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ORIGINAL ARTICLE

Genetically engineered hairy root cultures of *Hyoscyamus senecionis* and *H. muticus*: ploidy as a promising parameter in the metabolic engineering of tropane alkaloids

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Abstract

Key message **Tetraploidy improves overexpression of *h6h* and scopolamine production of *H. muticus,* while in *H. senecionis, pmt* overexpression and elicitation can be used as effective methods for increasing tropane alkaloids**.

Abstract The effects of metabolic engineering in a polyploid context were studied by overexpression of *h6h* in the tetraploid hairy root cultures of *H. muticus*. Flow cytometry analysis indicated genetic stability in the majority of the clones, while only a few clones showed genetic instability. Among all the diploid and tetraploid clones, the highest level of *h6h* transgene expression and scopolamine accumulation was interestingly observed in the tetraploid clones of *H. muticus*. Therefore, metabolic engineering of the tropane biosynthetic pathway in polyploids is suggested as a potential system for increasing the production of tropane alkaloids. Transgenic hairy root cultures of *Hyoscyamus senecionis* were also established. While overexpression of *pmt* in *H. senecionis* was correlated with a sharp increase in hyoscyamine production, the *h6h*-overexpressing clones were not able to accumulate higher levels of scopolamine than the leaves of intact plants. Applying methyl jasmonate was followed by a sharp increase in the expression of *pmt* and a drop in the expression of tropinone reductase II (*trII*) which consequently resulted in the higher biosynthesis of hyoscyamine and total alkaloids in *H. senecionis*.

Keywords Hairy roots · Metabolic engineering · Methyl jasmonate · Tetraploidy · Tropane alkaloids

Abbreviations

BSTFA	*N,O*-Bis (trimethylsilyl)trifluoroacetamide
GC	Gas chromatography
GC–MS	Gas chromatography–mass spectrometry
MeJa	Methyl jasmonate
MS	Murashige and Skoog
PMT	Putrescine *N*-methyltransferase
TMCS	Trimethylchlorosilane
TRI	Tropinone reductase I
TRII	Tropinone reductase II
TA	Tropane alkaloids

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Introduction

Tropane alkaloids (TAs) such as hyoscyamine and scopolamine are among the oldest drugs in medicine, with wide-ranging pharmaceutical applications for their mydriatic, antispasmodic, anticholinergic, analgesic, and sedative properties (Arroo et al. 2007; Yamada et al. 1990). Chemical synthesis of tropane alkaloids is not economically feasible, so commercial production of these compounds is entirely dependent on their isolation from various members of the Solanaceae family, including the genera *Atropa*, *Duboisia*, *Datura,* and *Hyoscyamus* (Mateus et al. 2000; Oksman-Caldentey and Arroo 2000). Scopolamine is the most valuable TA due to its higher pharmacological activity, fewer side-effects, and relatively limited supply (Rischer et al. 2013). The worldwide demand for scopolamine is about ten times greater than for hyoscyamine (Hashimoto et al. 1993; Oksman-Caldentey and Arroo 2000). Accordingly, there has been a long-standing interest in increasing the TA content of plants (especially scopolamine) and their in vitro cultured tissues by the conventional and biotechnological approaches (Dehghan et al. 2012; Palazón et al. 2003; Wang et al. 2011).

Hairy root cultures offer many advantages such as high genetic stability, rapid and hormone-free growth, and the ability to synthesize the same compounds as the roots of the intact plant. They have been considered as a promising approach to TA production in several solanaceous plants (Cardillo et al. 2010; Zhang et al. 2004). The biosynthetic pathway of tropane alkaloids and key enzyme genes has been identified (Kai et al. 2007; Li et al. 2006; Portsteffen et al. 1994) (Fig. S1). In the TA biosynthetic pathway, putrescine *N*-methyltransferase (PMT) is considered as the first rate-limiting upstream enzyme, while hyoscyamine 6β-hydroxylase (EC 1.14.11.11, H6H) is a key enzyme catalyzing the last two steps in scopolamine biosynthesis. Engineering of the tropane pathway genes, especially *h6h* and *pmt*, is reported to be a feasible approach to enhance the production of hyoscyamine and scopolamine in plants or in vitro cultures of *H. muticus* (Jouhikainen et al. 1999; Moyano et al. 2003), *H. niger* (Zhang et al. 2004, 2007), *Duboisia* hybrids (Palazón et al. 2003), *Atropa baetica* (Zárate et al. 2006), and *Atropa belladonna* (Wang et al. 2011).

Hyoscyamus senecionis var. *bipinnatisectus* is a rare herbaceous perennial medicinal plant belonging to the family Solanaceae, which grows at high altitudes in the Iranian plateau (Khatamsaz 1998). Our recent study indicated a higher content of scopolamine than its precursor hyoscyamine in *H. senecionis* (Dehghan et al. 2013); however, productivity of TA in its in vitro cultures has not been explored yet. Egyptian henbane (*H. muticus*) is another important TA-producing species, but with a high hyoscyamine and low scopolamine content. We previously reported the higher potential for scopolamine production in autotetraploid plants and hairy root cultures of this species, compared to their diploid counterparts (Dehghan et al. 2012). To explore the potential of metabolic engineering of the TA biosynthesis pathway, overexpression of *pmt* and *h6h* genes in hairy root cultures of *H. senecionis* and diploid and tetraploid plants of *H. muticus* (strain Cairo) was investigated. The expression levels of the key genes and metabolites of the TA pathway in methyl jasmonate-elicited hairy root cultures of *H. senecionis* were also studied.

Materials and methods

Bacterial strains

The *Agrobacterium tumefaciens* strain C58C1 (pRiA4), containing the *pmt* gene from *Nicotiana tabacum* L. under the control of the constitutive CaMV 35S promoter (Moyano et al. 2002, 2003), and *A. rhizogenes* strains, LBA9402, carrying pLAL21 plasmid, containing the *h6h* gene from *H. niger* under the control of CaMV 35S promoter (Jouhikainen et al. 1999) and A4 were used for plant transformation. The *Agrobacterium* strains were grown at 28 °C in YMB medium.

Establishment of hairy roots and culture conditions

Diploid and the seventh generation (C7) of induced autotetraploid plants of *H. muticus* (Dehghan et al. 2012) were grown from seeds under greenhouse conditions at 25 ± 2 °C, with a photoperiod of 16 h light/8 h darkness. Young leaves of diploid and tetraploid plants were used for transformation with the disarmed *A. tumefaciens* strain C58C1 carrying the pRiA4 of *A. rhizogenes* and the binary vector pBMI and *A. rhizogenes* strains, LBA9402, carrying the pLAL21 plasmid, and A4 as described before (Dehghan et al. 2012).

The transformed roots that developed at wound sites were excised and cultured individually in half-strength liquid MS medium (Murashige and Skoog 1962) containing 3% sucrose and 500 mg/l cefotaxime to eliminate the excess bacteria, and grown at 25 ± 2 °C on a gyratory shaker (Heidolph, Germany). Root clones were kept in darkness and subcultured by transferring about 125 mg fresh weight of young roots to 25 ml fresh medium at 4-week intervals.

Young sterilized leaves and nodal segments of *H. senecionis* plants grown under the stated greenhouse conditions were used for inoculation with *A. tumefaciens* strain C58C1 (pRiA4) and *A. rhizogenes* strains LBA9402 (carrying pLAL21 plasmid) and A4. Nodal segments were inoculated by the same aforementioned method for *H. muticus* and leaf explants were submerged in the bacterial

suspension for 10 min. Transformed roots appeared after 1–4 weeks of culture, and were grown under the conditions described above.

MeJa treatment

The 3-week-old *h6h*-transgenic and *pmt* transgenic clones of *H. senecionis* with a good growth rate and normal phenotype were incubated in half-strength liquid MS medium and subjected to duplicate 100-μM MeJa (Sigma) treatments of 0, 12, 24, and 48 h. A portion of each sample was harvested for RNA extraction and the remainder was freeze-dried for alkaloid analyses. Plants were grown in the same conditions as mentioned earlier and MeJa (100 μM) was sprayed on the intact plants twice a day. Leaf samples were flash-frozen in liquid nitrogen, freeze-dried, and kept at −80 °C prior to alkaloid extraction.

Polymerase chain reaction analysis

PCR analyses were performed on DNA (Saghai-Maroof et al. 1984) extracted from transgenic and control samples. PCR primers and the procedure for *rol*C were designed and conducted according to Dehghan et al. (2012). To avoid the interference of *Agrobacterium* in the PCR results, *Agrobacterium vir*D1 was used as negative control. The oligonucleotide primers of 5′-GAAAGATATATTTCTCAAGA-3′ and 5′-TCTCCAAATGAAATGAACTTC-3′, specific for the 35S promoter and 5′-GAACTCGTCAAGAAGGC-3′ and 5′-CAGGTTCTCCGGCCGCT-3′, specific for *npt*II, were used for the detection of transferred constructs in the transgenic *h6h* and *pmt* clones. Further confirmation was performed by the PCR primers of the *pmt* gene from *N. tabacum* L. according to Moyano et al. (2003). After separation by 1.2% agarose gel electrophoresis, amplified fragments were stained with DNA Green viewer and observed under UV light using a UVP transilluminator.

Flow cytometric (FCM) analysis

To determine the ploidy levels and genetic stability of transgenic and control hairy root cultures obtained from *H. senecionis* and diploid and tetraploid plants of *H. muticus,* flow cytometry analysis was conducted using a ploidy analyzer flow cytometer (Partec PA, Münster, Germany) equipped with a UV mercury arc lamp excitation source and filter combination for DAPI staining according to previously established protocol (Dehghan et al. 2012). Young leaves (about 2 cm^2) or young tip (about 1.5 cm) of the hairy roots was used for extracting nuclei from the cells. Tissues were chopped in the nuclei extraction buffer (Partec PA, Germany), followed by DAPI staining (Partec PA, Germany) and measurement by flow cytometer. Signal intensity of at least 10,000 nuclei was quantified using ModFit LT^{TM} program after removing debris and aggregates from the analysis.

Real-time RT-PCR analysis

Total RNA from transgenic *H. senecionis* and *H. muticus* clones as well as MeJa-treated and control samples was extracted and reverse transcribed to cDNA by procedures reported previously (Dehghan et al. 2013). Real-time quantitative PCR was performed using a Biorad Real-Time PCR System (C1000^{TM} Thermal Cycler, Biorad) and Maxima SYBR Green kit (Fermentas). Primer pairs for specific amplification of *pmt*, *h6h*, *trI,* and *trII* genes were designed as shown in Table S1. *ef-1α* was also used as the reference gene. The conditions were set as follows: an initial polymerase activation step of 94 °C for 10 min, followed by 40 cycles of 30 s at 94 °C for denaturation, 30 s at 58 °C for annealing, and 30 s of extension at 72 °C. The reactions were carried out as described previously (Dehghan et al. 2013).

Tropane alkaloid extraction and GC and GC–MS analyses

About 100 mg lyophilized samples of leaves and hairy root cultures of *H. senecionis* and *H. muticus* were extracted as previously described (Dehghan et al. 2013). Hyoscyamine, scopolamine, littorine, and 3-hydroxy littorine content of the samples were analyzed as previously described (Dehghan et al. 2013; Li et al. 2006) with the exception that both GC/MS identification of peaks and GC quantitation of alkaloids were carried out using a 30 m × 0.25 mm DB-23 column with 0.25 um film thickness (Agilent), temperature programmed from 160 to 240 °C at 4 °C per min.

Statistical analyses

Data were processed using GraphPad Prism 7.02 program. All the experiments were run at least in three replicates and the significance of the results was analyzed using an unpaired, two-tailed Student's *t* test where needed and data were presented as mean ± SD. A p value <0.05 was considered significant.

Results

Growth and morphology of the transgenic hairy roots

In both species, transgenic hairy roots emerged from the wounded sites about 1–4 weeks after *Agrobacterium* inoculation (Fig. S2). The transformation rate of leaves and

nodal segments of *H. senecionis* was about 50% (data not shown). Considerable variation was observed among clones in terms of morphology, growth, and biomass production. *Pmt* and *h6h* transgenic root clones of *H. muticus* showed a more normal morphology than those of *H. senecionis*. Several *pmt* clones of *H. senecionis* were dark brown in color with dense short-branch morphology, while *pmt* clones of *H. muticus* showed a light brown color and longer branches. While *h6h*-transgenic roots of *H. muticus* showed a normal phenotype, a range of changes, including severe callus formation, callus suspension formation, and deformed roots with wide branches, were observed among *h6h*-transgenic clones of *H. senecionis* (Fig. S2).

It is also worth noting that we observed a lower transformation rate of tetraploid (6%) *H. muticus* versus its diploid (40%) counterpart. The growth rate of tetraploid *pmt*-transgenic clones was significantly ($p < 0.05$) lower than in the diploid clones (data not shown). We obtained 20 *h6h*-transgenic tetraploid clones with normal appearance and growth, while only eight *pmt*-transgenic tetraploid clones were established. All the tetraploid *pmt*-transgenic clones had low growth rates and were not analyzed further, while a few h6h-transgenic clones with normal growth rate were selected for the further analyses. The tetraploid clones were also more friable than the diploid ones, and a number of them grew after a lag phase of about 10 days after subculturing.

The majority of the *pmt* and *h6h* transgenic clones of *H. senecionis* and diploid *H. muticus* showed a normal growth rate similar to that of the controls. Compared to *h6h*, *pmt* overexpression was in accordance with a lower growth rate and biomass production shown in several transgenic tetraploid clones of *H. muticus*.

Identification of transformed root lines through PCR analysis

The presence of heterologous *pmt* and *h6h* genes in the genome of hairy roots was confirmed by PCR. Amplification of a 612-bp fragment confirmed the presence of *rol*C in hairy root clones. The expected fragments were amplified using the 35s promoter, *npt*II, tobacco *pmt* and *h6h* (F-35s and R-*h6h*) in the transgenic clones, which did not show up in the DNA of the normal adventitious roots. Using specific primers, PCR experiments confirmed the presence of transgenes in the transformed hairy root clones (Fig. S3).

Genetic stability and ploidy level of transgenic clones

Flow cytometry experiments were conducted to determine the genetic stability and ploidy levels of the transgenic *h6h* and *pmt* clones, as well as the control normal adventitious roots of *H. senecionis* and diploid and tetraploid plants of *H. muticus*. We also attempted to find a correlation between the morphology, particularly in callus-producing clones, and genetic instability of the cultures. DAPI fluorescence intensity of 85 transgenic and control clones for the two above mentioned *Hyoscyamus* species was studied. As expected, the majority of the clones were stable and their fluorescence intensity was similar to the controls. We did, however, detect a small number of unusual clones with a different FCM profile. One of the *h6h*-transgenic clones of *H. senecionis*, with severe callus formation morphology, showed a lower DNA fluorescence intensity than the controls, indicating possible aneuploidy (Fig. 1a, b). However, the other ten callus-producing clones that we tested were normal.

Flow cytometry experiments confirmed the tetraploidy of the induced hairy roots from the C_7 generation of the tetraploid *H. muticus* (Fig. 1c, d). We also observed a tetraploid *pmt*-transgenic *H. muticus* clone that tended toward octaploidy and endoreduplication (Fig. 1f). This tetraploid clone was deformed, showed a low growth rate, and was not analyzed further.

Overexpression of *pmt* and *h6h* genes in transgenic clones of *H. senecionis*

Quantitative RT-PCR was conducted to reveal the level of expression of TA pathway genes in transgenic roots of both *Hyoscyamus* species. A considerable variation in the gene expression profiles of *pmt, h6h*, *trI,* and *trII* was observed among the transgenic clones. Transfer of *pmt* and *h6h* resulted in the overexpression of these genes in a number of the hairy root clones of *H. muticus* and *H. senecionis*. One of the *pmt*-transgenic clones of *H. senecionis* (P1) represented a 3100-fold increase in *pmt* expression. This clone was relatively dark brown and showed a moderate growth rate. The P4 and P5 clones also overexpressed the *pmt* gene about 40-fold higher than the controls (Fig. 2a). Considering the highest level of *pmt* expression, the amount of produced hyoscyamine and scopolamine was not as high as expected in the clone P1. However, this clone showed a higher level of littorine between all the studied *H. senecionis* clones (Fig. 2d). The highest content of hyoscyamine (6.3 mg/g DW) was observed in the clone P5 which was about 16.5-fold higher than in the intact plant leaves (0.4 mg/g DW) and sixfold higher than in the control non-transgenic samples. This clone also showed a good growth rate and the highest yield of hyoscyamine (56 mg/l) and total alkaloid (58.6 mg/l) among the studied *H. senecionis* clones (Fig. 2c, d).

When compared to the *pmt*-transgenic clones, transformation with *h6h* had a modest effect on *h6h*

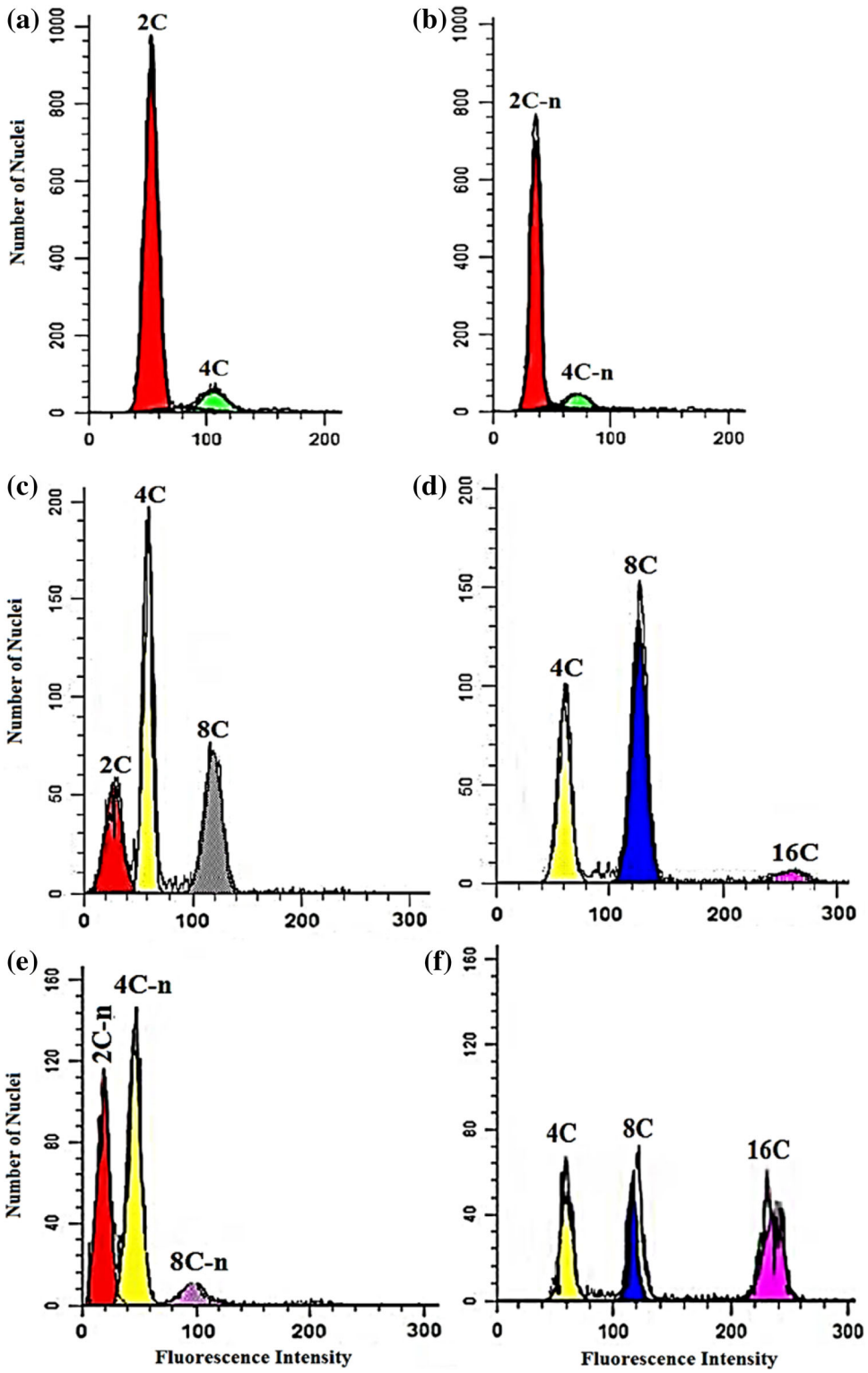

Fig. 1 Relative fluorescence intensity of DNA content of normal and unstable hairy root clones of *H. senecionis* and *H. muticus*. A normal *h6h*-transgenic hairy root clone of *H. senecionis* (**a**), a callus-forming hairy root clone of *H. senecionis* carrying the *h6h* transgene with genetic instability (**b**), the control diploid (**c**) and tetraploid (**d**) clones of *H. muticus*, a diploid *h6h*-transgenic clone with less DNA content (**e**) and a tetraploid *pmt*-transgenic clone of *H. muticus* showing a partial endoreduplication (**f**)

overexpression and conversion of hyoscyamine to scopolamine. The best clone, H2, showed a tenfold increase in *h6h* expression and scopolamine production compared to the control non-transgenic clones (Fig. 2b–e).

The effects of methyl jasmonate treatment on gene expression and tropane alkaloid accumulation

Having established the hairy root cultures of *H. senecionis*, we were interested to study TA productivity of in vitro

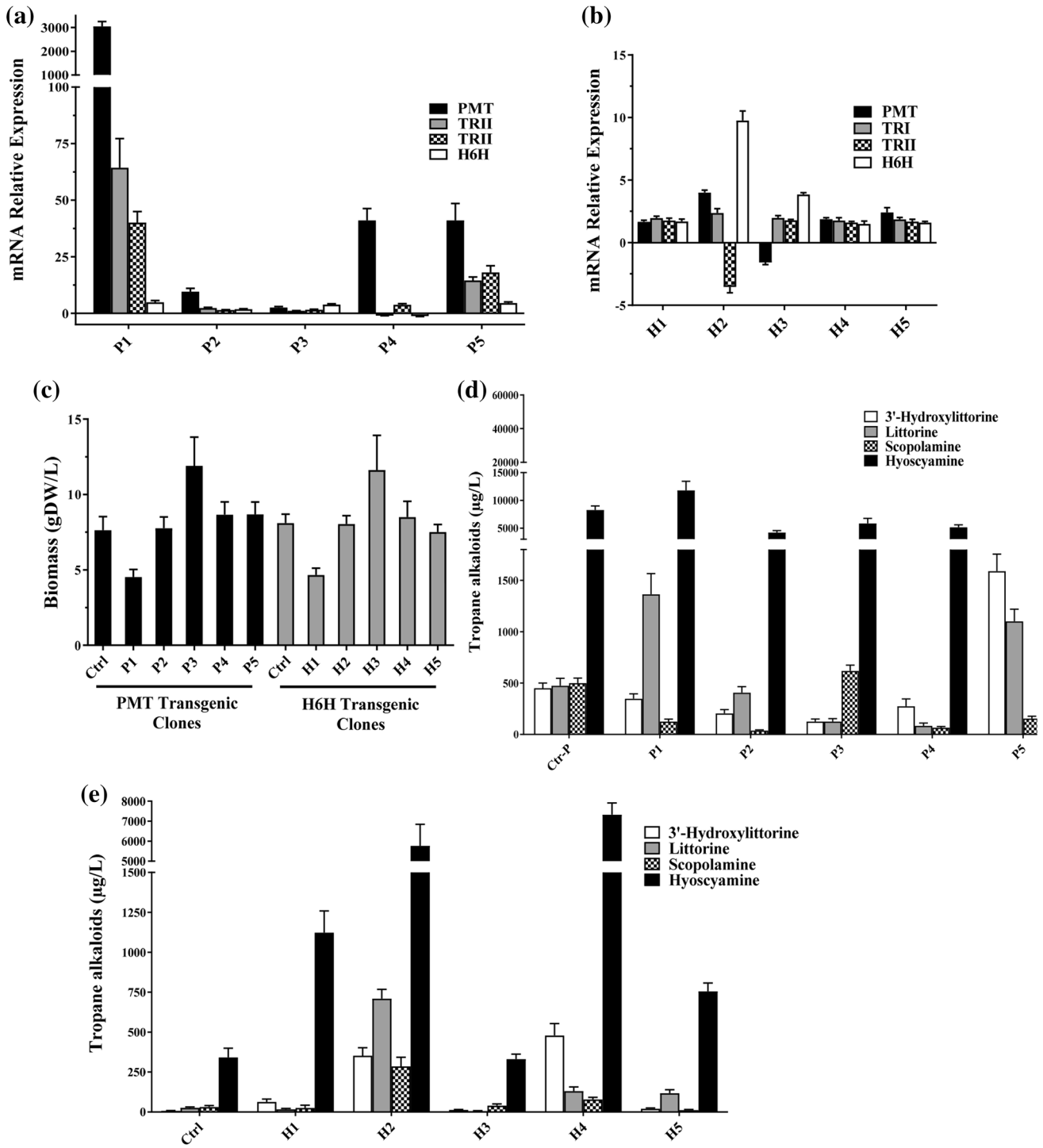

Fig. 2 Analysis of gene expression and tropane alkaloid productivity of transgenic *H. senecionis* hairy root clones. Relative gene expression of TA key enzymes in transgenic hairy root clones of *H. senecionis* showing up-regulation of *pmt* (**a**) and *h6h* (**b**), data are representative of three biological and three technical replicates normalized to the non-transgenic control hairy root lines. The production of biomass (g DW/l) in *pmt*- and *h6h*-transgenic hairy root cultures of *H. senecionis* (**c**). Productivity of littorine, 3′-hydroxylittorine, hyoscyamine, and scopolamine (µg/l) in *pmt*- (**d**) and *h6h*- (**e**) transgenic hairy root cultures of *H. senecionis*. Data are based on three replicates and the measurements are based on the dry weight per liter of growing media. All data are presented as mean ± SD

cultures in response to MeJa elicitation. The added MeJa changed the gene expression profile of the TA pathway (Fig. 3a) by up-regulating expression of *pmt* and decreasing expression of *trII*. While expression of *trI* was moderately reduced, elicitation did not show a significant change in h6h expression compared to the control (Fig. 3a). The transgenic hairy root clones also produced higher amounts of total alkaloids than the controls. The highest increase in TA accumulation that was observed after 12 h treatment was about tenfold increase in littorine and threefold increase in 3′-hydroxylittorine and hyoscyamine. Scopolamine accumulation was also increased about 3.5-fold after 24-h treatment, although the total amount still is less than that of the observed in the intact plants (Fig. 3b, c).

The profile of tropane alkaloid accumulation was also changed by MeJa elicitation of *H. senecionis* intact plants. The leaves of the intact plants, treated with MeJa for 64 h, accumulated significantly higher amount of scopolamine and total alkaloids compared to that of the control untreated ones (Fig. 3c).

Overexpression of *pmt* and *h6h* genes in transgenic clones of *H. muticus*

The selected diploid and tetraploid clones of *H. muticus* represented a relatively good growth rate and biomass production. In this study, three *h6h*-transgenic and one control tetraploid hairy root clones, with normal growth

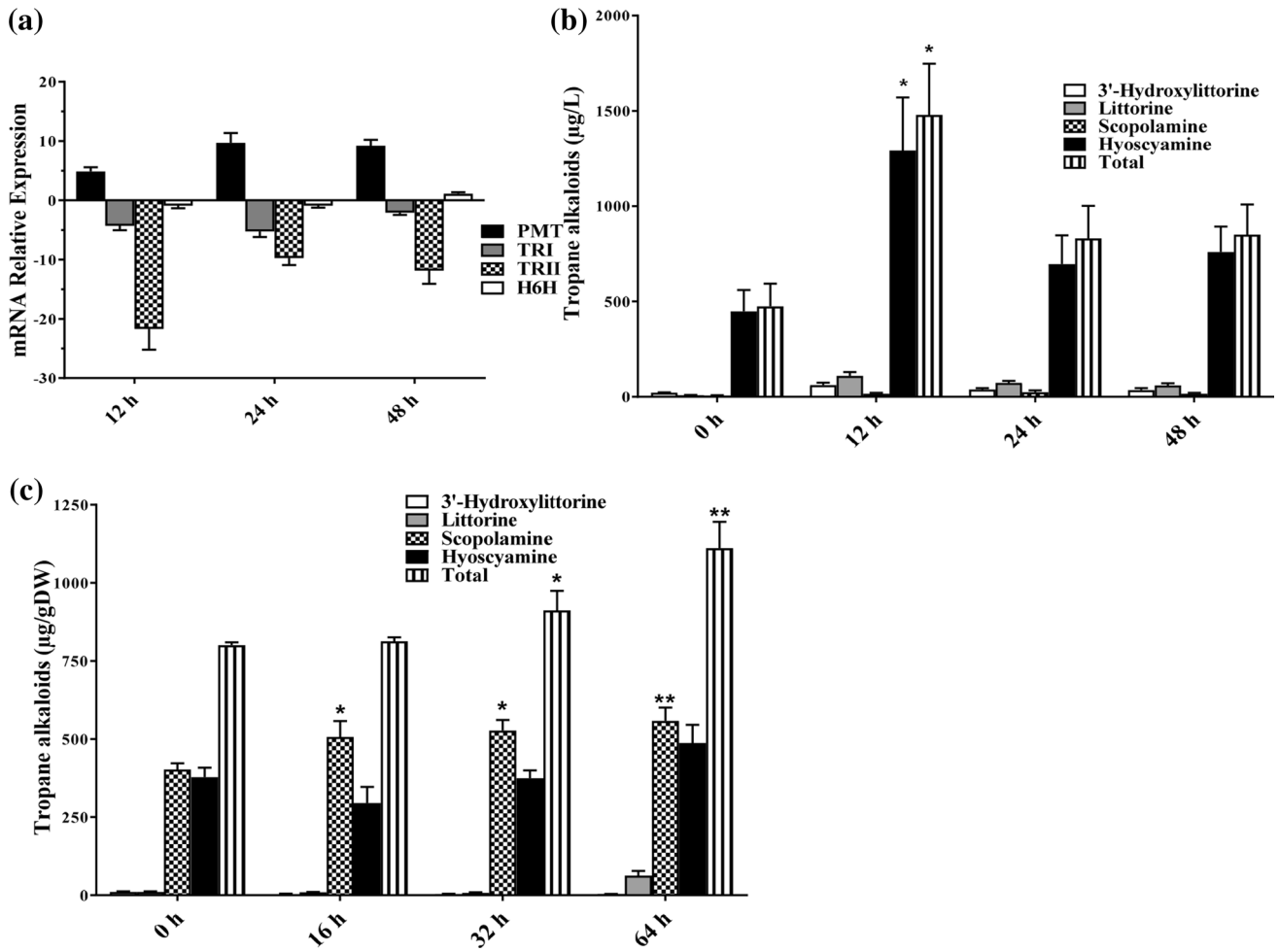

Fig. 3 Gene expression analysis and tropane alkaloid productivity of in vitro root hairy root culture and in vivo plants of *H. senecionis*. Relative gene expression of TA key enzymes in one of the transgenic hairy root clones of *H. senecionis* in different time points after treating by methyl jasmonate (100 µM). Data are based on three biological and three technical replicates normalized to the time 0 (**a**). Productivity of tropane alkaloids including littorine, 3′-hydroxylittorine, hyoscyamine, and scopolamine (µg/l) in the same clone and time points after treating by methyl jasmonate (100 µM). Data are based on three replicates and the measurements are based on the dry weight per litter of growing media (**b**). Accumulation of tropane alkaloids in leaf samples of *H. senecionis*, treated with 100 µM methyl jasmonate. Data are based on three replicates and the measurements are based on the µg of the compounds per gram dry weight of the samples (**c**). Statistical significance is indicated by asterisks (*$p < 0.05$ and **$p < 0.01$) between each specific treatment group and the control using two-tailed Student's *t* test. All data are presented as mean ± SD

rate and morphology, were selected and analyzed for gene expression patterns and alkaloid production. In contrary to the diploids, tetraploid *h6h*-transgenic clones of *H. muticus* showed a significantly ($p < 0.05$) higher growth rate than the obtained *pmt* overexpression clones (data not shown).

As shown in Figs. 4 and 5, a wide variation in gene expression and tropane alkaloid production was observed among the induced hairy root clones of diploid and tetraploid plants. A 45-fold increase in *pmt* expression and the highest hyoscyamine and total alkaloid production was observed in the P1 clone (Figs. 4a, 5a). Although *pmt* was up-regulated about 400-fold in P2, its alkaloid productivity was only two times higher than the control one thus remaining substantially lower than P1. In general, *pmt*-transgenic clones produced higher hyoscyamine, littorine, and 3′-hydroxylittorine than the *h6h*-transgenic clones ($p < 0.05$).

A sharp increase in *h6h* expression and scopolamine production was observed in H4 due to overexpression of the *h6h* gene under control of the 35S promoter (Fig. 4b), with scopolamine production being about 80-fold higher than in the control non-transgenic root clones (Fig. 5c). However, the hyoscyamine pool was not completely converted to scopolamine and the ratio of scopolamine to hyoscyamine did not increase more than 10%. The same phenomenon has been previously reported by Jouhikainen et al. (1999).

The ploidy level also had a great impact on the gene expression profile and scopolamine production of transgenic hairy roots. Comparing the expression level of the

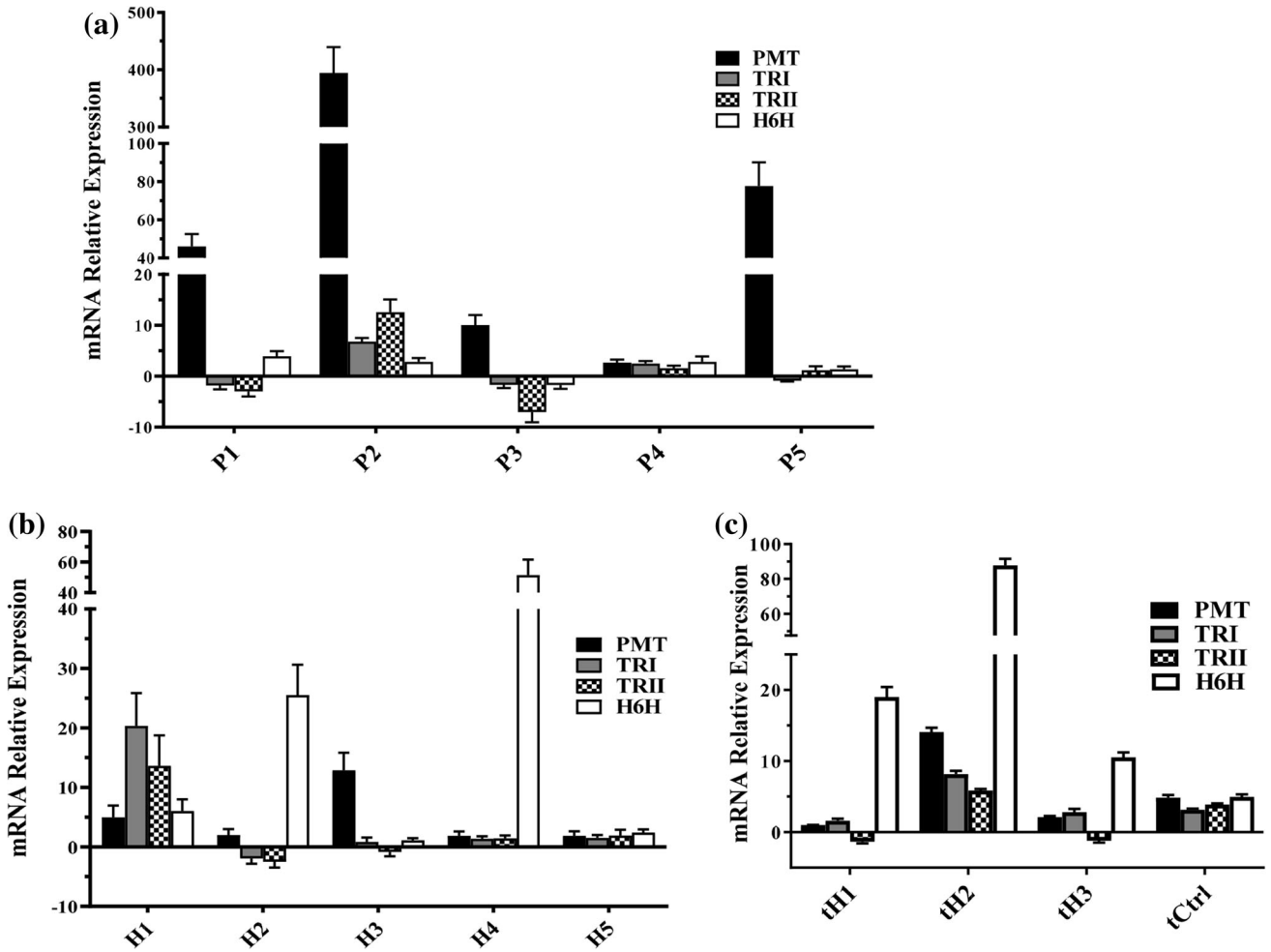

Fig. 4 Gene expression analysis of diploid and tetraploid clones of *H. muticus*. Real-time fluorescence quantitative PCR analysis of the expression of tropane alkaloid (TA) pathway genes in *pmt* (**a**) and *h6h* (**b**) transgenic clones of diploid *H. muticus*. Data are based on three biological and three technical replicates normalized to control non-transgenic counterparts. Expression level of TA pathway genes in the tetraploid control non-transgenic clone and three selected *h6h* overexpressing tetraploid clones (**c**). Data are based on three biological and three technical replicates normalized to the diploid *h6h*-transgenic clones. Statistical analysis (two-tailed Student's *t* test) showed a significant ($p < 0.05$) increase in *h6h* expression comparing all the *h6h*-transgenic clones of the tetraploid to the diploid ones. All data are presented as mean ± SD

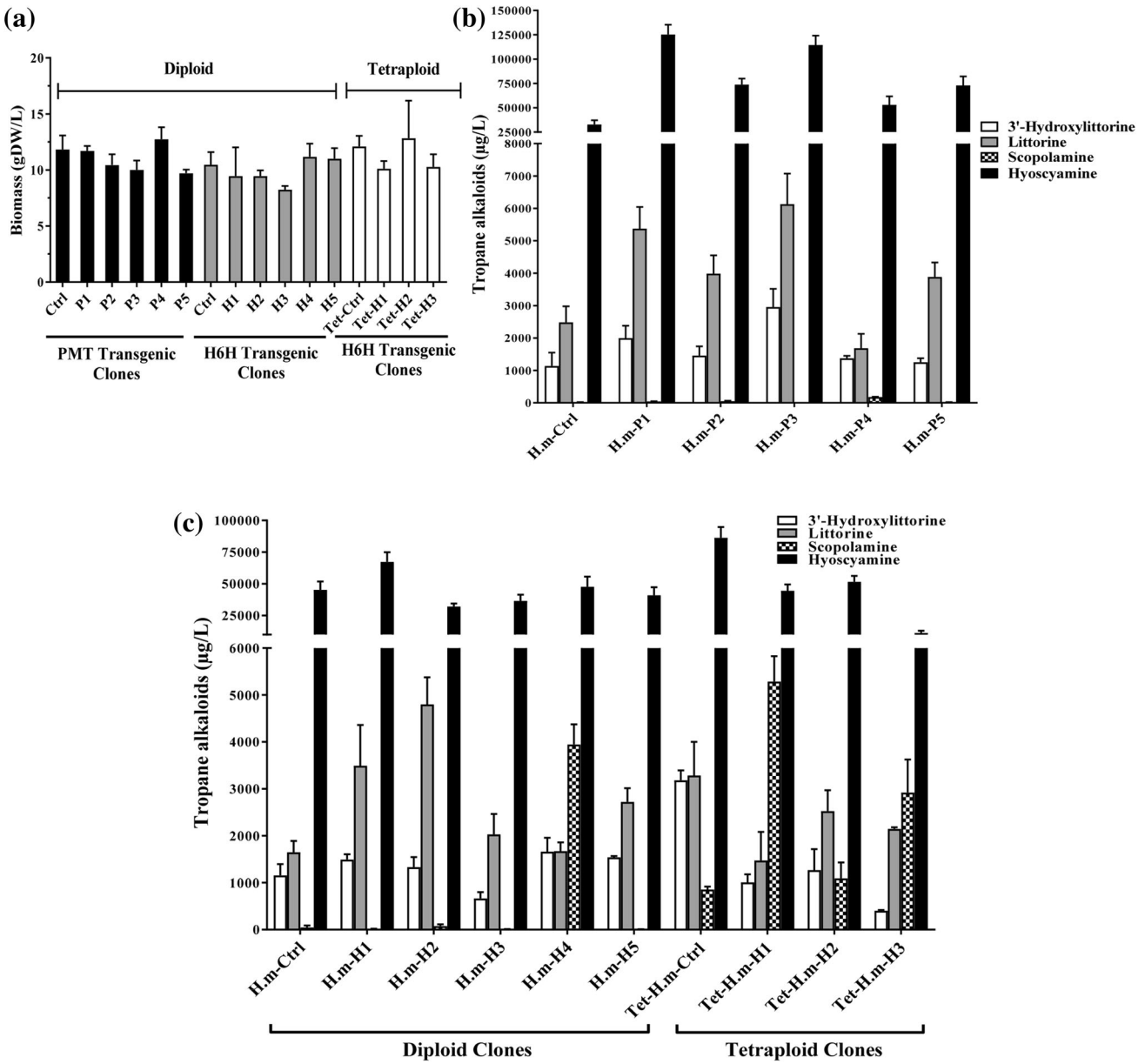

Fig. 5 Tropane alkaloid productivity of *pmt*- and *h6h*-transgenic hairy root cultures of diploid and tetraploid of *H. muticus.* The production of biomass (g DW/l) in diploid *pmt*- and diploid and tetraploid *h6h*-transgenic hairy root cultures of *H. muticus*. Data are based on three biological replicates (**a**). Productivity of littorine, 3′-hydroxylittorine, hyoscyamine, and scopolamine (µg/l) in diploid *pmt*- (**b**) and diploid and tetraploid *h6h*- (**c**) transgenic hairy root cultures of *H. muticus*. Data are based on three biological replicates and the measurements are based on the dry weight per litter of media. Statistical analysis (two-tailed Student's *t* test) showed a significant ($p < 0.05$) increase in scopolamine production comparing tetraploid clones to the diploid ones. All data are presented as mean ± SD

TA pathway genes in the tetraploid transgenic clones and non-transgenic control one with that of the diploid *h6h*-overexpressing clones, a significantly higher expression level of *h6h* ($p < 0.05$) was observed in all the tetraploid clones (Fig. 4c). Interestingly, the highest levels of *h6h* transgene expression and scopolamine accumulation were observed in the tetraploid clones (Figs. 4c, 5c). The mean scopolamine/hyoscyamine ratio was 0.4 and 13.5% in diploid and tetraploid *h6h*-overexpressing clones, respectively. Comparing non-transgenic clones of diploid and tetraploid, the tetraploid one produced 2.5- and 25-fold more hyoscyamine and scopolamine, respectively.

Discussion

Tropane alkaloids, especially scopolamine, are among the important medicines derived from natural sources with a wide range of pharmaceutical applications. With the growing demand for TAs, exploring unknown sources and

establishing new techniques to improve the quantity and quality of them will help to guarantee the long-term supply of TAs.

Here, we report establishment and metabolic engineering of hairy root cultures of *H. senecionis* and diploid and tetraploid clones of *H. muticus* overexpressing *pmt* and *h6h* transgenes, for the first time. In a number of *H. senecionis* hairy root clones, the presence of the *pmt* transgene was accompanied by morphological alterations such as rapid aging and browning. These changes have been reported in a number of previous studies on metabolic engineering of the TA pathway (Jouhikainen et al. 1999; Moyano et al. 2002, 2003).

Flow cytometry (FCM) confirmed the tetraploidy of induced hairy roots from the C_7 generation of tetraploid plants of *H. muticus*. The FCM analyses also indicated the genetic stability of the majority of transgenic clones and genetic instability of a few *h6h*-transgenic clones of *H. senecionis* and the diploid and tetraploid plants of *H. muticus* (Fig. 1). There was no correlation between the FCM profile and morphology, especially in the callus-forming clones of in vitro transgenic and non-transgenic hairy root cultures. The majority of the clones were genetically stable as it expected. These results differ from those reported by Ochatt et al. (2013) who claimed that insertion of the transgene does not exert any effect on the genetic stability of the transformed tissues.

Pmt overexpression in *H. senecionis* and *H. muticus* led to an increase in the production of hyoscyamine, littorine, and 3′-hydroxylittorine, suggesting that *pmt* overexpression might increase the total flux of the pathway by diverting the polyamine pool of the cells to the tropane alkaloid pathway. Our results agree with the study of Moyano et al. (2003), who reported an enhanced TA content in *pmt*-transgenic clones of *H. muticus* and *Datura metel*. However, overexpression of *pmt* in hairy root cultures of *Duboisia* hybrid, *H. niger,* and *A. belladonna* did not promote tropane alkaloid accumulation (Moyano et al. 2002; Sato et al. 2001; Zhang et al. 2007). This contradiction might be partly due to the difference in genetic regulation of the host plant pathway, including different posttranslational modifications and availability of different bottle necks through the pathways leading to production of TAs.

Although *pmt* overexpression led to a higher hyoscyamine and total alkaloid content in both *Hyoscyamus* species, no linear correlation was observed between these variables. This could be due to the role of the other genes of the pathway and the complex regulation of the TA biosynthetic pathway. For example, in the P2 clone of *H. muticus*, despite the enhanced metabolic flux of the pathway caused by *pmt* up-regulation, the higher expression of *trII* than *trI* led to the higher formation of calystegines (table S2) at the expense of TAs. Littorine mutase plays an important role in tropane alkaloid production. Although the expression level of the littorine mutase gene was not analyzed, its activity can be directly related to the accumulation of hyoscyamine and 3′-hydroxylittorine (Li et al. 2006). As represented in Figs. 2 and 5, the higher production of 3′-hydroxylittorine is usually associated with higher hyoscyamine and total alkaloid production. For example, the P1 clone of *H. senecionis* showed more than 3000-fold increase in *pmt* expression and a higher *trI*/*trII* expression ratio. The higher expression levels of *trI* than *trII* in P1 resulted in undetectable amount of calystegines and the greatest amount of littorine between all the *H. senecionis* clones but the 3′-hydroxylittorine and hyoscyamine content remained low, possibly because of lower activity of littorine mutase. Therefore, in addition to the *pmt* and *h6h* genes, the expression ratio of *trI*/*trII* and littorine mutase is other factors to be taken into account when trying to improve TA production and selecting elite clones.

When the TA content of three callus-forming hairy root lines of *H. senecionis* was analyzed, no correlation was observed between morphology and potential of TA production. This is in contrast with *H. muticus* hairy root cultures, where callus formation was associated with low alkaloid production in all cases (Jouhikainen et al. 1999). The observed difference may be due to organ-independent expression of TA pathway genes in *H. senecionis* (Dehghan et al. 2013). The established transgenic root cultures of *H. senecionis* showed great potential for hyoscyamine production but were unexpectedly weak in scopolamine production. Contrary to the level of *h6h* overexpression, the scopolamine content of hairy root cultures was lower than in *H. senecionis* aerial parts. The scopolamine/hyoscyamine ratio was about 1.50 in the leaves of *H. senecionis*, while it was about 0.04 in hairy root cultures. As previously discussed (Dehghan et al. 2013), this observation raises the question about the role of H6H in scopolamine production in green tissues and a hypothesis of de novo synthesis of TAs in aerial tissues (Kushwaha et al. 2013). Thus, we suggest future studies on the potential of scopolamine production in green hairy root cultures or in regenerated plants of *H. senecionis* from transgenic root cultures. Given that significant differences in scopolamine production have been reported between greenhouse and field grown plants of *H. muticus* (Oksman-Caldentey et al. 1987), the different scopolamine levels of *H. senecionis* root cultures and plants may also be a result of different growth conditions.

Rapid growth is seen as a beneficial feature when selecting clones, while a high content of products is usually associated with poor growth rate (Sevon and Oksman-Caldentey 2002). Thus, it is not surprising that most clones with a high content of alkaloids may be discarded in favor

of selection of the rapidly growing clones. We also concluded that the regulation of scopolamine biosynthesis may be more complicated than that of hyoscyamine and it seems to be more sensitive to environmental and culture conditions (Dehghan et al. 2012; Oksman-Caldentey et al. 1987).

Our results indicate that MeJa has a considerable effect in changing the gene expression and TA production of *H. senecionis*. It has been previously reported that TA production is activated by MeJa treatment (Kai et al. 2012; Kang et al. 2004), but in this study, the elicitor affected TA pathway genes in other ways. While *pmt* was strongly overexpressed, the other genes of the TA pathway, especially *trII*, were down-regulated by MeJa treatment. As observed in the elicited clone of *H. senecionis* (Fig. 3), increasing pathway flux by up-regulation of *pmt* and down-regulation of *trII* was accompanied by a sharp increase in TA production. To increase the simultaneous expression of key TA pathway genes, we suggest using a combination of appropriate elicitors. The enhanced overexpression of *pmt* and TA production in elicited hairy root cultures of *H. senecionis* indicate the key role of MeJa in regulating TA production. In contrast, Biondi et al. (2000) reported that MeJa treatment only slightly stimulated alkaloid production in *H. muticus*. To our knowledge, this is the first report on the up-regulation of *pmt* and concomitant down-regulation of *trII* in a TA-producing plant after exposure to MeJa.

Manipulation of ploidy level, alone or combined with metabolic engineering, sharply increased the expression of *h6h* and associated production of scopolamine (Figs. 4, 5). The higher expression level of *h6h* in tetraploid control clone was consistent with our previous study, as tetraploid plants of *H. muticus* produced about 200% more scopolamine than their diploid counterparts (Dehghan et al. 2012). Interestingly, the insertion of a transgene of *h6h* in tetraploid plants was able to further improve *h6h* up-regulation and the conversion of hyoscyamine to scopolamine. In other words, tetraploid hairy roots represent a higher potential for transgene expression, and consequently, tetraploid plants of Egyptian henbane are a preferred genetic context for metabolic engineering and overexpression of *h6h*. To our knowledge, this is the first report about metabolic engineering of a secondary metabolite pathway in an artificially induced polyploid plant.

Conclusion

Genetically modified hairy root cultures of *H. senecionis* and *H. muticus* with an altered gene expression profile and metabolite accumulation were obtained by overexpression of two key enzyme genes, *pmt* and *h6h*. As scopolamine production in hairy root cultures of *H. senecionis* was moderate in comparison with green parts of the plant, we concluded that aerial parts might have an important role in scopolamine biosynthesis and accumulation. Elicitation with MeJa increased the flux thorough the tropane alkaloid pathway in *H. senecionis* root cultures by up-regulation of *pmt* and down-regulation of *trII*, which led to a modest increase in tropane alkaloid production. Although the amounts of the produced alkaloids in the in vitro conditions are not yet compatible to the commercially available *Duboisia* hybrid cultivation system, the in vitro systems not only are applicable as an alternative approach to provide tropane alkaloid resources, but also has opened a strong research tool to increase our knowledge about different aspects of biosynthesis and regulation of the TAs pathway. Applying polyploidy as a source of genetic variation, we suggest metabolic engineering of polyploid plants to increase the quality of TAs in the intact plants of *Hyoscyamus* or other TAs producing species. As mentioned for the P1 clone of *H. senecionis*, manipulation of other potential rate-limiting steps, such as littorine mutase, is worth trying along with the other enzymes in the future studies. In summary, the results of this study emphasize the importance of metabolic engineering, elicitation, and ploidy manipulation for increasing tropane alkaloid production in *H. senecionis* and *H. muticus*.

Author contribution statement ED and FS designed the experiments; ED and ZH performed the experiments; DWR and PSC conducted GC and GC–MS analyses; JP prepared the binary vector pBMI; K-MO-C provided *h6h* construct; ED, K-MO-C, and JP wrote the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Arroo R, Woolley J, Oksman-Caldentey K-M (2007) *Henbane*, *Belladonna*, *Datura* and *Duboisia*. In: Pua E, Davey M (eds) Biotechnology in agriculture and forestry, Transgenic Crops VI, vol 61. Springer, Berlin, pp 189–204

Biondi S, Fornale S, Oksman-Caldentey KM, Eeva M, Agostani S, Bagni N (2000) Jasmonates induce over-accumulation of methylputrescine and conjugated polyamines in *Hyoscyamus muticus* L. root cultures. Plant Cell Rep 19:691–697

Cardillo AB, Otalvaro AAM, Busto VD, Talou JR, Velasquez LME, Giulietti AM (2010) Scopolamine, anisodamine and hyoscyamine production by *Brugmansia candida* hairy root cultures in bioreactors. Process Biochem 45:1577–1581

Dehghan E, Häkkinen S, Oksman-Caldentey K-M, Shahriari Ahmadi F (2012) Production of tropane alkaloids in diploid and tetraploid plants and in vitro hairy root cultures of Egyptian

henbane (*Hyoscyamus muticus* L.). Plant Cell Tissue Organ Cult 110:35–44

Dehghan E, Shahriari Ahmadi F, Ghotbi Ravandi E, Reed DW, Covello PS, Bahrami AR (2013) An atypical pattern of accumulation of scopolamine and other tropane alkaloids and expression of alkaloid pathway genes in *Hyoscyamus senecionis*. Plant Physiol Biochem 70:188–194

Hashimoto T, Yun D, Yamada Y (1993) Production of tropane alkaloids in genetically engineered root cultures. Phytochemistry 32:713–718

Jouhikainen K, Lindgren L, Jokelainen T, Hiltunen R, Teeri TH, Oksman-Caldentey K-M (1999) Enhancement of scopolamine production in *Hyoscyamus muticus* L. hairy root cultures by genetic engineering. Planta 208:545–551

Kai G et al (2007) Molecular cloning and characterization of a new cDNA encoding hyoscyamine 6-beta-hydroxylase from roots of *Anisodus acutangulus*. J Biochem Mol Biol 40:715–722

Kai G, Yang S, Zhang Y, Luo X, Fu X, Zhang A, Xiao J (2012) Effects of different elicitors on yield of tropane alkaloids in hairy roots of *Anisodus acutangulus*. Mol Biol Rep 39:1721–1729

Kang SM, Jung HY, Kang YM, Yun DJ, Bahk JD, Yang JK, Choi MS (2004) Effects of methyl jasmonate and salicylic acid on the production of tropane alkaloids and the expression of PMT and H6H in adventitious root cultures of *Scopolia parviflora*. Plant Sci 166:745–751

Khatamsaz M (1998) Flora of Iran, vol 24. Research Institute of Forests and Rangelands, Iran

Kushwaha AK, Sangwan NS, Trivedi PK, Negi AS, Misra L, Sangwan RS (2013) Tropine forming tropinone reductase gene from *Withania somnifera* (Ashwagandha): biochemical characteristics of the recombinant enzyme and novel physiological overtones of tissue-wide gene expression patterns. PLoS One 8:e74777

Li R, Reed DW, Liu EW, Nowak J, Pelcher LE, Page JE, Covello PS (2006) Functional genomic analysis of alkaloid biosynthesis in *Hyoscyamus niger* reveals a cytochrome P450 involved in littorine rearrangement. Chem Biol 13:513–520

Mateus L, Cherkaoui S, Christen P, Oksman-Caldentey KM (2000) Simultaneous determination of scopolamine, hyoscyamine and littorine in plants and different hairy root clones of *Hyoscyamus muticus* by micellar electrokinetic chromatography. Phytochemistry 54:517–523

Moyano E, Fornalé S, Palazón J, Cusidó RM, Bagni N, Piñol MT (2002) Alkaloid production in *Duboisia* hybrid hairy root cultures overexpressing the *pmt* gene. Phytochemistry 59:697–702

Moyano E, Jouhikainen K, Tammela P, Palazon J, Cusido RM, Piñol MT, Teeri TH, Oksman-Caldentey K-M (2003) Effect of *pmt* gene overexpression on tropane alkaloid production in transformed root cultures of *Datura metel* and *Hyoscyamus muticus*. J Exp Bot 54:203–211

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497

Ochatt S, Jacas L, Patat-Ochatt EM, Djenanne S (2013) Flow cytometric analysis and molecular characterization of *Agrobacterium tumefaciens*-mediated transformants of *Medicago truncatula*. Plant Cell Tissue Organ Cult 113:237–244

Oksman-Caldentey K-M, Arroo R (2000) Regulation of tropane alkaloid metabolism in plants and plant cell cultures. In: Verpoorte R, Alfermann A (eds) Metabolic engineering of plant secondary metabolism. Kluwer, Dortrecht, pp 253–281

Oksman-Caldentey K-M, Vuorela H, Isenegger M, Strauss A, Hiltunen R (1987) Selection for high tropane alkaloid content in *Hyoscyamus muticus* plants. Plant Breed 99:318–326

Palazón J, Moyano E, Cusidó RM, Bonfill M, Oksman-Caldentey KM, Piñol MT (2003) Alkaloid production in *Duboisia* hybrid hairy roots and plants overexpressing the *h6h* gene. Plant Sci 165:1289–1295

Portsteffen A, Dräger B, Nahrstedt A (1994) The reduction of tropinone in *Datura stramonium* root cultures by two specific reductases. Phytochemistry 37:391–400

Rischer H, Häkkinen ST, Ritala A, Seppänen-Laakso T, Miralpeix B, Capell T, Christou P, Oksman- Caldentey K-M (2013) Plant cells as pharmaceutical factories. Curr Pharm Design 19:5640–5660

Saghai-Maroof MA, Soliman K, Gorgensen RA, Allard RW (1984) DNA spacer length polymorphism in Barley: Mendelian inheritance, chromosomal location and population genetics. Proc Natl Acad Sci USA 81:8014–8018

Sato F, Hashimoto T, Haciya A, Tamura K, Choi K-B, Morishige T, Fujimoto H, Yamada Y (2001) Metabolic engineering of plant alkaloid biosynthesis. Proc Natl Acad Sci USA 98:367–372

Sevon N, Oksman-Caldentey K-M (2002) *Agrobacterium rhizogenes* mediated transformation: root cultures as a source of alkaloids. Planta Med 68(10):859–868

Wang X, Chen M, Yang C, Liu X, Zhang L, Lan X, Tang K, Liao Z (2011) Enhancing the scopolamine production in transgenic plants of *Atropa belladonna* by overexpressing *pmt* and *h6h* genes. Physiol Plant 143:309–315

Yamada Y, Hashimoto T, Endo T (1990) Biochemistry of alkaloid production in vitro. In: Charlwood B, Rhodes MJC (eds) Secondary products from plant tissue culture. Clarendon Press, Oxford, pp 201–225

Zárate R, Jaber-Vazdekis N, Medina B, Ravelo Á (2006) Tailoring tropane alkaloid accumulation in transgenic hairy roots of *Atropa baetica* by over-expressing the gene encoding hyoscyamine 6β-hydroxylase. Biotechnol Lett 28:1271–1277

Zhang L et al (2004) Engineering tropane biosynthetic pathway in *Hyoscyamus niger* hairy root cultures. Proc Natl Acad Sci USA 101:6786–6791

Zhang L, Yang B, Lu B, Kai G, Wang Z, Xia Y, Ding R, Zhang H, Sun X, Chen W, Tang K (2007) Tropane alkaloids production in transgenic *Hyoscyamus niger* hairy root cultures over-expressing putrescine *N*-methyltransferase is methyl jasmonate-dependent. Planta 225:887–896