAs) biosynthesis pathway in *C. roseus*. Enzymes involved in MIAs pathway: STR strictosidine synthase, SGD strictosidine β-glucosidase, THAS tetrahydroalstonine synthase, SAT stemmadenine-*O*-acetyltransferase, HL1 hydrolase 1, HL2 hydrolase 2

Transformed hairy roots (line #1 of each construct) and wild type hairy root were used for the experiment. Figure 4a showed that *CrTPT2* was successfully overexpressed in *CrTPT2* hairy root line, while the expression of *CrTPT2* in WT, pBIGD, and pBI121 hairy root lines were at same levels. Besides *CrTPT2*, other five important pathway genes (*STR*, *THAS*, *SAT*, *HL1*, *HL2*) were also examined for their expression in different *C. roseus* hairy root lines. Not surprisingly, no big variations could be observed for all five selected pathway genes in four different hairy root lines (Fig. 4b–f), which indicates that contemporaneous catharanthine transfer by CrTPT2 from where it is being biosynthesized should be critical to promote catharanthine production in *C. roseus* hairy roots.

Discussion

Plants synthesize large and diverse groups of secondary metabolites for adapting to the ever-changing environment (Yang et al. 2018). Some of these compounds are high-value pharmaceuticals with very small amounts accumulated in host plants, which has primed a desire for promoting the efficiency of their production in various organisms. With the development of synthetic biology and pathway elucidation of valuable plant secondary metabolites, microorganisms were gradually considered as new hosts for producing these compounds according to their clear genetic background and well-controlled cultivation environment (Gandhi 2019). However, the complex pathway construction in microorganisms and toxicity of these compounds to host cells are still big challenges that need to be addressed at first view. Although a big breakthrough of artemisinic acid production by yeast cells has been successfully conducted recently (Paddon et al. 2013; Ro et al. 2006), so far, the high production cost still limits its industrialized application (Shen et al. 2018), which indicates that plants are still the major organism for these compounds production.

In host plants, these valuable metabolites are often stored in particular sites, which are spatially distinct from where they were synthesized (St-Pierre et al. 1999; Bird et al. 2003; Samanani et al. 2005; Yu and De Luca 2014). This necessitates trafficking of biosynthetic intermediates or final products between different organelles or cell types (Yu and De Luca 2014). Furthermore, this compartmental feature for plant secondary metabolites biosynthesis makes the whole pathway more complicated for manipulating these compounds production.

Recently, more and more plant secondary metabolite transporters have been identified, which greatly promotes the new emerging field of transport engineering for regulating accumulation levels of these valuable compounds (Lv et al. 2016). Application of these specific transporters could

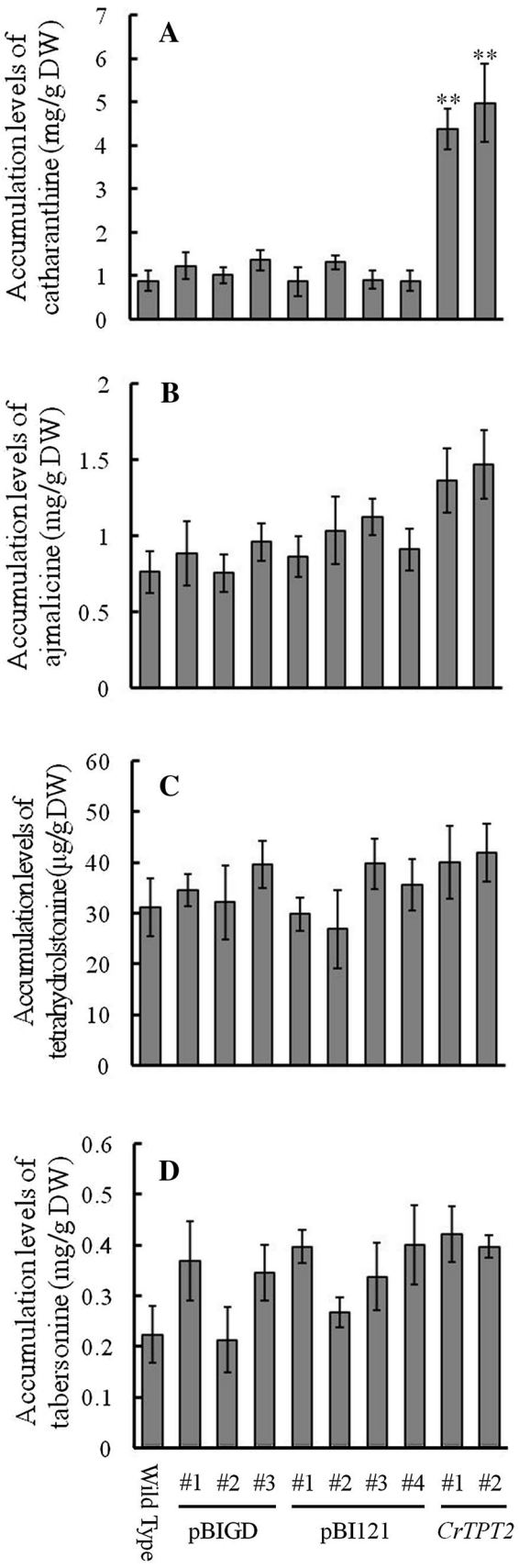

Fig. 3 Accumulation levels of catharanthine (**a**), ajmalicine (**b**), tetrahydrolstonine (**c**), and tabersonine (**d**) in wild type, pBIGD (empty vector control), pBI121 (*GUS* overexpression control), and *CrTPT2* (*CrTPT2* overexpression) hairy root lines. Hairy roots were harvested at day 20 after sub-culture and then dried in 50 °C oven for 24 h. After MIAs extraction by methonal, alkaloid contents were determined by HPLC analysis. Error bars represent standard errors from three biological replicates. Data were analyzed using the Student's *t* test and the asterisks indicate statistically significant differences of MIAs levels in transformed hairy roots compared with that in wild type hairy roots. $^{**}P < 0.01$

regulate metabolic flux to the desired pathway branch that leads to high accumulation of target compounds in the original host plants. Meanwhile, as mentioned previously, synthetic biology intends engineering new biological processes and has shown great potential for these valuable compounds production in microorganisms. However, low yield, which could be caused by feedback inhibition and toxicity of final products, has always been a big problem that needs to be solved prior to real industrial application of synthetic biology for production of valuable plant secondary metabolites (Lv et al. 2016). Therefore, utilization of transport engineering to translocate these compounds in storage compartments or outside cells by specific transports may achieve high yield production.

In this work, *C. roseus* hairy roots were selected for investigating improved target metabolite production under transport engineering strategy. Utilizing previously identified specific catharanthine transporter, CrTPT2 (Yu and De Luca 2013), transformed *C. reseus* hairy root with overexpressing *CrTPT2* was constructed. HPLC results indicate that catharanthine accumulation levels are successfully promoted about fivefold higher than that in WT, pBIGD and pBI121 control hairy roots, while the accumulation levels of other selected three MIAs from different pathway branches do not show obvious variations in all different transformed hairy root lines. Since CrTPT2 has specifically export activity for transferring catharanthine to the leaf surface in *C. roseus* (Yu and De Luca 2013), overexpression of *CrTPT2* in *C. roseus* hairy roots should accelerate removal of catharanthine from its synthesis site to storage site, which promotes catharanthine production by weakening the effect of feedback inhibition of catharanthine during enzymatic reactions or toxicity of catharanthine to hairy root cells. Previous report indicated that alkaloids could be observed in both hairy roots and medium in *C. roseus* hairy cultures (Cai et al. 2012), which pointed out that culture medium could be potential destination for alkaloids transport by *C. roseus* hairy roots. In order to examining whether CrTPT2 transports catharanthine to the culture medium, HPLC was performed for analyzing alkaloids accumulation in *C. roseus* hairy roots culture medium. Unfortunately, we did not detect any alkaloids accumulated in the medium, which indicated

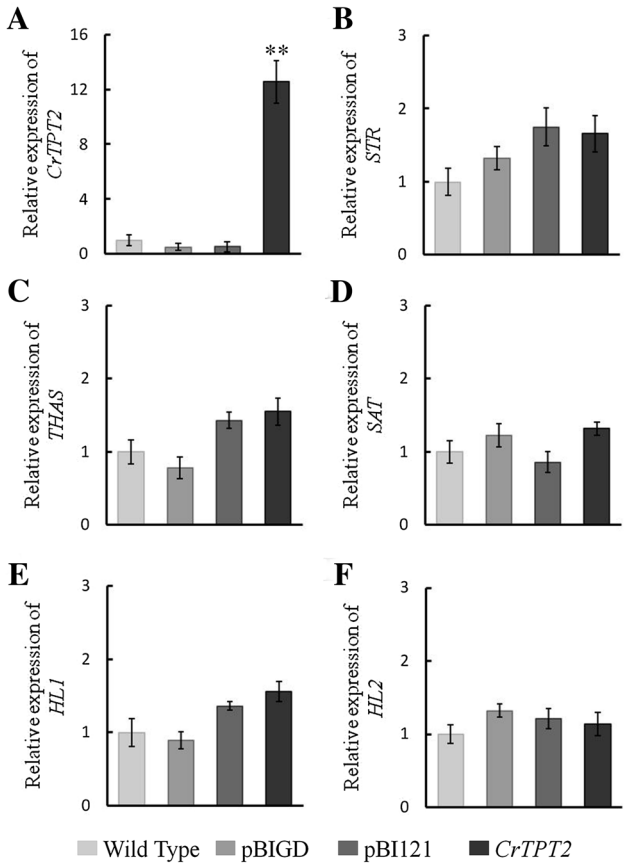

Fig. 4 Relative expression of *CrTPT2* (**a**) and MIAs biosynthesis pathway genes, *STR* (**b**), *THAS* (**c**), *SAT* (**d**), *HL1* (**e**), and *HL2* (**f**) in wild type, pBIGD (empty vector control), pBI121 (*GUS* overexpression control), and *CrTPT2* (*CrTPT2* overexpression) hairy root lines. Results are normalized to *C. roseus ACTIN3* and are shown relative to the level in Wild Type. Error bars represent standard variations from three technical replicates. Data were analyzed using the Student's *t* test and the asterisks indicate statistically significant differences of gene expression levels in transformed hairy roots compared with that in wild type hairy roots. $^{**}P < 0.01$

that culture medium is not catharanthine transport destination by CrTPT2 in *C. roseus* hairy roots. By considering liposoluble and acidic water-soluble feature of catharanthine, vacuole or intercellular space could be the possible storage sites for catharanthine accumulation that needs to be further investigated in future work. Since similar expression levels of pathway genes in all hairy root lines through qRT-PCR analysis have been observed, enforced transporter-mediated catharanthine trafficking could be responsible for high yield of catharanthine in *C. roseus* hairy roots.

In this case, transport engineering was shown as an effective strategy for promoting certain secondary metabolite production. However, this regulation strategy is still limited so far for large-scale application to improve target metabolites production since only few specific plant secondary metabolite transporters are identified. Fortunately, the speed of identifying transporters of plant secondary metabolites keeps increasing according to rapid development of new techniques (Chen et al. 2010, 2012). So far, although application of transport engineering is restricted to the number of identified transporters of plant secondary metabolites, this regulatory strategy still shows great potential for significantly promoting valuable compounds production and benefits development of synthetic biology for breaking through the bottleneck of low yield of target products.

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Author contributions WY, JS, and YF designed research; WY and YB performed research; WY, YB, ZM, JS, and YF analyzed data; and WY and YF wrote the paper.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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