Molecular cloning and characterization of two tropinone reductases in *Anisodus acutangulus* and enhancement of tropane alkaloid production in AaTRI-transformed hairy roots

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Tropane alkaloids are used medicinally as anticholinergic agents with increasing market demand, so the improvement and production of active components from medicinal plants using molecular biotechnology show great potential for applications that should benefit human healthcare. Two tropinone reductases constitute a branching point in the biosynthesis of tropane alkaloids. In the present paper, we report for the first time the cloning and characterization of two full-length cDNAs encoding TRI (tropinone reductase I) (GenBank® accession number EU424321) and TRII (tropinone reductase II) (GenBank® accession number EU424322) from the solanaceous plant *Anisodus acutangulus* by rapid amplification of cDNA ends. Sequence comparison indicated that AaTRI (*A. acutangulus* TRI) and AaTRII (*A. acutangulus* TRII) had high homology with other tropinone reductases from *Hyoscyamus niger*, *Datura stramonium* etc., but AaTRI and AaTRII showed identity of only 60.8%. Phylogenetic-tree analysis showed that AaTRI and AaTRII belong to different clusters and have the closest relationship with *H. niger* TRI and TRII respectively. Expression-pattern analysis showed that AaTRI and AaTRII were expressed in all tissues tested, including root, stem and leaf, but the transcript level of AaTRI was much lower than AaTRII. Expression of AaTRI and AaTRII could be enhanced by methyl jasmonate, with a weak effect for AaTRI and a strong effect for AaTRII. AaTRI-transformed hairy-root lines were accompanied by a mean 1.87-fold higher level of hyoscyamine and a mean 8-fold higher level of scopolamine compared with control roots, indicating that AaTRI is a promising target for genetic engineering to increase tropane alkaloid in *A. acutangulus*.

Introduction

A few genera of the plant family Solanaceae, including *Anisodus*, *Atropa*, *Datura*, *Duboisia*, *Hyoscyamus* and *Scopolia* are able to produce biologically active tropane alkaloids simultaneously. Tropane alkaloids have been used as established drugs for medical treatment for many years. The tropane alkaloids hyoscyamine (its racemic form being atropine) and scopolamine are used medicinally as anticholinergic agents that act on the parasympathetic nervous system to treat spasms, to sedate patients and for dilation of the pupil (mydriasis) [1,2]. However, a new group of nortropane alkaloids, calystegines, was revealed 15 years ago. Calystegines resemble monosaccharides in structure, and it is not surprising that they have proved to be strong glycosidase inhibitors [3].

Two stereospecific reductases, TRI (tropinone reductase I; Enzyme Commission (EC) accession number EC 1.1.1.206) and TRII (tropinone reductase II; EC 1.1.1.236) constitute a branching point in the biosynthesis of tropane alkaloids. TRI and TRII present as the first putative metabolite branch specific to hyoscyamine and calystegines respectively (Scheme 1). TRI converts tropinone into tropine (also known as 3α-hydroxytropane), whereas TRII converts tropinone into pseudotropine (also known as ψ-tropine or 3β-hydroxytropane). Hyoscyamine is derived from tropine, then converted into scopolamine by H6H (hyoscyamine 6β-hydroxylase), whereas calystegines are metabolites of pseudotropine. In contrast with hyoscyamine and scopolamine, calystegines are not esterified [4]. Both

Key words: *Anisodus acutangulus*, bioinformatics, expression pattern, transformation, tropane alkaloid, tropinone reductase (TR).

Abbreviations used: AaTR, *A. acutangulus* tropinone reductase; AtPTR, *Arabidopsis thaliana* putative TR; AtSDR, *A. thaliana* short-chain dehydrogenase/reductase; AUAP, Abridged Universal Amplification Primer; CsTR, *Calystegia sepium* TR; DsTR, *Datura stramonium* TR; H6H, hyoscyamine 6β-hydroxylase; HnTR, *Hyoscyamus niger* TR; MeJA, methyl jasmonate; NCBI, National Center for Biotechnology Information; ORF, open reading frame; PMT, putrescine *N*-methyltransferase; PtTR, *Populus trichocarpa* unknown protein; RACE, rapid amplification of cDNA ends; RT–PCR, reverse-transcription PCR; StTR, *Solanum tuberosum* TR; TRI, tropinone reductase I; TRII, tropinone reductase II; VvPro, *Vitis vinifera* unnamed protein product.

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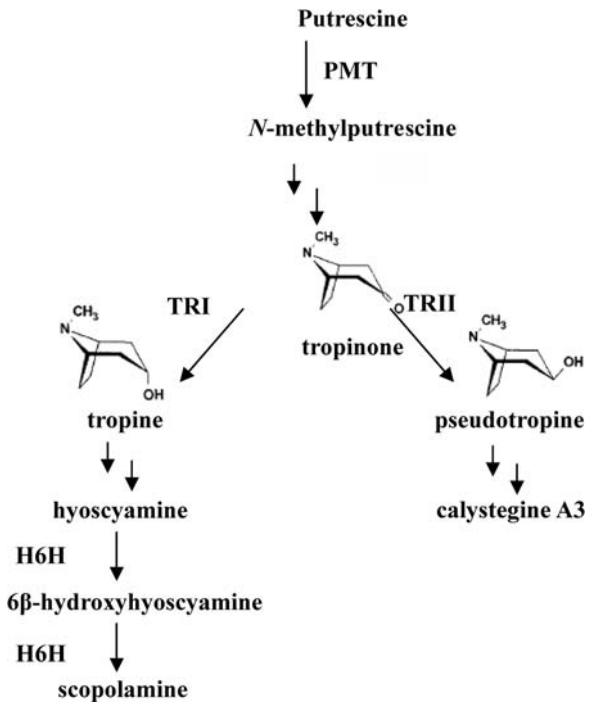
The nucleotide sequence data reported will appear in GenBank®, EMBL, DDBJ and GSDB Nucleotide Sequence Databases under the accession numbers EU424321 and EU424322.



Scheme 1 The tropane-alkaloid biosynthesis pathway

TRI and TRII are present together in any given tropane-alkaloid-producing species [5]. The ratios of TRI and TRII activities vary considerably depending on the species and may determine the flux of metabolites at the branching point, because no interconversion between tropine and pseudotropine has been observed *in vivo* [6].

Two TR genes have been isolated from the Solanaceae species *Hyoscyamus niger* and *Datura stramonium*, which can produce hyoscyamine and scopolamine [7,8]. It has been reported that overexpression of the TRI gene can considerably enhance the production of tropane alkaloids in the root cultures of *Atropa belladonna* [4]. A TRII cDNA that does not accumulate the classical tropane alkaloids was also cloned from potato [9]. TRs are members of a short-chain dehydrogenase family with TR-homologous genes represented in plant species (e.g. *Arabidopsis thaliana*, *Solanum tuberosum*, *Cochlearia officinalis*) that do not produce tropane alkaloids, suggesting that these enzymes may function in otherwise unrelated metabolic pathways [9,10].

Anisodus acutangulus is a solanaceous perennial plant that is endemic to China and classified as an endangered species in China. As it contains tropane alkaloids such as hyoscyamine and scopolamine, it has been used as a traditional herbal anaesthetic medicine in Yunnan Province for several hundred of years [11]. It has been reported previously that total plant alkaloids reached levels of $>1.2\%$ of dry weight in wild *A. acutangulus*, which is much higher than in the common Solanaceae species, such as *A. belladonna*, *D. stramonium* and *H. niger*, so it has been an attractive resource-plant species for the production of tropane alkaloids in China [12,13,14].

In the present study, we report for the first time the cloning and characterization of TRI and TRII from *A. acutangulus* by RACE (rapid amplification of cDNA ends). The expression of both genes in various tissues and the expression pattern after MeJA (methyl jasmonate) treatment were investigated. A significant increase of tropane alkaloids was observed after TRI single-gene transformation in *A. acutangulus*. This work provides new useful information for further understanding the role of TRI and TRII in tropane alkaloid biosythesis and molecular regulation of tropane alkaloid production in *A. acutangulus*.

Materials and methods

Materials and RNA isolation

A. acutangulus plants, collected from Yunnan Province, China, were grown in pots in the greenhouse of our laboratory at 25 °C with a 16 h light period (white fluorescent tubes; irradiance 350 μmol $\cdot$ m^{-2} $\cdot$ s^{-1}) and relative air humidity of 50%. For investigating induction by MeJA elicitor, sterile cotton dipped into 100 μM MeJA (Sigma–Aldrich), was placed into pots. Plants (4 weeks old) in pots were treated for 12 and 24 h by the volatilization of MeJA. Untreated plants were used as controls. Roots, stems and leaves of *A. acutangulus* plants were collected and immediately used for RNA extraction using the method reported previously [15]. The quality and concentration of RNA was checked and stored as described in [16,17]. *A. acutangulus* hairy roots were obtained as described previously [13]. Hairy roots (21 days old) were subjected to various treatments, including 500 and 200 μM MeJA respectively, using untreated hairy roots as controls. The samples were harvested 0, 12, 24 and 48 h after treatment and RNA was extracted for further analyses of AaTR (*A. acutangulus* TR) expression by RT–PCR (reverse-transcription PCR). Each treatment was repeated three times.

Molecular cloning of the full-length TRI and TRII cDNAs by RACE

First-strand cDNA was synthesized by the 3′-RACE System (Gibco BRL) as described by Kai et al. [12]. For amplification of the 3′-end of AaTRI and AaTRII, the specific primers TRIF2 (5′-ATTCGGGTCAATTCAGTTGCTCC-3′) and TRIIF2 (5′-TAAAGGTTTTAATGTTGAAGC-3′) were designed according to the conserved region of several known TRI and TRII gene sequences from particular Solanaceae species such as *H. niger*, *D. stramonium* and *S. tuberosum*. The 3′-RACE was performed using primers TRIF2 and TRIIF2 as the forward primer and the

AUAP (Abridged Universal Amplification Primer: 5′-GGCCACGCGTCGACTAGTAC-3′) as the reverse primer in a total volume of 50 μl containing 2 μl of cDNA, 10 μM F2 primer, 10 μM AUAP, 10 μmol of dNTPs, 1 $\times$ Ex PCR buffer and 5 units of Ex Taq™ polymerase (TaKaRa). PCR was performed using the following protocol: the template was denatured at 94 °C for 3 min followed by 35 cycles of amplification (94 °C for 50 s, 58 °C for 50 s, 72 °C for 90 s) and by a final 10 min at 72 °C. The PCR product was subcloned into the pMD18-T vector (TaKaRa) and sequenced.

The first-strand cDNA synthesis in 5′-RACE was performed using the SMART™ RACE cDNA Amplification Kit according to the manufacturer's instructions (Clontech) using the 5′-RACE CDS Primer (5′-T25N-1N-3′). Based on the sequence of the 3′-RACE product, the complementary reverse gene-specific primer TRIR2 (5′-TGAGGATTTTTCTTAATTGCAG-3′) and AaTRIIR2 (5′-ATAAGTGGTAAGCAGCCTCAAAG-3′) were designed to amplify the 5′-end of the TRs. The 5′-RACE PCR was carried out using the R2 primers (TRIR2 and TRIIR2 respectively) and Universal Primer A Mix provided by the kit under the conditions described in [12].

Based on the nucleotide sequences of the 3′- and 5′-RACE products, the full-length cDNA sequences of AaTRI and AaTRII were obtained and the cDNAs were subsequently amplified by PCR using a pair of primers, AaTRIF1 (5′-AAAGGAAAACCAATTGTCACATTTG-3′) and AUAP, and AaTRIIF1 (5′-AAATCAACTCGAGGACCACTTCA-3′) and AUAP; the PCRs were repeated three times. The amplified full-length cDNA of AaTRI and AaTRII were used for molecular characterizations such as sequence homology, the presence of conserved motifs and phylogenetic-tree reconstruction.

Bioinformatics analyses

The ORF (open reading frame) Finder graphical analysis tool on the NCBI (National Center for Biotechnology Information) website (www.ncbi.nlm.nih.gov/projects/gorf/) was used to predict coding sequences and the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to find similarity for AaTR cDNAs with other TR cDNAs in online databases (http://www.ncbi.nlm.nih.gov). ClustalX was used for sequence alignment and phylogenetic analysis. A phylogenetic tree was constructed by the neighbour-joining method and the reliability of each node was established by bootstrap methods using MEGA2 software (http://www.megasoftware.net).

Expression-profile analysis

One-Step RT–PCR (TaKaRa) was carried out to investigate the expression profiles of TRs in different tissues including roots, stems and leaves of *A. acutangulus* treated with and without MeJA-elicitor treatment, as well as hairy roots treated with different concentrations (500 and 200 μM) of MeJA for various time periods. All RNA templates were digested with RNase-free DNase I (Qiagen). Aliquots of total RNA (0.5 μg) extracted from roots, stems, leaves and hairy roots respectively of *A. acutangulus* were used as templates in one-step RT–PCR reactions. The primers TRIKF1 (5′-ATGGGAGAATCAAAAGTTTACAT-3′) and TRIKR1 (5′-TCAAAATCCACCATTAGCTGTGA-3′) for AaTRI, and TRIIKF1 (5′-ATGGCAGGAAGGTGGAATCTTGA-3′) and TRIIKR1 (5′-TTAAAACCCACCATTAGCCATAA-3′) for AaTRII, were used. Meanwhile, RT–PCR reactions for a housekeeping gene (the 18S rRNA gene, which is highly conserved in plants), using the specific primers 18SF (5′-GTGACAATGGAACTGGAATGG-3′) and 18SR (5′-AGACGGAGGATAGCGTGAGG-3′) designed on the basis of the conserved regions of plant 18S rRNA genes, were performed to estimate whether equal amounts of RNA among samples were used as an internal control.

Construction of AaTRI expression vector and plant transformation

The vectors pBI121 (Clontech) and pCAMBIA1304 (Dr R. Jefferson, CAMBIA) were double-digested with HindIII and EcoRI. The purified HindIII/EcoRI small DNA fragment containing a GUS expression cassette from pBI121 was cloned into the large pCAMBIA1304 fragment to generate the recombinant plasmid pCAMBIA1304+. Full-length TRI cDNA was used to replace the GUS gene of the GUS gene expression cassette from pBI121 to generate the recombinant expression vector pCAMBIA1304+-AaTRI. The vector pCAMBIA1304+ contained the TRI gene of *A. acutangulus* under the control of the CaMV (cauliflower mosaic virus) 35S promoter. The disarmed *Agrobacterium tumefaciens* strain C58C1 harbouring both the *Agrobacterium rhizogenes* Ri plasmid pRiA4 and pCAMBIA1304+, containing a single TRI gene, was used for plant transformation [2]. The blank vector pCAMBIA1304+ without the AaTRI gene was used as a control.

Sterile leaf sections of *A. acutangulus* were inoculated with *A. tumefaciens* strain C58C1 (containing pRiA4 and pCAMBIA1304+) carrying the AaTRI gene. Wild-type plants were grown in the same growth chamber. Hairy roots generated at cutting edges after 4 weeks of co-cultivation were excised and cultivated individually on solid, hormone-free B5 medium (PhytoTechnology) supplemented with 500 mg/l cefotaxime and 100 mg/l kanamycin (Asia Pioneer). Rapidly growing root lines that showed hygromycin resistance with no bacterial contamination were used to establish hairy-root lines. Approx. 100 mg of fresh roots were inoculated into 150-ml conical flasks containing 50 ml of liquid 1/2 MS (Murashige and Skoog) medium and

maintained on an orbital shaker at 100 rev./min and 25 °C in the dark. The hairy-root clones were routinely subcultured every 2 weeks as described previously [13,18].

PCR analysis and alkaloid analysis

Genomic DNA was isolated from hairy-root samples using the CTAB (cetyltrimethylammonium bromide) method [19]. The DNA was then used in PCR analysis for detecting the presence of *Agrobacterium* rolB, rolC and AaTRI genes in transgenic hairy roots, following the method described previously [20,21]. The PCR primers used for TRI detection were TRIKF1 and TRIKR1. PCR was performed as described above, the only condition change being a 50-s extension.

Hairy roots were examined for their alkaloid production after 6 weeks of culture in liquid 1/2 MS medium. Tropane alkaloids (hyoscyamine and scopolamine) were extracted and analysed by HPLC as described by Li et al. [13].

Statistical analysis

Results are presented as mean values ± S.D. calculated from three sets of independent experiments. The statistical significance of alkaloid difference was analysed by Student's *t* test using SPSS 17.0 software (SPSS, Inc.). A *P*-value of $P < 0.05$ was considered to indicate a statistically significant difference in alkaloid compared with the control.

Results and discussion

Cloning and sequencing of the full-length cDNAs of AaTRI and AaTRII

Based on the highly conserved sequences of plant TRs, the full-length cDNAs of AaTRI and AaTRII were isolated from young roots of *A. acutangulus*, using one-step RT–PCR. The cloned full-length cDNA of AaTRI (GenBank® accession number EU424321) was 1188 bp, which contained a 822-bp ORF encoding a deduced protein of 273 amino acid residues. The cloned full-length cDNA of AaTRII (GenBank® accession number EU424322) was 1116 bp, which contained a 783-bp ORF encoding a deduced protein of 260 amino acid residues. The deduced protein AaTRI had a pI of 6.45 and a calculated molecular mass of approx. 29.5 kDa. The deduced protein AaTRII had a pI of 5.35 and a calculated molecular mass of approx. 28.3 kDa. A BLAST search revealed that cDNAs of AaTRs showed high homology (>86% identity) to some known TR cDNAs from Solanaceae species such as *H. niger*, *D. stramonium* and *S. tuberosum*.

Comparative and bioinformatic analysis of AaTRs

The deduced amino acid sequences of AaTRs were submitted to NCBI for PSI-BLAST searching (http://www.ncbi.nlm.nih.gov/BLAST/) and the results showed that AaTRs had high homology with TR sequences from other plant species. At the amino acid level, AaTRI was 90%, 87%, 86% and 57% identical with HnTRI (*H. niger* TRI; NCBI accession number BAA13547), DsTRI (*D. stramonium* TRI; NCBI accession number AAA33281), StTRI (*S. tuberosum* TRI; NCBI accession number CAC34420) and CsTRI (*Calystegia sepium* TRI; NCBI accession number CAD20555) respectively, and AaTRI was 96%, 94%, 95% and 74% similar to HnTRI, DsTRI, StTRI and CsTRI respectively. At the amino acid level, AaTRII was 97%, 97% and 93% identical with HnTRII (NCBI accession number P50164), StTRII (NCBI accession number CAB52307) and DsTRII (NCBI accession number AAA33282) respectively, and was 98%, 98% and 96% similar to HnTRII, StTRII and DsTRII respectively (Figure 1). AaTRs showed high similarity to other TRs from some solanaceous species, indicating that there is high conservation among plant TRs.

The deduced amino acid sequences of AaTRs also shared significant similarities with enzymes in nonsolanaceous species, such as AtPTR (*Arabidopsis thaliana* putative TR; NCBI accession number AAC02738), AtSDR (*A. thaliana* short-chain dehydrogenase/reductase; NCBI accession number NP_850131), VvPro (*Vitis vinifera* unnamed protein product; NCBI accession number CAO15693) and PtTR (*Populus trichocarpa* unknown protein; NCBI accession number ABK94936). Typical motifs of the SDRs are evident in AaTR sequences. The catalytic tyrosine in the sequence YXXXK is present at position 171 (Figure 1); this residue was found to be essential for SDRs, judging from crystal structures and site-directed mutations [22]. The orientation of the substrate in the active centres is crucial for the stereospecific reduction of tropinone to pseudotropine [23,24]. Pseudotropine-forming TRs contain a tyrosine at position 113 and a valine at position 169 (labelled by black arrows in Figure 1), whereas TRI has His^{113} and Glu^{169}.

Molecular-evolution analysis

At the amino acid level, AaTRI shows high similarity to TRI of other Solanaceae plants, but has only 60.8% identity with AaTRII, therefore it would be interesting to investigate its evolutionary position in the phylogenetic tree of various TRs and SDRs. A phylogenetic tree was constructed as described above. As shown in Figure 2, the result showed that AaTRII, HnTRII, DsTRII and StTRII were grouped into one cluster as expected (Group I), whereas AaTRI, HnTRI, DsTRI and StTRI were naturally classified into another cluster (Group II). Unexpectedly, CsTRI formed one cluster independently (Group III). SDRs from plants that do not produce tropane alkaloids, such as PtTR, AtPTR and AtSDR, belong to a fourth cluster (Group IV) which is different from TRs of tropane-producing plants. AaTRI and AaTRII belong to different clusters, but solanaceous TRI and TRII were derived from an ancestor.

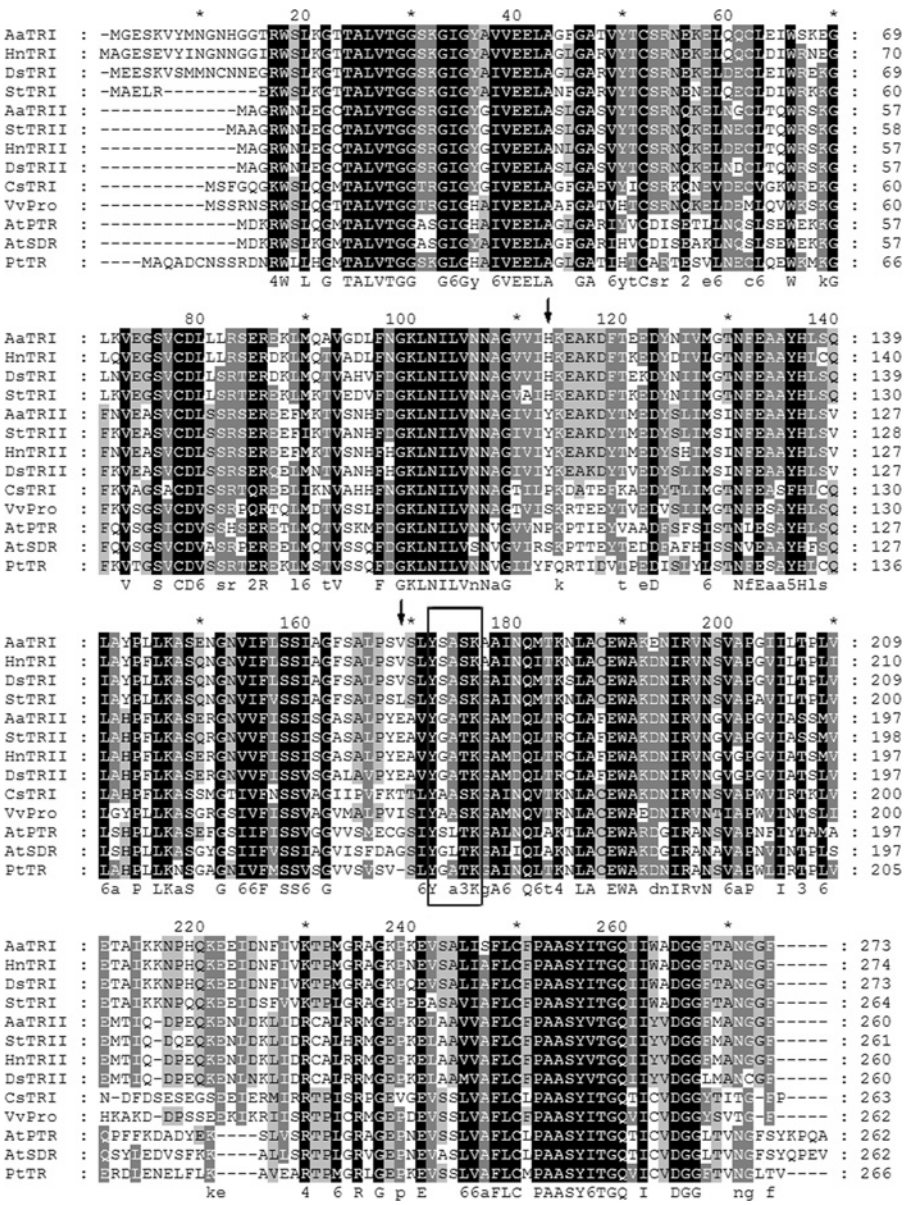


Figure 1 Multiple sequence alignments of AaTRs with other known plant TRs and SDRs

HnTRI, DsTRI, StTRI, CsTRI, HnTRII, StTRII, DsTRII, AtPTR, AtSDR, VvPro and PtTR were used for alignments. Black boxes and grey boxes indicate the completely identical residues and the conserved residues among the aligned sequences respectively.

It has been reported that two TRs with different stereospecificities are short-chain dehydrogenases that evolved from a common ancestor. Gene duplication and mutation produced two specialized TRs with strict specificity [8]. In the *A. thaliana* genome, 19 'putative TRs' are annotated, two of which are pseudogenes (http://www.arabidopsis.org). One putative TR was expressed in *Escherichia coli*, but it did not reduce tropinone or nortropinone [10]. It is more likely that the TR genes are widely present but active only in a few species that produce tropane alkaloids. TRI from *C. sepium* does not belong to Group I or Group II. Calystegines have been reported from three families of vascular plants, Convolvulaceae, Solanaceae and Moraceae. Solanaceae and Convolvulaceae are closely related and are in the order Solanales. Since *C. sepium* belongs to the Convolvulaceae [25], it is not difficult to understand that TRI from *C. sepium* appears adjacent to Solanaceae TRs.

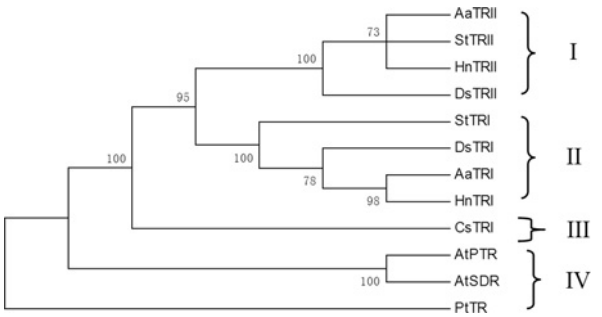


Figure 2 Phylogenetic-tree analysis of AaTRs with other plant TRs and SDRs

HnTRI, DsTRI, StTRI, CsTRI, HnTRII, StTRII, DsTRII, AtPTR, AtSDR and PtTR were used for molecular-evolution analysis.

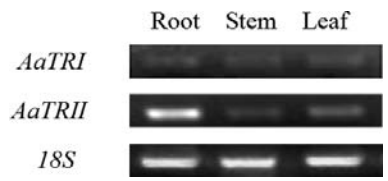


Figure 3 Expression-pattern analyses of AaTRs in different tissues of *A. acutangulus*

Total RNA (0.5 μg) was isolated from root, stem and leaf respectively and subjected to one-step RT–PCR amplification. The 18S rRNA gene was used as a control to show the normalization of the templates in PCR reactions (lower panel).

Expression-pattern analyses of AaTRs in different tissues of *A. acutangulus*

To investigate the expression profile of AaTRs in different tissues of *A. acutangulus*, total RNA was isolated from root, stem and leaf tissues and subjected to one-step RT–PCR using primers TRIKF1 and TRIKR1 for TRI, and TRIIKF1 and TRIIKR1 for TRII. As shown in Figure 3, both AaTRI and AaTRII gene transcripts could be detected in all the tested tissues, suggesting that TRI and TRII are constitutively expressed genes. The transcript level of TRI in all tissues was low, whereas TRII transcripts could be detected at high levels in all tissues. In total, the TRI transcription level in the three tissues was much lower than TRII. This fact is in accordance with the observations in other Solanaceous species such as *H. niger* and *A. belladonna* [26,27]. TRII activity was found to be stronger in many Solanaceae tissues; shortly after application of tropinone, pseudotropine accumulated faster than tropine [22,28]. The converse was found in most organs of *D. stramonium*: TRI activities were higher than TRII activities [5], reflecting that the complexity of AaTR expression may diverge significantly in different plant species.

The belowground organs (roots) of tropane-alkaloid-producing plants, such as *A. acutangulus* and *H. niger*, are usually used as the main source for tropane-alkaloid extraction, as the tropane-alkaloid content in belowground tissues is higher than that in aerial parts of plants [29,30]. Hence, it would be interesting to know whether AaTR expression is positively correlated with the tropane-alkaloid content in different parts of the *A. acutangulus* plant. It has been reported that strong TRI and TRII activities were found in the root tissues of *H. niger*, *A. belladonna* and *D. stramonium* [5]. As shown in Figure 3, AaTRI and AaTRII gene transcripts could be detected in all the tested tissues but at different transcription levels. TRI transcripts did not show any obvious difference in all the tested tissues, whereas TRII transcripts were detected at highest level in roots, which was similar to the observation in other solanaceous plants reported previously [5]. H6H and PMT (putrescine *N*-methyltransferase) genes, which encode the key enzyme involved in biosynthesis of tropane alkaloids, were successfully cloned in *A. acutangulus* [12,31]. Interestingly, AaH6H and AaPMT expression were detected at the highest level in roots, which is similar to AaTRII.

Expression profiles of AaTRs under induction of MeJA elicitor

The action of JA (jasmonic acid) and MeJA as elicitors of secondary metabolites in plants is well known. Furthermore, the accumulation of several classes of alkaloids is strongly stimulated upon treatment with MeJA [32]. One-step RT–PCR analysis was carried out to monitor the changes of AaTRI and AaTRII expression upon MeJA-elicitor treatment. The result showed that the expression of AaTRII varied with treatment time (Figure 4A). AaTRII transcription was strongly induced by MeJA, reaching the highest level after 24 h of treatment in root and stem. The AaTRII transcription level was highest in leaf after 12 h of MeJA treatment. However, AaTRI seemed to be induced more weakly by MeJA in all tissues compared with AaTRII (Figure 4B). Overall, AaTRI transcription in all conditions still maintained the lower level. The results suggested that AaTRII is a MeJA-responsive gene and can be effectively elicited, at least at the transcriptional level, reflecting that MeJA may stimulate the accumulation of calystegines and not hyoscyamine or scopolamine in *A. acutangulus*. In addition, AaH6H expression was also strongly elicited by MeJA at least in root and leaf, while no obvious change was observed in stem (Figure 4C). By comparing the expression of AaTRs and AaH6H as discussed above, it seemed to be the case that AaTRI was not obviously induced by MeJA in the tested tissues.

The effects of MeJA at different concentrations (500 μM and 200 μM) on the expression of AaTRI and AaTRII in *A. acutangulus* hairy roots for different treatment times were investigated. Unexpectedly, the results showed

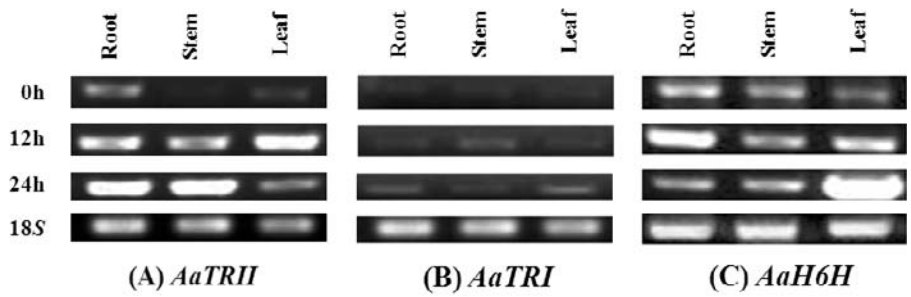


Figure 4 Expression profiles of AaTRs and AaH6H after 50 μM MeJA-elicitor treatment for 0 h, 12 h and 24 h

(**A**) AaTRII transcript levels in root, stem and leaf after MeJA treatment. (**B**) AaTRI transcript levels in root, stem and leaf after MeJA treatment. (**C**) AaH6H transcript levels in root, stem and leaf after MeJA treatment.

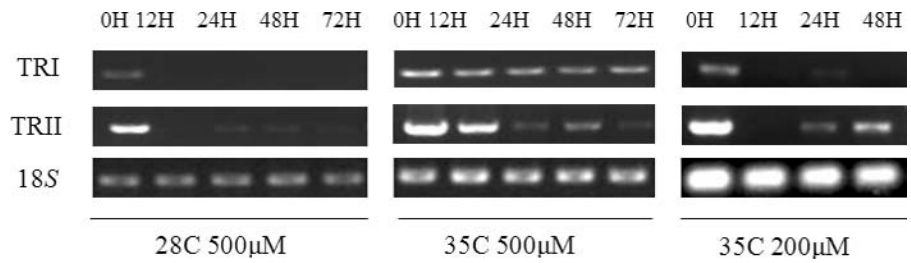


Figure 5 Expression profiles of AaTRs in hairy roots after 500 μM and 200 μM MeJA-elicitor treatment for 0 h, 12 h, 24 h 48 h and 72 h

28C, 28 cycles; 35C, 35 cycles.

that MeJA seemed to obviously inhibit expression of AaTRI and AaTRII to various extents in *A. acutangulus* hairy roots (Figure 5), in contrast with the results in plant tissues (Figure 4). This may be due to a different, tissue-specific regulation of the endogenous genes in response to the foreign elicitor at the transcriptional level.

The MeJA treatments always increased alkaloid accumulation in many tropane-alkaloid-producing species, such as *H. niger*, *D. stramonium* and *Scopolia parviflora* [18,33,34]. PMT, TRI and H6H are committed enzymes in tropane-alkaloid biosynthesis. PMT is the first committed enzyme in the biosynthetic pathways of tropane alkaloids. It is observed that PMT is inducible by MeJA in *A. acutangulus* [12]. However, the AaTRI gene is induced very weakly by MeJA, so it may be deduced that there is limited contribution to enhanced tropane alkaloids after MeJA treatment in *A. acutangulus*. Considering the observation that the TRI transcription level was much lower than TRII, it was deduced that it would be possible to improve tropane alkaloid production in *A. acutangulus* by overexpressing AaTRI.

Molecular analysis of genetically engineered hairy roots

In total, 39 TRI hairy-root lines were generated, of which 18 lines survived the subsequent subculture process. Control hairy-root lines generated from blank-vector transformations were denoted as C. All of the hairy roots contained the rol genes (rolB, rolC) as revealed by PCR analysis (Figures 6A and 6B). The integration of TRI in transformed hairy roots was also confirmed (Figure 6C). The TRI-PCR-positive hairy-root lines amounted to 55.56% (10/18). None of the checked DNA bands were amplified from the control wild-type root and control hairy roots.

Tropane-alkaloid-production analysis of genetically engineered hairy roots

The capacities of transgenic root lines to biosynthesize hyoscyamine and scopolamine are shown in Figure 7. Hyoscyamine and scopolamine production in control hairy-root lines was 2.90 mg per g of dry weight and 0.01 mg per g of dry weight (mean values) respectively. Hyoscyamine accumulation in TRI-single-gene transgenic hairy-root lines (117, 119, 120, 122 and 138) reached 4.60, 4.29, 5.79, 9.11 and 3.35 mg per g of dry weight respectively, and the mean hyoscyamine production of the above lines was 5.43 mg per g of dry weight. The hyoscyamine level in lines 117, 120 and 122 was significantly higher than that in the control ($P < 0.05$). Among all of the transgenic lines, the best line, 122, showed that the hyoscyamine content was 3.14-fold higher than the control. Scopolamine accumulation in these

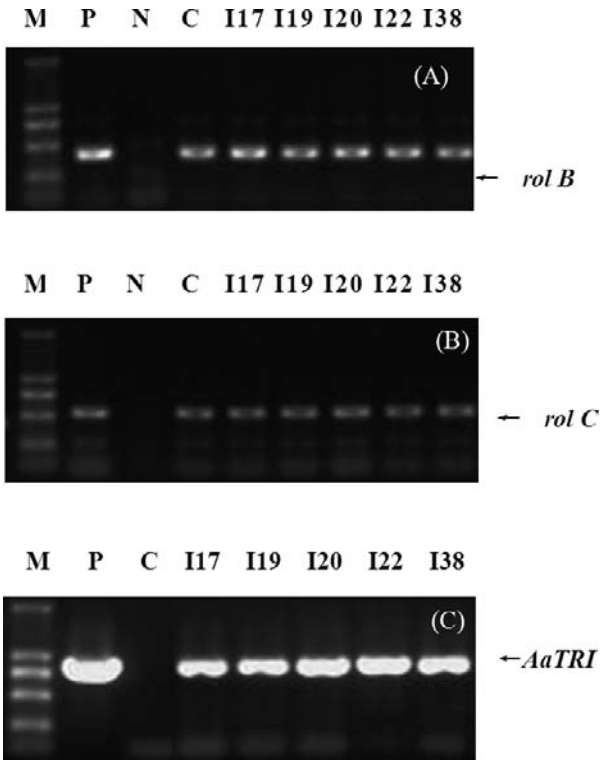


Figure 6 Representative PCR analyses for determining the presence of rolB, rolC and TRI genes in transgenic hairy-root lines

(**A**) Detection of the rolB gene. (**B**) Detection of the rolC gene. (**C**) Detection of the TRI gene. M, DL 2000 DNA Marker (TaKaRa; 100–2000 bp); P, pRiA4 (positive control); N, wild-type *A. acutangulus* root (negative control); C, control hairy root generated from blank-expression-vector transformations; I, different TRI-transformed hairy-root lines of *A. acutangulus*.

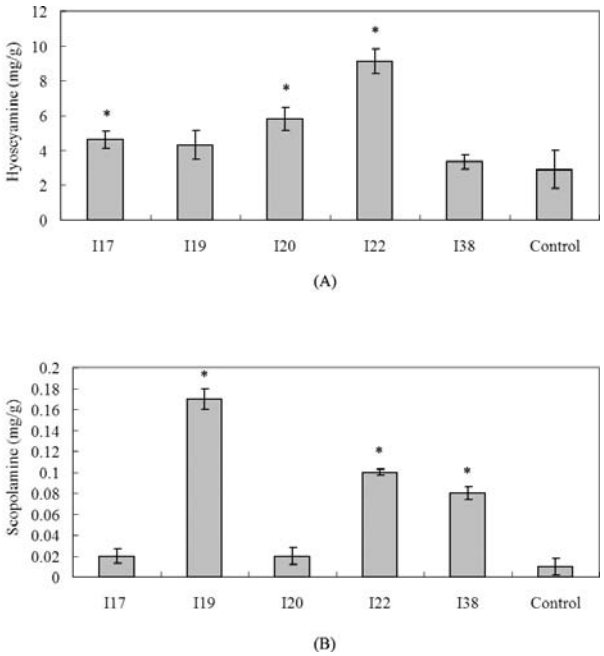


Figure 7 (A) Hyoscyamine and (B) scopolamine production in various hairy-root lines analysed by HPLC

Control hairy-root lines were generated from blank transformations. I, different TRI-transformed hairy-root lines of *A. acutangulus*. The values are means ± S.D of triplicate analyses. Asterisks indicate a result significantly different from that of the control ($P < 0.05$).

transgenic hairy-root lines reached 0.02, 0.17, 0.02, 0.10 and 0.08 mg per g of dry weight respectively, and the mean scopolamine production of the above lines was 0.08 mg per g of dry weight, among which scopolamine was increased 17-fold compared with control hairy root in the best line, I19. Root lines I19, I22 and I38 displayed significantly higher scopolamine content than the control line ($P < 0.05$).

In the present study, alkaloids (hyoscyamine and scopolamine) produced in the TRI-transformed hairy-root lines (I lines) are produced at much higher levels compared with the control lines, which indicated that AaTRI is a promising target site of genetic engineering to increase tropane alkaloids in *A. acutangulus*. The mean hyoscyamine production increased by 87% and scopolamine also increased up to 8-fold in the TRI-transformed root lines compared with control. It is obvious that although scopolamine content in some control hairy-root lines was too low to be detected, scopolamine could be detected clearly in all TRI-transformed root cultures. Scopolamine production was improved obviously in TRI-transformed roots. This phenomenon could be explained by the fact that the content of hyoscyamine is impressively high, but scopolamine is very limited, in wild *A. acutangulus*, which is a traditional medicine plant in China [13]. Our observation is consistent with the results of TRI single-gene overexpression in *A. belladonna*. In the TRI-transformed hairy-root lines of *A. belladonna*, hyoscyamine increased up to nearly 3-fold and scopolamine also increased up to 5-fold [4]. The increased scopolamine production in *A. acutangulus* and *A. belladonna* indicated that probably more hyoscyamine was available for the H6H to produce scopolamine. Until now, a significant increase in tropane alkaloids after TRI single-gene transformation had been observed only in *A. acutangulus* and *A. belladonna* [4].

The production of tropane alkaloids could be significantly increased by various approaches, such as overexpressing genes encoding the key enzymes in the biosynthetic pathway. Several genes such as PMT, TRI and H6H in the tropane-alkaloid biosynthesis pathways have been cloned [7,8,12,31], providing possibilities to enhance the yield of these medicinal alkaloids by metabolic engineering. Being the first committed step in the tropane-alkaloid biosynthesis pathway, PMT-gene overexpression can enhance total alkaloid production in *Hyoscyamus muticus* [35], but not effectively in *A. belladonna* [36], *H. niger* [18] and *Duboisia* hybrid [36]. In most of the above tested plants,

overexpression of the PMT gene did not boost tropane-alkaloid biosynthesis effectively, which may be explained by the fact that PMT was an earlier step and its effect could be influenced by later committed steps in the pathway. The H6H gene encodes the enzyme that catalyses hyoscyamine to scopolamine, which is the final committed step in the tropane-alkaloid biosynthetic pathway; it has been well documented that scopolamine production is greatly enhanced by H6H overexpression in *H. niger*, *H. muticus* and *Atropa baetica* [2,37,38]. The strong total alkaloid augmentation observed after simultaneous transformation of *H. niger* hairy-root cultures with PMT and H6H is impressive. It should also be noted that overexpressing multiple biosynthetic genes is crucial for improved production of useful plant secondary metabolites [2].

TRI and TRII constitute a branching point in the biosynthesis of tropane alkaloids. How the metabolite flux is controlled by these reductases is an interesting question, particularly in view of our observation that the transcript level of TRI was much lower than TRII in *A. acutangulus*. The present study highlights the enhancement effects of total alkaloid production in TRI-overexpressing *A. acutangulus* hairy roots, which was consistent with that in *A.belladonna* reported by Richter et al. [4], suggesting that the TRI gene is a new promising target site for guiding the metabolic flux towards the tropane-alkaloid pathway (target pathway) away from the pathway involved in calystegine production (side pathway). Most importantly, the AaH6H and AaTRI genes have been cloned by our laboratory and these two genes are the final genes involved in the formation of scopolamine instead of calystegines [31], whereas PMT participates in earlier steps of the pathway [12]. To enhance the levels of the most important tropane alkaloids (hyoscyamine and scopolamine), co-overexpression of multiple biosynthetic genes such as AaTRI and AaH6H in *A. acutangulus* is a promising strategy in the near future. This co-expression should boost the accumulation of scopolamine, an alkaloid that is medicinally more interesting and in higher demand.

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