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An Optimized Tobacco Hairy Root Induction System for Functional Analysis of Nicotine Biosynthesis-Related Genes

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Abstract: *Rhizobium rhizogenes*-mediated plant hairy root induction is a convenient method for functional study of root-specific genes. To develop an optimized tobacco hairy root induction system and study gene function in nicotine biosynthesis, we investigated hairy root induction by three *R. rhizogenes* strains, R1601, K599, and LBA9402, on different media with leaf discs from plants of different ages, and we observed that the strain LBA9402 used for explant infection exhibited the highest hairy root induction rate with 4 and 8 week old leaf discs of the tobacco 'Coker176' on 2/3MS medium, and it could also be used as a cargo delivering foreign genes to hairy roots. Overexpression of *MsSPL12* gene, an alfalfa (*Medicago sativa*) *SQUAMOSA* promoter binding protein-like (SPL) transcription factor, significantly improved nicotine production in transgenic hairy roots, reaching 1.38–1.85 mg/g compared to 0.5 mg/g of the controls. Expression analysis of the nicotine biosynthesis and transport-related genes responding to methyl-jasmonate (MeJA) treatment revealed a significant upregulation of *NtMPO2* responsible for increased nicotine biosynthesis in *MsSPL12* transgenic hairy roots. Our results establish a high-throughput approach for gene functional characterization in the hairy roots of a tobacco elite cultivar, 'Coker176', as well as suggest a system for efficiently manipulating tobacco nicotine biosynthesis.

Keywords: *Rhizobium rhizogenes*; hairy root; nicotine biosynthesis; tobacco

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1. Introduction

Rhizobium rhizogenes, previously referred to as *Agrobaterium rhizogenes*, is a soil-borne Gram-negative microbe that can infect dicots and induce hairy roots at the infected site [1]. The hairy roots produce secondary metabolites, which can be synthesized in intact plants [2,3]. *R. rhizogenes*-mediated plant transformation has been widely used in the production of secondary metabolites and is also increasingly applied to studying plant secondary metabolism and/or gene expression regulation pathways due to the simple transformation procedure and the rapid growth of the hairy root [4,5].

Hairy root induced by *R. rhizogenes* infection has also been used in tobacco for functional gene investigation [3]. Co-expression of basic helix–loop–helix protein (bHLH) and transcriptional activator *Myb* genes reportedly induced anthocyanin production in tobacco hairy roots [6]. Tobacco hairy roots expressing stilbene synthase from *Vitis vinifera* and a transcription factor AtMYB12 from *Arabidopsis* exhibited enhanced biosynthesis of stilbenes [7]. Tobacco hairy roots could also be used as a convenient system for studying the function of genes in circadian rhythm [8]. Similarly, a tobacco hairy root culture system was established to investigate the function of genes related to aluminum [9] or cadmium tolerance [10]. Considering the culturing time needed, induction of hairy root by *R. rhizogenes* has many advantages over traditional transgenic technology in the study of gene function and regulation of secondary metabolism [9].

Similar to *Agrobacterium*-mediated plant transformation, the efficiency of hairy root induction by *R. rhizogenes* is affected by many factors [11,12], including plant species, developmental stages of explant, *R. rhizogenes* strains, medium composition, and culture environment among others [13,14]. For example, when using *R. rhizogenes* strain MTCC 532 to infect *Aegle marmelos, Boerhavia diffusa, Datura innoxia,* and *Solanum xanthocarpum*, the hairy root induction efficiency (HRIE) of *Solanum xanthocarpum* was 75%, whereas that of *Boerhavia diffusa* was only 60% [15]. When using different strains to infect the same plant, variation in HRIE was also noticed [13]. In addition, the *R. rhizogenes* strains and culture media also have a synergistic effect on hairy root induction [16] as observed in soybean; the hairy root induction efficiency was significantly affected by the cocultivation medium [17]. Hence, the establishment of a highly efficient system for hairy root induction necessitates an optimized composition of the culture medium [5,18].

Nicotine is the most important secondary metabolite of tobacco. mainly synthesized in the root and transported by xylem sap to the above-ground part of the plant where it is mostly stored in the vacuoles of the leaf cells [19,20]. Jasmonic acid (JA) and MeJA play important regulatory roles in nicotine biosynthesis of tobacco as previously demonstrated in our investigation of genes involved in nicotine biosynthesis and JA-induced nicotine accumulation in tobacco [21]. Exogenous application of JA or MeJA can stimulate the rapid expression of nicotine biosynthesis-related genes such as *NtPMT* and *NtQPT* in intact plants, cultured cells, and roots [22–25]. Genes involved in JA metabolism or signaling transduction can also affect nicotine biosynthesis [25]. *SQUAMOSA* promoter binding protein-like (SPL) proteins are plant-specific transcription factors, which regulate plant growth, development, and secondary metabolism, as well as respond to exogenous hormone treatments, such as JA [25]. In *Arabidopsis*, SPL9 was reported to negatively regulate JA response through interaction with JAZ proteins [26]. In tobacco, the expression level of miR156 and other miRNAs was significantly repressed by topping treatment [27], suggesting that overexpression of a miR156-targeted *SPL* gene could benefit nicotine biosynthesis. The *MsSPL12* gene is a target gene of miR156 in alfalfa (*Medicago sativa*) and was reported to respond to various stresses [28]. This raises an interesting question regarding the role that *MsSPL12* may play in plant secondary metabolism. To this end, we investigated whether overexpression of the *MsSPL12* gene would affect nicotine accumulation and the expression of nicotine biosynthesis-related genes in a heterologous system.

In this study, we firstly optimized the hairy root induction system of tobacco ('Coker176') by investigating the effects of explant age, *R. rhizogenes* strains, and medium compositions on hairy root induction efficiency. The optimized system was then used for functional characterization of the alfalfa *MsSPL12* gene. We showed that *MsSPL12* overexpression in tobacco hairy root significantly improved nicotine biosynthesis.

2. Materials and Methods

2.1. Plant Materials and Culture Conditions

Seeds of the tobacco (*Nicotiana tabacum)* 'Coker176' used in this study were rinsed in 75% (*v*/*v*) ethanol for 60 s, sterilized in 5% (*v*/*v*) sodium hypochlorite (NaClO) for 10 min, washed five times in sterile water, and then placed on solid 2/3 MS medium (pH 5.8) containing 20 g/L sucrose and 8.0 g/L agar (Sigma) [29] for germination. The plant materials were cultured at 25 °C under a photoperiod of 16 h light/8 h dark, with a light intensity of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$, unless otherwise mentioned. The leaves of the 2 week old, 4 week old, and 8 week old tobacco plants (Figure 1a–c) were used as explants for *R. rhizogenes* infection.

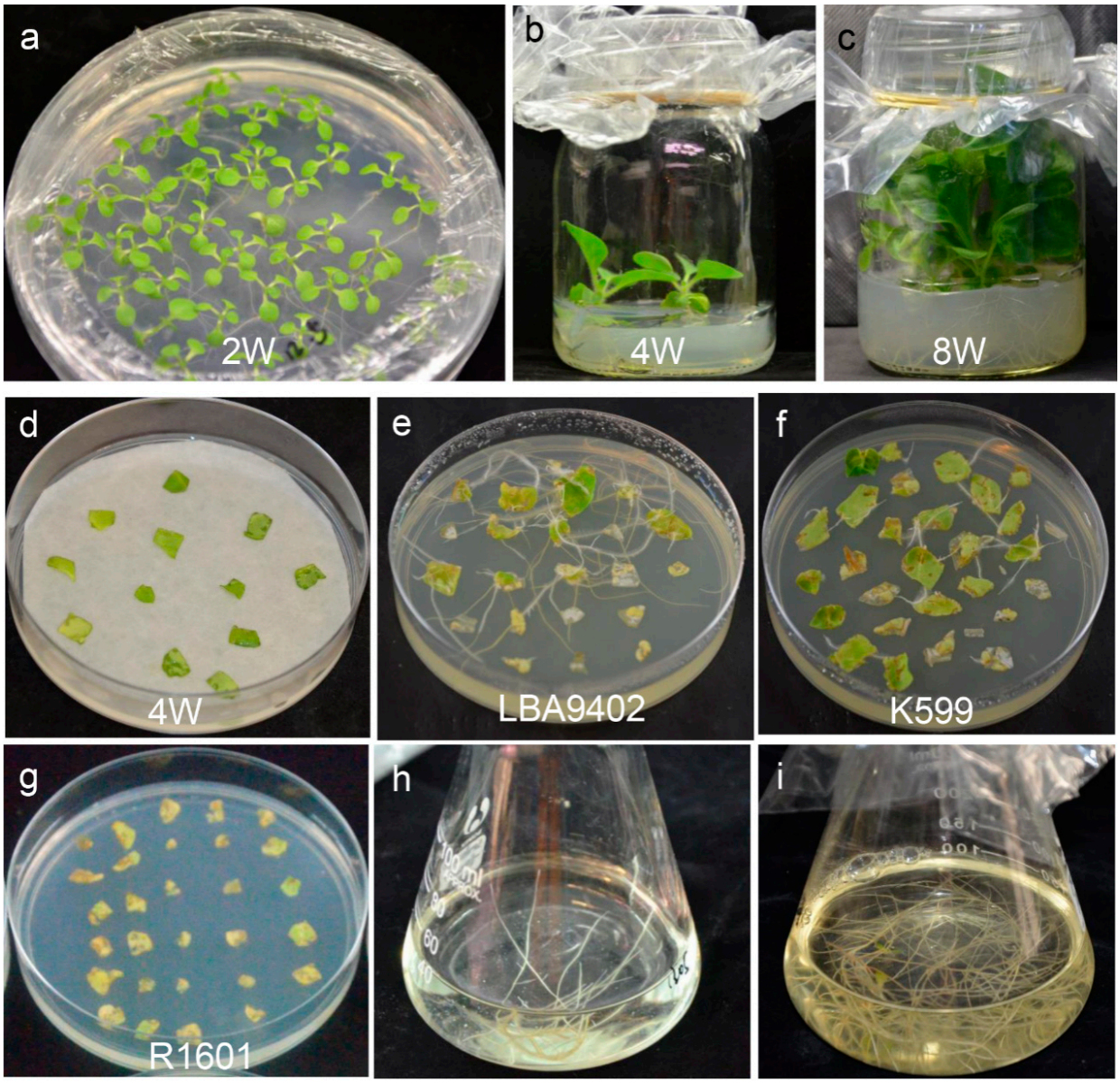

Figure 1. *R. rhizogenes*-mediated tobacco hairy root induction: (**a**) 2 week old tobacco plants' (**b**) 4 week old tobacco plants; (**c**) 8 week old tobacco plants; (**d**) a typical photograph of 4 week old tobacco leaf discs after cocultivation for 3 days' (**e**) induction tobacco hairy roots with *R. rhizogen* strain LBA9402 for 1 month; (**f**) induction tobacco hairy roots with *R. rhizogen* strain K599 for 1 month; (**g**) induction tobacco hairy roots with *R. rhizogen* strain R1061 for 1 month; (**h**) hairy roots inoculated in 2/3 MS liquid medium; (**i**) growth state of hairy roots cultured in liquid medium for 1 month.

2.2. R. rhizogenes Strains and Hairy Root Induction

Three *R. rhizogenes* strains, R1601 (kanamycin resistance), K599 (streptomycin resistance), and LBA9402 (rifampicin resistance), which could effectively induce hairy root on peanut [30], soybean [31], and *Papaver somniferum* (Le Flem-Bonhomme et al., 2004), respectively, were used to infect tobacco leaf discs. Single colonies of the three *R. rhizogenes* strains were incubated in YEP liquid medium [32] (containing kanamycin 100 mg/L, streptomycin 50 mg/L, and rifampicin 50 mg/L) in a shaker (Taichang THZ-C-1, Jiangsu, China) at 28 °C, 200 r/min. When the optical density at 600 nm wavelength (OD_{600}) measured by an ultraviolet/visible spectrophotometer (PGeneral, Beijing, China) reached 0.8, *R. rhizogenes* bacteria were collected by centrifuge at 3000 rpm for 10 min, and then resuspended in liquid 2/3MS medium to 0.6 at OD_{600}, followed by the addition of 100 μM acetosyringone.

To compare the effects of leaf age and different *R. rhizogenes* strains on hairy root induction, leaf discs (about 0.5 × 0.5 cm^2) of 2, 4, and 8 week old tobacco plants (Figure 1a–c) were used as explants for *R. rhizogenes* infection. After cutting with a scalpel in a laminar flowhood, the explants were soaked in *R. rhizogenes* suspension for 20 min, and then blotted on a sterilized paper towel. Cocultivation was performed in the dark on 2/3MS solid medium with 20 g/L sucrose and 100 μM acetosyringone for 3 days. One layer of sterilized filter paper was placed on the surface of the cocultivation medium to avoid bacteria overgrowth (Figure 1d). After cocultivation, the explants were cleaned with sterile water containing 500 mg/L cefotaxime, briefly dried on sterilized filter paper, and then transferred onto 2/3MS solid medium with 500 mg/L cefotaxime for culture in dark, at 25 °C, until hairy roots were induced.

To compare the effects of organic ingredients on hairy root induction, 2/3MS medium supplemented with 0.5 g/L glutamine (2/3MS-g) or 0.5 g/L glutamine and 0.5 g/L casein acid hydrolysate (2/3MS-gh) was used for hairy root induction after *R. rhizogenes* strain LBA9402 infection of the 4 week old explants. The number of leaf discs that had a hairy root and the total number of induced hairy roots were recorded to calculate the hairy root induction rate and mean number of hairy root per leaf disc, respectively, 4 weeks after root induction. Three independent infection experiments were treated as three biological replicates with each one having at least 30 leaf discs.

2.3. Expression of Foreign Genes in Tobacco Hairy Root

To test whether *R. rhizogenes* strain LBA9402 could be used as a cargo for foreign gene delivery into hairy roots, *R. rhizogenes* strain LBA9402 harboring the binary vector pCAMBIA1305.1 or pZH01-*MsSPL12* was used to infect the 4 month old leaf discs to introduce and express foreign genes in tobacco hairy root. The pZH01-*MsSPL12* vector was constructed by inserting the full-length cDNA of *MsSPL12* (XM_003601719.2) between the restriction sites *Bam*HI and *Kpn* I of the vector pZH01 [33]. *MsSPL12* was amplified by a PCR reaction with a pair of primers, *MsSPL12-Bam*HI-F (5′–GGATCCATGGAGTGGAACGTGAAATCTCCCG–3′) and *MsSPL12-Kpn*I-R (5′–GGTACCTTAATCCAGCTGGTTGCAAGGGAAAC–3′). When grown to 2–3 cm long, the hairy root was separated from the leaf disc and cultured on the same solid medium containing 10 mg/L hygromycin for selection. The selected hairy root grown to about 5 cm long was transferred into a 150 mL Erlenmeyer containing liquid selective medium and cultured at 70 r/min in an incubation shaker for further growth (25 °C, dark). The liquid selective medium was renewed every 2 weeks.

2.4. Transgenic Hairy Root Verification

Genomic DNA of the hairy roots was extracted by the CTAB method [34] for PCR analysis to verify the resistant hairy root containing the *GUS* gene or *MsSPL12* gene. The primers used were designed using software Primer premier 5 [35] and are listed in Table S1. The PCR-positive hairy roots transformed with the pCAMBIA1305.1 vector were also subjected to a GUS staining assay [36]. The number of hairy roots that showed blue color was counted. The transformation efficiency was calculated according to the GUS staining assay results of the hairy roots transformed with the pCAMBIA1305.1 vector (http://www.cambia.org accessed on 12 December 2022)and the PCR test results for the transformation with the pZH01-*MsSPL12* vector.

2.5. RNA Extraction and Quantitative RT-PCR

Total RNA of the hairy roots was extracted with Trizol reagent (Thermo Fisher Scientific, San Jose, CA, USA). One microgram of total RNA was used for reverse transcription to synthesize the first strand of the cDNA according to the manufacturer's instructions (PrimeScript RT Reagent Kit with gDNA Erase, TAKARA, Dalian, China RR047A). The cDNA was used as template to analyze the expression of genes related to nicotine synthesis by real-time quantitative PCR (RT-qPCR) using SYBR green supermix (Takara, Dalian, China RR420). To test the effects of JA on the expression of nicotine biosynthesis-related

genes, 100 µM MeJA (Sigma-Aldrich, St. Louis, MO, USA) was added into the hairy root culture medium for 1 h, and the hairy roots were then collected for RNA extraction [21]. The primers used for RT-qPCR were designed using Primer premier 5 software and are listed in Table S2. The relative expression level of nicotine biosynthesis-related genes with 100 µM MeJA treatment compared with no MeJA treatment was calculated using the $2^{-\Delta\Delta Ct}$ method [37]. A tobacco *Actin* gene (X63603) was used as an internal control to normalize gene expression [21]. Data from at least three biological replicates with each one having at least 30 leaf discs were used for statistical analyses in the experiments.

2.6. Determination of Nicotine Content

Nicotine content of the hairy roots was determined by gas chromatography–mass spectrometry using an Agilent HP6890 GC-FID system [38]. Two month old hairy root samples were collected and dried at 65 °C for 72 h, and then ground to fine powder. Fifty milligrams of powder of each sample was weighted for nicotine content measurement (YC/T 383-2010) in Yuxi Jiahui Detection Technology Co. Four independent tests (replicates) were performed for each sample.

3. Statistical Analysis

All data were collected from at least three biological replicates. One-way analysis of variance (ANOVA) was used for data analysis. Means were compared by Duncan's multiple range test ($p < 0.05$). The proc GLM for ANOVA of SAS 8.2 (SAS Institute, Cary, NC, USA) was used for the analyses.

4. Results

4.1. Effects of the Explant Age and R. rhizogenes Strains on Hairy Root Induction

Upon 1 month of cultivation, obvious hairy roots grew out from the edges of the leaf discs. The explants infected with LBA9402 showed the best hairy root growth, followed by K599 and R1601 (Figure 1e–g). We noticed the explants from the 2 week old leaves became necrotic easily after cocultivation, and the hairy root induction rate was the lowest compared to the other transformations (Figure 2). The hairy root induction rates of the 4 and 8 week old explants showed no significant difference when infected with the same *R. rhizogenes* strain, whereas the hairy root induction rates with the infection of different *R. rhizogenes* strains showed a significant difference. The *R. rhizogenes* strain LBA9402 exhibited the highest hairy root induction rate (up to 80%) using the 4 and 8 week old tobacco leaf disc explants, followed by the *R. rhizogenes* strain K599 with a hairy root induction rate of about 50%. The highest hairy root induction rate obtained using the *R. rhizogenes* strain R1601 was only 15% (Figure 2). The results suggest that the strain LBA9402 is more suitable for hairy root induction for tobacco 'Coker176'.

4.2. Effects of Medium Additives on Hairy Roots Induction

We studied the effects of organic ingredients on the efficiency of hairy root induction for 4 week old leaf discs by *R. rhizogenes* strain LBA9402 infection. Glutamine (2/3MS-g) or glutamine and casein acid hydrolysates (CH) (2/3MS-gh) were added to the 2/3MS medium during hair root induction and further growth. Intriguingly, the additional organic components reduced the hairy root induction compared to 2/3MS medium (Figure 3). The hairy root induction rate and the number of hairy roots per leaf disc generated on 2/3MS medium without additives were significantly higher than those on medium supplied with additives. Thus, 2/3MS medium without additional CH or glutamine was optimal for tobacco hairy root induction.

4.3. R. rhizogenes Strain LBA9402 as a Cargo for Foreign Gene Delivery and Expression in Hairy Root

The resistant hairy roots resulting from the transformation by *R. rhizogenes* strain LBA9402 harboring the binary vector pCAMBIA1305.1 were subjected to PCR targeting

the *GUS* gene for transformation analysis. As shown in Figure 4a, 12 out of 14 randomly selected hairy root lines showed the target band. The PCR-positive hairy roots also showed a strong blue color in GUS staining assay (Figure 4b), indicating that the foreign gene was stably expressed. About 85% of hairy roots stained positively in the GUS assay (Figure 4c).

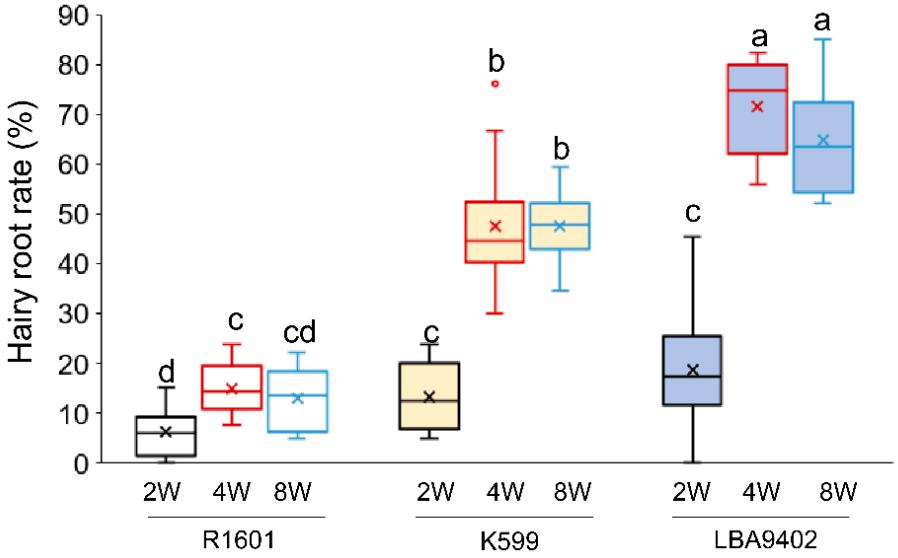

Figure 2. Effects of explant age and *R. rhizogenes* strain on hairy root generation efficiency. The data were statistically analyzed using one-way ANOVA. The data are presented as the mean ± standard error (SE) (n = 3). Different letters indicate a statistically significant difference according to Duncan's multiple range test ($p < 0.05$).

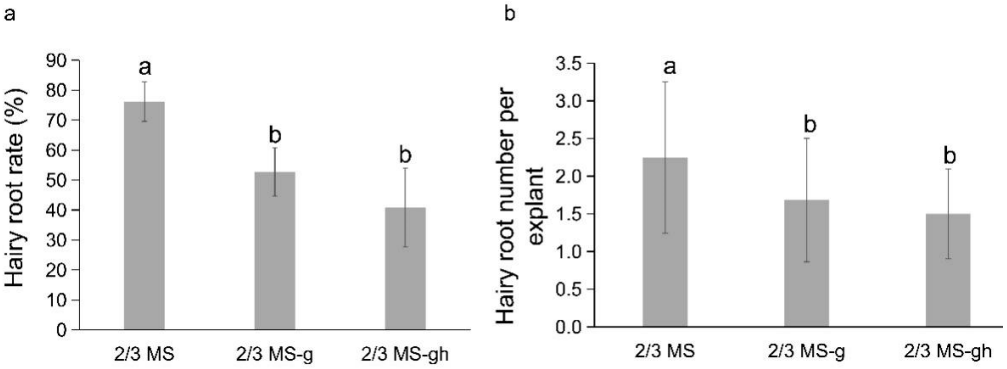

Figure 3. Effects of medium supplements on the *R. rhizogenes* strain LBA9402 hairy root induction: (**a**) effects of medium supplements on hairy root generation efficiency; (**b**) number of hairy roots that emerged per leaf disc on different media. The data are presented as mean ± standard error (SE) (n = 3) and were statistically analyzed using one-way ANOVA. The comparison of treatments was conducted using Duncan's multiple range test ($p < 0.05$). Different letters indicate a statistically significant difference ($p < 0.05$).

4.4. Expression of Genes Related to Nicotine Biosynthesis in Hairy Roots

JA has previously been reported to induce the expression of nicotine biosynthesis-related genes in tobacco. To examine how JA would impact gene expression in hairy roots, we applied 100 µM MeJA to the transgenic lines harboring pCAMBIA1305.1 and analyzed the expression of 11 nicotine synthesis-related genes before and 1 h after MeJA treatment by RT-qPCR. The results showed that MeJA treatment led to a significant upregulation of the genes tested (Figure 5), of which *NtAO*, *NtODC2*, *NtPMT1a*, and *NtQS* were strongly upregulated, while *NtA622*, *NtERF189*, *NtMATE1*, *NtMATE2*, and *NtQPT* were moderately upregulated, and *NtMPO1* and *NtBBLa* were slightly upregulated.

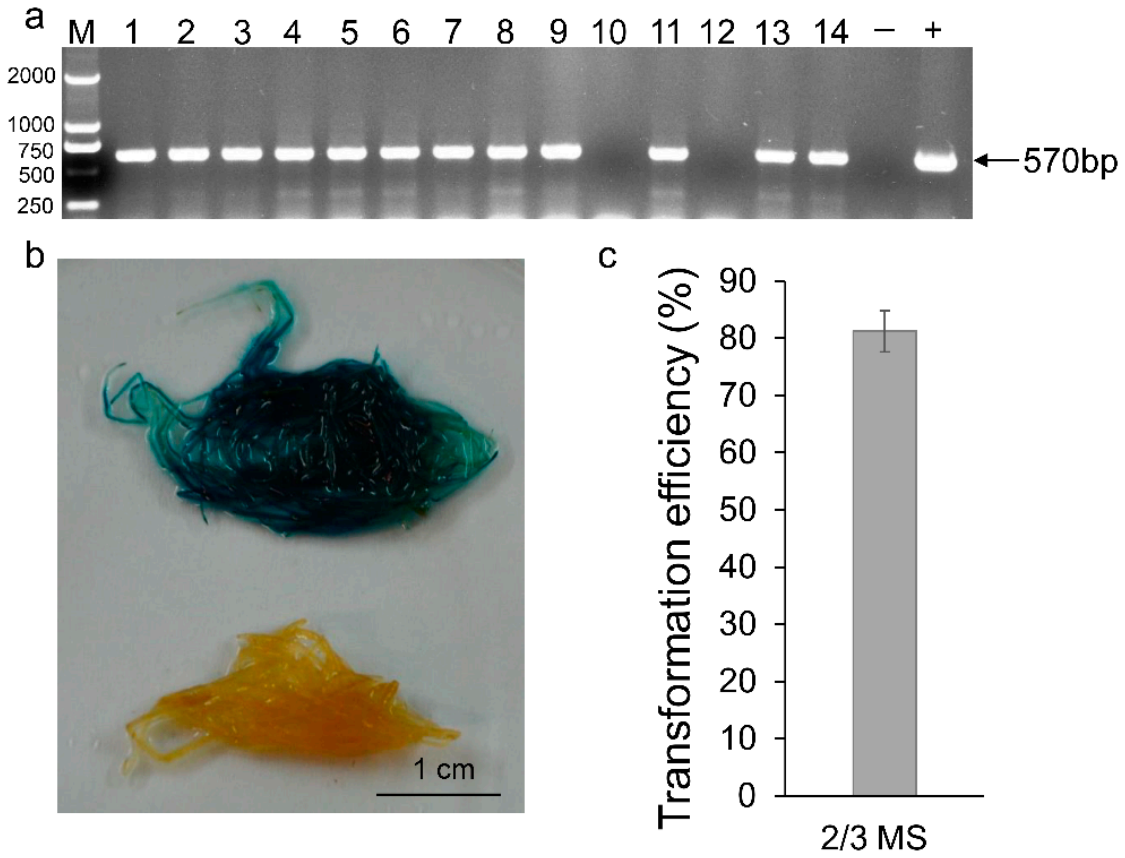

Figure 4. Efficiency of the hairy root transformation by *R. rhizogenes* strain LBA9402 containing a binary vector pCAMBIA1305.1: (**a**) PCR targeting *GUS* gene in hairy roots; (**b**) a typical picture showing positive (blue) and negative (brown) GUS staining of the hairy roots; (**c**) efficiency of tobacco hairy root transformation mediated by *R. rhizogenes* strain LBA9402 cultured on 2/3MS medium.

4.5. Effects of Overexpression of MsSPL12 on Nicotine Biosynthesis in Tobacco Hairy Roots

To test the effects of *MsSPL12* overexpression on nicotine biosynthesis, we generated *MsSPL12* transgenic hairy roots by *R. rhizogenes* strain LBA9402-mediated transformation. Of the nine potential transgenic hairy root lines randomly selected for PCR, seven contained the *MsSPL12* gene (Figure 6a). Analysis of the two transgenic lines (OE-1 and OE-2) with the highest *MsSPL12* expression (Figure 6b) revealed that, upon 4 weeks of culture, the hairy roots of the OE-1 and OE-2 were much browner and exhibited less growth, but significantly higher nicotine accumulation than those of the WT. The nicotine contents of the tested transgenic line OE-1 and OE-2 were 1.38 mg/g and 1.85 mg/g, respectively, while that of WT was only 0.51 mg/g. Although insignificant, *MsSPL12* expression in OE-2 was higher than that in OE-1, which correlated well with the significantly higher nicotine accumulation in OE-2 than in OE-1. Clearly, overexpression of the *MsSPL12* gene significantly improved the nicotine biosynthesis in the transgenic tobacco hairy roots. Expression analysis of the nicotine biosynthesis-related genes indicated that *NtMPO2* was induced significantly, *NtBBLa* and *NtERF189* had no significant changes, and *NtA662*, *NtAO*, *NtERF91*, *NtMATE1*, *NtMATE2*, *NtMPO1*, *NtODC2*, *NtPMT1a*, *NtQPT2*, *NtQS*, and *NtMYC2a* were significantly downregulated in OE-1 and OE-2 compared to WT (Figure 6d). Obviously, overexpression of *MsSPL12* had profound effects on the expression of the nicotine biosynthesis-related genes.

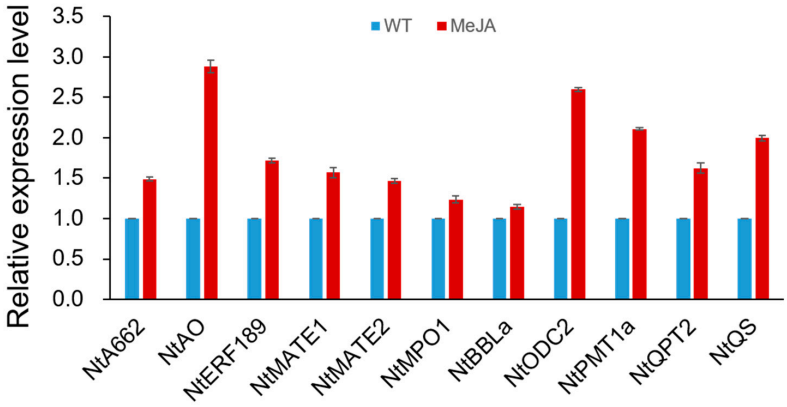

Figure 5. Relative expression of nicotine biosynthesis and transport-related genes 1 h after hairy roots were treated with 100 μM MeJA.

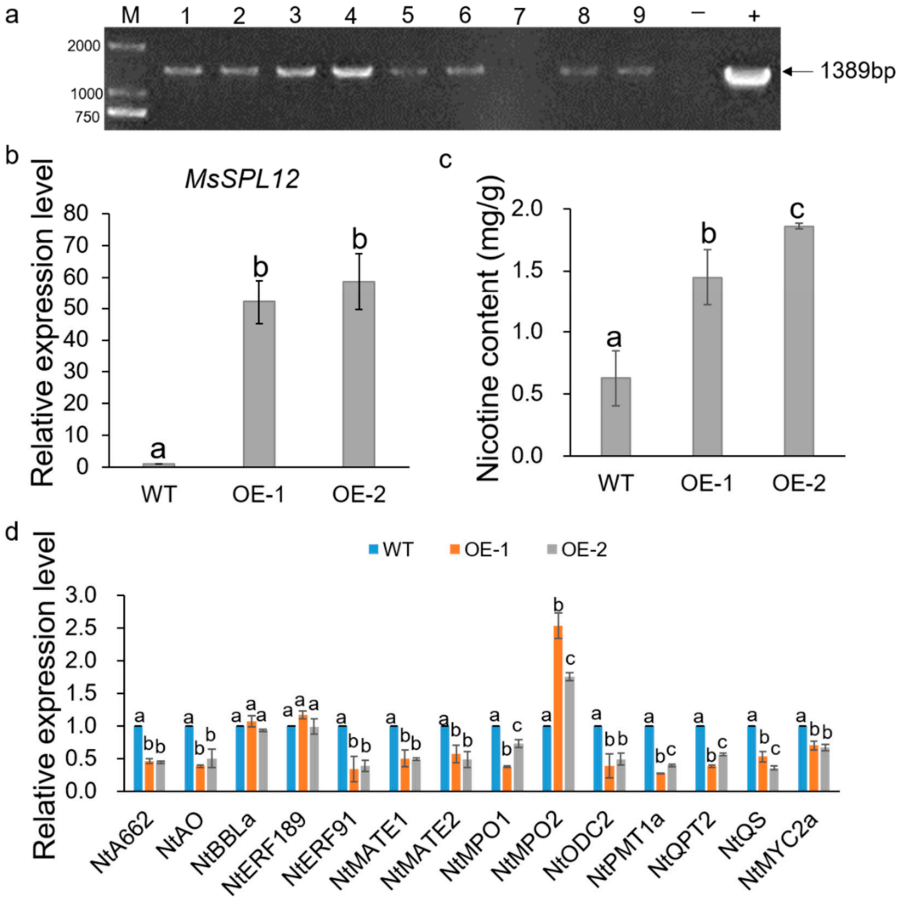

Figure 6. Overexpression of *MsSPL12* in tobacco hairy roots led to enhanced nicotine production and altered expression of nicotine biosynthesis and transport related genes: (**a**) PCR targeting *MsSPL12* in tobacco hairy roots; (**b**) RT-qPCR test revealing the relative expression level of *MsSPL12* in different lines of hairy roots; (**c**) the content of nicotine in hairy roots; (**d**) RT-qPCR test revealing the relative expression of nicotine synthesis and transport related genes in wild-type roots (control) and transgenic hairy roots overexpressing *MsSPL12*. The data are presented as the mean ± standard error (SE) ($n = 3$) and were statistically analyzed using one-way ANOVA. The comparison of treatments was conducted using Duncan's multiple range test ($p < 0.05$). Different letters indicate a statistically significant difference ($p < 0.05$).

5. Discussion

Tobacco 'Coker176' is highly resistant to tobacco mosaic virus (TMV) and suitable for use in research. A fast and highly efficient hairy root culture system is desirable for high-throughput gene function studies in tobacco. To this end, we investigated the effects of *R. rhizogenes* stain, explant age, and medium additives on hairy root induction in 'Coker176' and established an optimized system for hairy root generation and genetic transformation.

Leaves are often used as explants for *R. rhizogenes*-mediated transformation in different species [39,40]. In this study, we observed that hairy roots were initiated from the vein at the edges of the leaf discs, and the age of explants affected hairy root induction. Four and 8 week old plants had well developed leaves and leaf veins, which benefited the induction of hairy roots. On the contrary, the 2 week old tobacco plants had younger leaves and leaf vascular bundles which may not be ideal for hairy root induction. The hairy induction rate for 2 week old tobacco leaf as explants was less than 15%, even with the best-performing strain LBA9402. Consistent with our observations, hairy roots were previously reported to be often formed from vascular bundles [39,40]. For an optimized experimental turnover, it is recommended to use leaves of the 4 week old plants instead of those of the 8 week old ones as explants, as they performed similarly in hairy root induction.

R. rhizogenes strains used for explant infection were also found to significantly impact hairy root induction [14,41]. It is, therefore, interesting to examine hairy root induction efficiency on different media. Hairy roots are normally induced using hormone-free MS solid medium [42,43], although liquid medium has also been used for hairy root proliferation [44]. In this study, the transgenic hairy roots grew well in liquid medium supplemented with hygromycine, and foreign gene insertion and expression were also confirmed by PCR and GUS staining assays. The results suggested that the optimized tobacco hairy root induction system could be used for gene function study.

JA and MeJA are both regulators of nicotine synthesis and can quickly induce the expression of genes encoding enzymes in nicotine biosynthesis [45,46]. In this experiment, 11 nicotine biosynthesis and transport-related genes were upregulated to certain degrees after JA treatment. The expression of four genes (*NtAO*, *NtODC2*, *NtPMT1a*, and *NtQS*) was significantly induced. Putrescine *N*-methyltransferase (PMT) and ornithine decarboxylase (ODC) play important roles in the creation of the pyrrolidine ring of nicotine. Aspartate oxidase (AO), quinolinate synthase (QS), and quinolinate phosphoribosyltransferase (QPT) are responsible for the formation of the pyridine ring of nicotine [47,48]. Expression manipulation of the genes encoding these enzymes could serve as an alternative way to control tobacco nicotine biosynthesis.

The SPL transcription factors have previously been implicated in regulating plant growth and stress resistance, as well as the production of secondary metabolites [49,50]. Overexpression of *SmSPL6* gene induced phenolic acid biosynthesis and affected root development in *Salvia miltiorrhiza* [25]. Overexpression of *AaSPL2* increased the artemisinin content of transgenic plants [51]. Intriguingly, we found that the hairy roots of *MsSPL12* transgenic lines easily turned brown compared to the control hairy roots expressing GUS in liquid culture medium, suggesting the *MsSPL12* transgenic lines might produce more abundant secondary metabolites [50]. Overexpression of *MsSPL12* significantly increased nicotine content of tobacco hairy roots. However, unlike *NtMPO2* which was significantly upregulated compared to WT controls, the expression of *NtBBLa* and *NtERF189* exhibited no significant change, and other nicotine biosynthesis-related genes showed significantly downregulated expression compared to WT controls. MPO is responsible for regulating the deamination of *N*-methylputrescine as one of the steps converting the ornithine ring to a pyrrolidine ring [52]. BBL acts on the final stage of condensation of the two rings to produce nicotine. ERF189 is a transcription factor that responds to JA [53,54]. Considering that the expression of all the other tested genes in the nicotine synthesis pathway was declined, upregulation of *NtMPO2* expression was probably responsible, at least partially, for the increased nicotine accumulation in *MsSPL12* transgenic hairy roots. It was previously reported that overexpression of *NtAOC2*, *NtPMT1a*, and *NtQPT2* did not significantly change

the nicotine content [55,56]. Nicotine biosynthesis, therefore, could be synergistically regulated at many levels. The expression of genes in the nicotine synthesis pathway could be feedback-regulated by the nicotine itself [25]. Overexpression of *MsSPL12* caused nicotine accumulation in hairy roots, but the detailed regulatory mechanism is yet to be revealed.

Overall, our study suggests that highly efficient tobacco hairy root induction and genetic transformation could be achieved using 4 week old 'Coker176' tobacco leaf discs on 2/3MS medium with *R. rhizogenes* strain LBA9402 infection. The key genes in the nicotine biosynthesis pathway could be significantly induced using MeJA treatment, and overexpression of the *MsSPL12* gene in tobacco hairy roots could increase nicotine biosynthesis. The system reported here could also be used for the functional characterization of genes involved in nicotine biosynthesis and metabolism.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy12020348/s1, Table S1: Primers used for PCR tests; Table S2: Sequence-specific primers used for qPCR tests.

Author Contributions: B.W. and W.Z. designed the experiments; S.Q., Y.L., J.Y. and S.L. performed the experiments; S.Q., Y.L., B.W. and W.Z. analyzed the data; S.Q., W.Z. and B.W. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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