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ORIGINAL RESEARCH

Topping and grafting affect the alkaloid content and gene expression patterns of tobacco (*Nicotiana tabacum* L.)

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Abstract

In this study, we aimed to determine molecular mechanisms underlying the effects of topping and grafting in tobacco (*Nicotiana tabacum* L.) by comparing the alkaloid contents and enrichment pathways of differentially expressed genes (DEGs) among plants subjected to different combinations of topping and grafting treatments. Plants of the tobacco variety "Zhongyan 100" and eggplant (*Solanum melongena* L.) were grafted in four combinations as scions and rootstocks, respectively. The four treatment groups were tobacco with topping, tobacco without topping, topped tobacco grafted onto an eggplant rootstock, and non-topped tobacco grafted onto an eggplant rootstock. Tobacco leaves were collected on the day of topping, at 7 days after topping, and after flue curing, the alkaloid contents of the collected leaves were determined. Leaves of plants subjected to the different treatments were collected for RNA sequencing and screened for DEGs, which were subsequently subjected to functional enrichment analyses. Analyses revealed reductions in the leaf alkaloid contents of tobacco subjected to combined topping and eggplant grafting. Gene annotation indicated that topping influences biological processes such as starch metabolism and stress response, whereas grafting affected the biosynthesis and metabolic pathways of secondary metabolites. Downregulated DEGs between non-topped tobacco and eggplant-grafted topped tobacco and between topped and non-topped tobacco are mainly involved in inositol phosphate metabolic and biosynthetic processes. Downregulated DEGs between different grafting methods (eggplant-grafted non-topped tobacco vs. non-topped tobacco and eggplant-grafted topped tobacco vs. topped tobacco) are mainly involved in sesquiterpene synthase activity and photosynthesis. The findings of this study provide important insights into the molecular mechanisms underlying the effects of topping and grafting on tobacco plants.

KEYWORDS
alkaloid; enrichment analysis; grafting, nicotine; tobacco; topping

1 | INTRODUCTION

Tobacco (*Nicotiana tabacum* L) is widely grown as an important economic crop. However, more than 5 million people die each year from smoking-related causes (Picciotto & Kenny, 2021). Nicotine, one of the numerous alkaloids synthesized in tobacco (Henry et al., 2019), acts in the brain via neuronal nicotinic acetylcholine receptors, which are implicated in nicotine addiction (Wills & Kenny, 2021). It is accordingly reasoned that by reducing the amounts of nicotine in tobacco, it might be possible to reduce nicotine dependence. However, when reducing nicotine content, it is necessary to ensure that the yield and quality of tobacco are maintained (Henry et al., 2019).

An important direction with respect to reducing the nicotine content in tobacco is variety improvement (Lewis, 2019). However, due to high levels of genetic variation in nature and different agronomic techniques, the quality of tobacco is variable and cannot be directly commercialized (Lewis et al., 2015). Tillage methods (appropriate ventilation and drainage) also have an effect on nicotine levels. However, in addition to influencing nicotine contents, no-till and deep tillage can also affect yields; consequently, the effects of tillage practices tend to be variable and unpredictable (Henry et al., 2019). Planting density can also have an influence on nicotine levels in tobacco. High-density planting will increase competition for nutrient resources and results in a reduction in nicotine synthesis, although this effect tends to be limited to the middle and upper leaves. In contrast, low-density planting, promotes increases in nicotine content, albeit with a corresponding reduction in yields (Miner, 1980).

Grafting is a widely used agronomic practice that can be applied to improve tobacco yield (Ren et al., 2020). Nicotine is synthesized in tobacco roots and transported to leaves, and it has previously been found that the leaves of tobacco grafted onto a tomato rootstock were characterized by no significant accumulation of nicotine (Dawson, 1942). Similarly, the leaves of tobacco that had been grafted onto an eggplant rootstock were found to contain very low levels of nicotine (Ren et al., 2020).

Topping is considered an indispensable procedure in the cultivation of tobacco, which, by suppressing apical dominance, can facilitate the transition from the seed propagation stage to the leaf vegetative stage. This operation also has the effect of increasing the nicotine content of leaves(Qin et al., 2020). In this study, we sought to assess the combined effects of topping and grafting on the tobacco transcriptome and leaf nicotine contents. We believe that the findings of this study will provide a theoretical basis for reducing nicotine contents during the cultivation of tobacco.

2 | MATERIALS AND METHODS

2.1 | Plant materials and experimental design

This study was carried out in Xiangcheng County, Xuchang, Henan Province, China, in 2021. Eggplant (*Solanum melongena* L.) was used as a rootstock and plants of the tobacco variety "Zhongyan 100" were used as scions.

Tobacco (on the 65th day after seed planting) and eggplant seedlings (on the 72nd day after seed planting) at the five- to six-leaf stage without lignified stems were selected for grafting. Shoots bearing the two or three uppermost leaves of tobacco seedlings were taken as scions, and eggplant rootstocks were cut at approximately 5 cm above the seedling roots. The scion was then inserted into an incision cut in the rootstock and was gently pressed flat with the thumb and forefinger prior to being secured with a grafting clip. Each treatment combination comprised 40 grafted plants. The grafted seedlings were initially insulated from light and maintained under damp conditions. The relative humidity during the first 3 days post-grafting was approximately 90%, and that of the next was approximately 80%. After 3 days, the seedlings were exposed to light, and the grafting clip was removed after 7 days.

For the purposes of this study, we established four treatment groups, for each of which, we assessed three replicates. For the first group, "Zhongyan 100" plants were used as both scion and rootstock and were subjected to topping (tobacco with topping group). For the second group, "Zhongyan 100" plants were again used as both scion and rootstock, although were left untopped (tobacco without topping group). In both the third and fourth groups, "Zhongyan 100" and eggplant were used as scions and rootstocks, respectively, with the difference between the two groups being that in the third group, the tobacco was topped (tobacco was topped after grafted onto an eggplant stock group), whereas in the fourth group, plants were left untopped (tobacco was non-topped after grafted onto an eggplant rootstock group).

2.2 | Sample collection

Grafting treatments were performed when tobacco seedlings had reached the approximate six-leaf stage of growth. On the 65th day after grafting, the tobacco was topped, and leaves were collected 6 h after topping for transcriptome sequencing and assay of alkaloid contents. Similarly, leaf samples collected 7 days after topping and subsequently at the stage of tobacco maturation (106 days after topping) were used for alkaloid assays.

2.3 | Determination of alkaloid levels

Fresh tobacco leaves collected during the course of tobacco plant growth were initially cured at 105°C, and then dried at 60°C. The leaves of mature tobacco plants were cured after harvesting without initial fixation. Alkaloids were extracted from crushed dried leaf material using methyl tertbutyl ether under alkaline conditions (Zhang et al., 2022). A gas chromatograph incorporating a hydrogen flame ionization detector (Agilent 7890A; Agilent Technologies, USA) was used for quantitative analysis of alkaloid content, with n-hexadecane

used as an internal standard (Guan et al., 2021). The initial column temperature was set to 190°C, increased to 230°C at a rate of 20°C/min, and then held for 1 min. The temperatures of the injector and detector were 250°C and 275°C, respectively. Quantitative data were obtained using the internal standard method.

For qualitative analysis, an ultrahigh-performance liquid chromatograph coupled with a Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) system equipped with an ACQUITYUPLCHSS T3 column (Waters Corporation, Milford, MA, USA) was used to perform the liquid chromatography–mass spectrometry analysis as previously described (Zhang et al., 2022).

2.4 | RNA extraction and sequencing

Total RNA was extracted from tobacco leaves using a Qiagen Plant RNA Mini kit and RNase-free DNase I (TaKaRa, Dalian, China) was used to remove DNA contamination. Oligo (dT)-immobilized magnetic beads were used to enrich mRNA with polyA tails. The enriched mRNA was fragmented by ultrasound, and using random primers, the fragmented mRNA was used as a template for the synthesis of first-strand cDNA using an m-MuLV reverse transcriptase system. Following the degradation of residual RNA using RNaseH, second-strand cDNA was synthesized from dNTPs using a DNA polymerase I system. The purified double-stranded cDNA was ligated with sequencing adapters, and AMPure XP beads were used to screen for cDNAs of approximately 200 bp in size. The cDNAs thus isolated were PCR-amplified, and the PCR products were subsequently purified using AMPure XP beads to obtain a sequencing library. To ensure library quality, RNA integrity and DNA contamination were assessed based on agarose gel electrophoresis. A NanoPhotometer spectrophotometer was used to determine RNA purity, quantification of RNA was performed using a Qubit2 fluorometer, and RNA integrity was assessed using an Agilent 2100 bioanalyzer. Sequencing was conducted using an Illumina Nova-seq 6000 sequencer.

2.5 | Quality control

The raw sequencing reads were subjected to quality control using fastp (Chen et al., 2018) and filtered to remove low-quality reads. Specifically, reads containing adapter sequences or more than 10% of N bases were removed. Thereafter, reads comprising all adenines and low-quality reads (bases with Q ≤ 20 accounting for more than 50% of the entire read) were also filtered out. HISAT2 (Kim et al., 2015) was used to align sequences obtained by paired-end sequencing to the reference genome (solgenomics_sierro_et_al_2014_K326). In addition, we used the short reads alignment tool bowtie2 (Langmead & Salzberg, 2012) to align the clean reads to the ribosome database for this tobacco species, removed the aligned ribosome reads showing mismatches, and used the retained unmapped reads for subsequent transcriptome analysis.

On the basis of the HISAT2 alignment results, we reconstructed the transcripts using Stringtie and determined the expression of all genes in each sample using RSEM. To ensure the accuracy of the subsequent analysis, we initially corrected the sequencing depth and then the length of the gene or transcript to obtain the per kilobase of exon per million reads mapped (FPKM) value of the gene prior to performing subsequent analysis. FPKM values were calculated using the following formula:

$$\mathrm{FPKM} = \frac{10^6 c}{NL/10^3},$$

where C represents the number of fragments aligned to the gene, N represents the number of fragments aligned to the reference genome, and L represents the number of bases comprising the gene.

2.6 | Statistical analysis

Using the gene expression data, we used the R package (http://www.r-project.org/) to perform principal component analysis (PCA) for assessments of distance relationships between samples based on dimensionality reduction.

Genes that were differentially expressed between groups (DEGs) were screened using DESeq2 software (Love et al., 2014). The Gene Ontology (GO) database (http://www.geneontology.org/) was used for the enrichment analysis of DEGs. DEGs were mapped to GO terms, and the number of genes enriched for each term was calculated. Genes were similarly screened for Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.kegg.jp/) pathway enrichment. Terms with a false discovery rate (FDR) < .05 and |log2 fold change (FC)| > 1 were considered significant. To further analyze the pathways with differential gene enrichment, we performed gene set enrichment analysis (GSEA) (Subramanian et al., 2005).

3 | RESULTS

3.1 | Alkaloid contents

We assessed the leaf alkaloid contents of tobacco plants in the different groups at different time points (Table 1). Plants in all groups showed increases in alkaloid content subsequent to flue curing. With the exception of anabasine, the contents of alkaloids in tobacco in the groups with topping were higher than those in tobacco without topping, thereby indicating that topping can promote an increase in the content of alkaloids in tobacco leaves. For example, on day 7 after topping, the nicotine content of leaves with tobacco as a rootstock was 1.994%, whereas at the same time point, the nicotine content of the samples without topping was 1.845%, the difference between which was found to be significant ($p < .05$). Comparatively, the nicotine content of topped tobacco grafted onto eggplant rootstocks was

TABLE 1 The alkaloid content of tobacco in different treatment groups

Grafting method	Topping treatment	Time of sampling	Nicotine (%)	Nornicotine (%)	Anabasine (%)	Anatabine (%)	Total alkaloids (%)
No grafting	Non-topped	Topping day	1.827 ± .0143 dC	.052 ± .0005 dC	.008 ± .0003 dCD	.087 ± .0026 dD	1.974 ± .0394 dC
		Seven days after topping	1.845 ± .0207 dC	.056 ± .0006 cC	.008 ± .0003 dCD	.090 ± .0031 dD	1.999 ± .0262 dC
		After curing	2.269 ± .0835 bAB	.127 ± .0024 bB	.013 ± .0005 bB	.153 ± .0054 bB	2.562 ± .0684 bB
	Topping	Topping day	1.895 ± .0421 dC	.052 ± .0004 dC	.008 ± .0004 dCD	.078 ± .0024 dD	2.033 ± .0229 dC
		Seven days after topping	1.994 ± .0689 cB	.056 ± .0008 cC	.009 ± .0003 cC	.117 ± .0048 cC	2.176 ± .0434 cC
		After curing	2.920 ± .0586 aA	.147 ± .0069 aA	.017 ± .0005 aA	.214 ± .0085 aA	3.298 ± .0973 aA
Grafting on eggplant	Non-topped	Topping day	.084 ± .0005 gF	NA	.006 ± .0002 eD	NA	.092 ± .0003 fE
		Seven days after topping	.086 ± .0009 gF	NA	.007 ± .0001 deD	NA	.092 ± .0015 fE
		After curing	0.103 ± .0024 eDE	NA	.007 ± .0003 deD	.008 ± .0001 eE	.119 ± .0072 efDE
	Topping	Topping day	.085 ± .0014 gF	NA	.006 ± .0001 eD	NA	.092 ± .0021 fE
		Seven days after topping	.098 ± .0026 fE	NA	.006 ± .0004 eD	NA	.104 ± .0011 fE
		After curing	.119 ± .0039 eD	NA	.008 ± .0001 dCD	.002 ± .0000 eE	.128 ± .0095 eD

Note: Data are presented as the means ± SD.
Different uppercase letters in the same column among different treatments indicate significant differences at the .05 level, and different lowercase letters indicate significant differences at the .01 level.
NA indicates that the value is below the detection threshold.

.098%, which was significantly higher than that of tobacco that was not topped (.086%) ($p < .05$). Seven days after topping, the nicotine content of the grafted tobacco was significantly lower than that of non-grafted tobacco ($p < .01$). These findings thus indicate that using eggplant as a rootstock can effectively reduce the nicotine content of tobacco leaves. Furthermore, we found that other types of alkaloids showed patterns similar to those observed for nicotine. In summary, whereas grafting tobacco plants onto eggplant rootstocks can contribute to a reduction in the alkaloid contents of tobacco leaves, topping promotes increases in leaf alkaloids.

3.2 | Sequencing data analysis

Subsequent to quality control, we obtained at least 99.75% clean reads for subsequent analysis, with the number of clean reads ranging from 37,800,214 to 47,774,374 (Supplementary Table S1). On the basis of comparisons of these clean reads with the tobacco reference genome, we obtained successful mapping percentages ranging from 93.22% to 94.26% (Supplementary Table S2).

3.3 | Samples analysis

In PCA ordination plots, the closer the distance between sample data points, the more similar the sample compositions. The PCA results obtained in this study revealed that samples from topped tobacco and topped tobacco grafted onto an eggplant rootstock group were clustered separately. Comparatively, samples obtained from non-topped grafted tobacco and the tobacco without topping were closer, showing relatively higher similarity (Figure 1a).

FIGURE 1 Principal components analysis plot. T–T: Tobacco with topping; T-NT: Tobacco without topping; G–T: Topped tobacco grafted onto an eggplant rootstock; G–NT: Non-topped tobacco grafted onto an eggplant rootstock.

3.4 | Differential gene expression between groups

Comparing non-topped and topped tobacco grafted onto an eggplant rootstock, we detected a total of 5,297 DEGs, among which 3,045 and 2,252 were upregulated and downregulated, respectively, whereas a total of 4,941 DEGs (2,689 upregulated and 2,252 downregulated) were obtained for the comparison between topped and non-topped tobacco. Furthermore, 906 DEGs (293 upregulated and 613 downregulated) were obtained for the comparison between non-topped grafted tobacco and non-topped tobacco, and there were 3,339 DEGs (1,817 upregulated and 1,522 downregulated) between topped grafted tobacco and topped tobacco (Figure 2).

3.5 | GO enrichment analysis

Among the three GO categories molecular function, cellular component, and biological process, we found that DEGs between non-topped and topped tobacco grafted onto eggplant rootstocks are mainly involved in plastid organization, starch metabolic process, oxidoreductase activity, and oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen (Figure 3). Comparison of the enrichment of DEGs between non-topped grafted tobacco and non-topped tobacco indicated that these genes are mainly involved in the response to stress, aminoglycan metabolic process, hydrolase activity, hydrolyzing *O*-glycosyl compounds, hydrolase activity acting on glycosyl bonds, and extracellular region

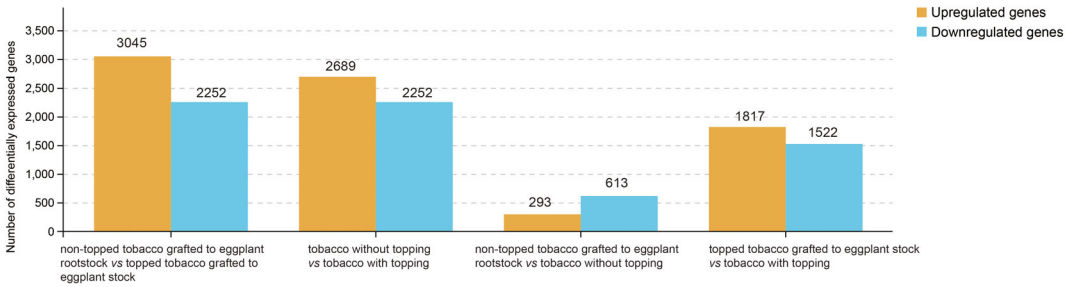
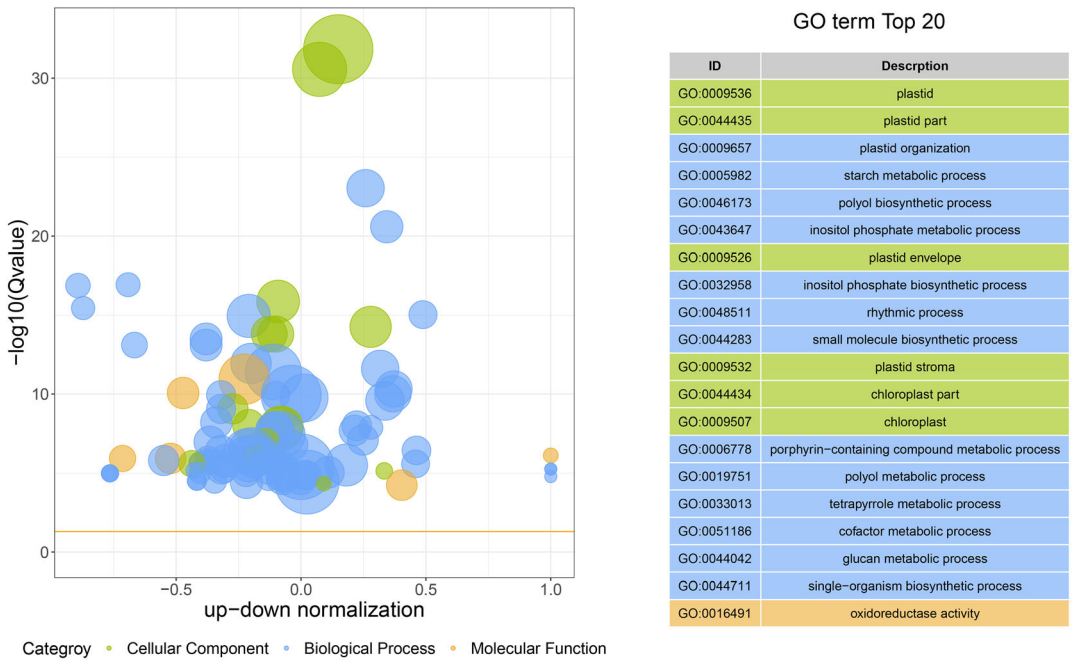


FIGURE 2 Genes differentially expressed between groups

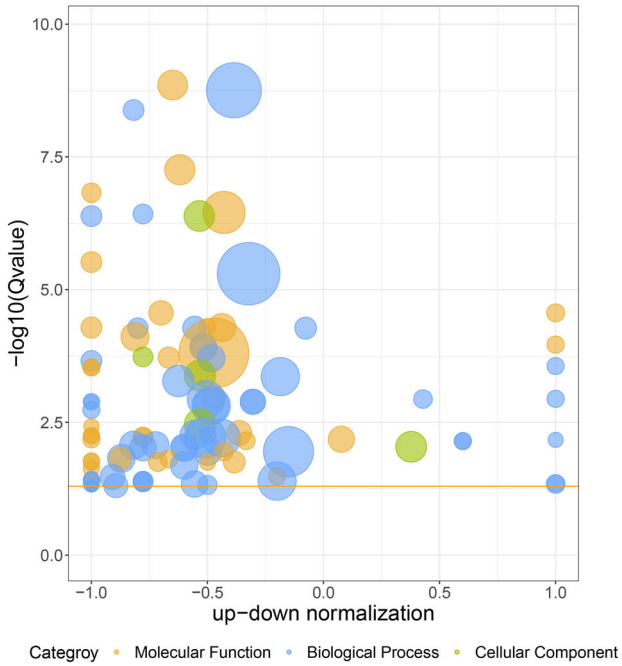


GO term Top 20

ID	Descrption
GO:0009536	plastid
GO:0044435	plastid part
GO:0009657	plastid organization
GO:0005982	starch metabolic process
GO:0046173	polyol biosynthetic process
GO:0043647	inositol phosphate metabolic process
GO:0009526	plastid envelope
GO:0032958	inositol phosphate biosynthetic process
GO:0048511	rhythmic process
GO:0044283	small molecule biosynthetic process
GO:0009532	plastid stroma
GO:0044434	chloroplast part
GO:0009507	chloroplast
GO:0006778	porphyrin−containing compound metabolic process
GO:0019751	polyol metabolic process
GO:0033013	tetrapyrrole metabolic process
GO:0051186	cofactor metabolic process
GO:0044042	glucan metabolic process
GO:0044711	single−organism biosynthetic process
GO:0016491	oxidoreductase activity

FIGURE 3 Gene ontology enrichment analysis of genes differentially expressed between non-topped and topped tobacco grafted onto an eggplant rootstock. The colors in the table correspond to the colors in the plot. Each bubble represents a term. The size of the bubble represents the number of genes contained in that term. Terms with false discovery rate (FDR) < .05 and |log2 fold change (FC)| > 1 were considered as significant terms. Top20 terms are displayed.

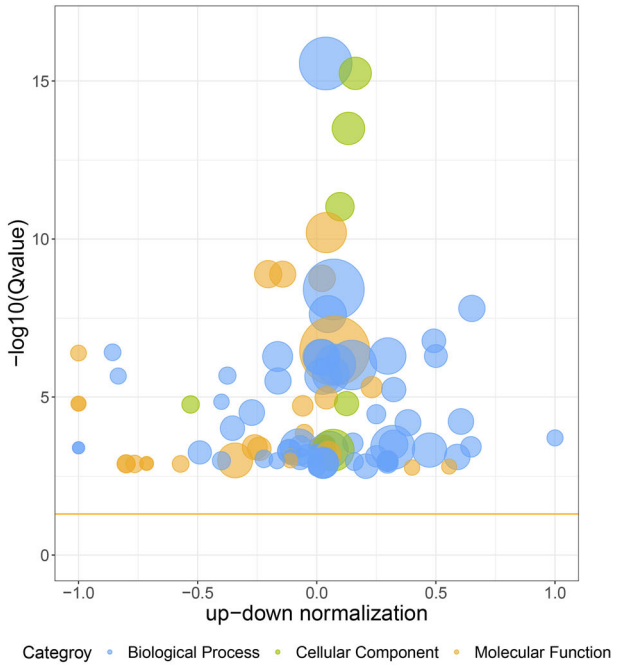
(Figure 4). The GO terms for DEGs between topped grafted tobacco and topped tobacco mainly included response to stress, response to stimulus, oxidoreductase activity, hydrolase activity, hydrolyzing *O*-glycosyl compounds, external encapsulating structure, and cell periphery (Figure 5), whereas those between non-topped and topped tobacco were mainly enriched in polyol biosynthetic process, inositol



GO term Top 20

ID	Descrption
GO:0004553	hydrolase activity, hydrolyzing O−glycosyl compounds
GO:0006950	response to stress
GO:0006022	aminoglycan metabolic process
GO:0016798	hydrolase activity, acting on glycosyl bonds
GO:0010334	sesquiterpene synthase activity
GO:0016491	oxidoreductase activity
GO:0006026	aminoglycan catabolic process
GO:0005576	extracellular region
GO:0046246	terpene biosynthetic process
GO:0010333	terpene synthase activity
GO:0050896	response to stimulus
GO:0004645	phosphorylase activity
GO:0016209	antioxidant activity
GO:0015603	iron chelate transmembrane transporter activity
GO:0016838	carbon−oxygen lyase activity, acting on phosphates
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen
GO:0009891	positive regulation of biosynthetic process
GO:0008643	carbohydrate transport
GO:1901136	carbohydrate derivative catabolic process
GO:0046906	tetrapyrrole binding

FIGURE 4 Gene ontology enrichment analysis of genes differentially expressed between non-topped tobacco grafted onto an eggplant rootstock and non-topped tobacco. The colors in the table correspond to the colors in the plot. The size of the bubble represents the number of genes contained in that term. Terms with false discovery rate (FDR) < .05 and |log2 fold change (FC)| > 1 were considered as significant terms. Top20 terms are displayed.



GO term Top 20

ID	Descrption
GO:0006950	response to stress
GO:0030312	external encapsulating structure
GO:0071944	cell periphery
GO:0005576	extracellular region
GO:0016491	oxidoreductase activity
GO:0004553	hydrolase activity, hydrolyzing O−glycosyl compounds
GO:0016798	hydrolase activity, acting on glycosyl bonds
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen
GO:0050896	response to stimulus
GO:0009642	response to light intensity
GO:0001101	response to acid chemical
GO:0009698	phenylpropanoid metabolic process
GO:0003824	catalytic activity
GO:0006022	aminoglycan metabolic process
GO:0010334	sesquiterpene synthase activity
GO:0009699	phenylpropanoid biosynthetic process
GO:1901700	response to oxygen−containing compound
GO:0006820	anion transport
GO:0051707	response to other organism
GO:0043207	response to external biotic stimulus

FIGURE 5 Gene ontology enrichment analysis of genes differentially expressed between topped tobacco grafted onto an eggplant rootstock and topped tobacco. The colors in the table correspond to the colors in the plot. The size of the bubble represents the number of genes contained in that term. Terms with false discovery rate (FDR) < .05 and |log2 fold change (FC)| > 1 were considered as significant terms. Top20 terms are displayed.

phosphate metabolic process, oxidoreductase activity, oxidoreductase activity acting on paired donors, with incorporation or reduction of molecular oxygen, and chloroplast (Figure 6).

3.6 | KEGG enrichment analysis

To identify the pathways in which DEGs might be involved, we performed a KEGG enrichment analysis. DEGs between the non-topped and topped grafted tobacco and between non-topped and topped tobacco were found to be mainly involved in pathways associated with the biosynthesis and metabolism of secondary metabolites (Supplementary Figures S1 and S2), thereby indicating that topping appears to have notable effects on the biosynthesis of secondary metabolites. We also found that under conditions of no topping, grafting (non-topped tobacco grafted onto an eggplant rootstock vs. non-topped tobacco) can have an influence on genes associated with the biosynthesis of secondary metabolites (Supplementary Figure S3). Further analysis revealed that under the conditions of topping, grafting (topped grafted tobacco vs. topped tobacco) influences genes that are mainly involved in sesquiterpenoid and triterpenoid biosynthesis and plant–pathogen interactions (Supplementary Figure S4).

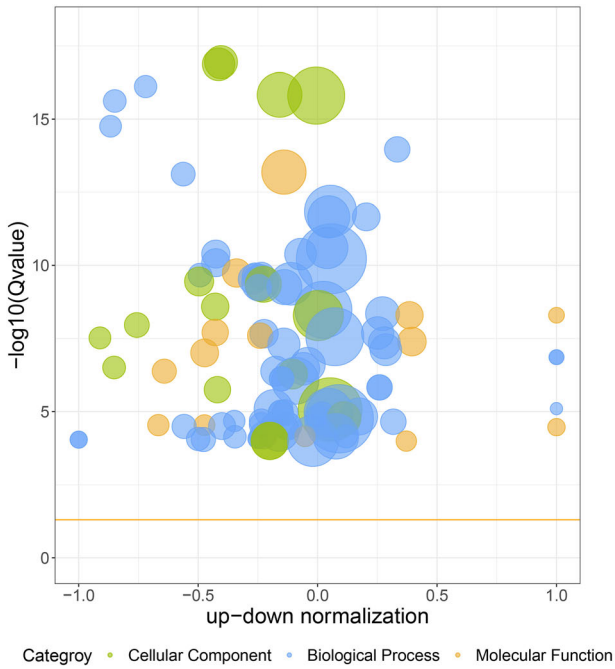
3.7 | Gene set enrichment analysis of DEGs

GSEA results indicated that genes showing downregulated differential expression between the non-topped and topped grafted tobacco are mainly involved in inositol phosphate metabolic and biosynthetic process, monooxygenase activity, oxidoreductase activity, and regulation of chlorophyll metabolic process (Supplementary Table S4). For non-grafted tobacco, downregulated DEGs between plants with and without topping were mainly enriched in inositol phosphate metabolic and biosynthetic process, photosystem, regulation of photosynthesis, and polyol biosynthetic process categories (Supplementary Table S4). Under conditions of no topping, the downregulated DEGs between grafted and non-grafted tobacco were mainly enriched in the categories sesquiterpene synthase activity, terpene biosynthetic process, terpene synthase activity, and monosaccharide transmembrane transporter activity (Supplementary Table S4). Among tobacco plants in the two topped groups, the downregulated DEGs between the grafted and non-grafted tobacco were found to be mainly involved in the photosynthetic electron transport chain, sesquiterpene synthase activity, photosynthesis, light reaction, and monosaccharide transmembrane transporter activity (Supplementary Table S4).

4 | DISCUSSION

Although topping and grafting are two common agronomic practices that are widely applied in tobacco cultivation, the molecular mechanisms that underlie the effects of these measures on tobacco alkaloid contents have yet to be sufficiently clarified. In this study, we applied combined topping and grafting treatments to investigate their effects on the alkaloid contents and transcriptome profiles of tobacco leaves.

In the two treatment groups in which tobacco served as the scion, we found that with the exception of anabasine, compared with non-topped plants, the leaves of topped plants had higher alkaloid



GO term Top 20

ID	Descrption
GO:0009507	chloroplast
GO:0044434	chloroplast part
GO:0046173	polyol biosynthetic process
GO:0044435	plastid part
GO:0009536	plastid
GO:0043647	inositol phosphate metabolic process
GO:0032958	inositol phosphate biosynthetic process
GO:0048511	rhythmic process
GO:0016491	oxidoreductase activity
GO:0019751	polyol metabolic process
GO:0009628	response to abiotic stimulus
GO:0005982	starch metabolic process
GO:0001101	response to acid chemical
GO:1901700	response to oxygen−containing compound
GO:0006778	porphyrin−containing compound metabolic process
GO:0009743	response to carbohydrate
GO:0050896	response to stimulus
GO:0033013	tetrapyrrole metabolic process
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen
GO:0015979	photosynthesis

FIGURE 6 Gene ontology enrichment analysis of genes differentially expressed between non-topped and topped tobacco. The colors in the table correspond to the colors in the plot. The size of the bubble represents the number of genes contained in that term. Terms with false discovery rate (FDR) < .05 and |log2 fold change (FC)| > 1 were considered as significant terms. Top20 terms are displayed.

contents, which is broadly consistent with the findings of previous studies (Zhao et al., 2018). Topping is an operation that suppresses the apical dominance of plants and promotes the rapid development of lateral branches (Zhang et al., 2022). In addition, topping can have the effects of delaying tobacco leaf senescence and promoting the accumulation of secondary metabolites in leaves (Zhao et al., 2018). Moreover, topping has been established to promote the synthesis and transport of alkaloids in tobacco roots, thereby contributing to further increases in the alkaloid contents of tobacco leaves (Zhao et al., 2018). The negligible differences between the two groups with respect to leaf anabasine contents in response to topping can probably be ascribed to the low levels of this alkaloid in tobacco (von Weymarn et al., 2016).

We also found that the alkaloid contents of tobacco grafted onto eggplant as a rootstock were lower than those in non-grafted tobacco plants, regardless of whether these plants were topped. Similar rootstock effects have been reported previously. For example, an approximate 90% reduction in nicotine contents has been detected in the leaves of tobacco grafted onto a tomato rootstock (Ruiz et al., 2005). In tobacco, alkaloids are synthesized in the roots and subsequently transported to leaves (Nölke et al., 2021), and thus grafting tobacco onto an eggplant rootstock effectively removes an important source of synthesized alkaloids. Consequently, the leaves of tobacco growing on an eggplant rootstock had lower alkaloid contents compared with those of the non-grafted tobacco. In addition, grafting serves as an important method for studying mechanisms associated with the transport of secondary metabolites (Dong et al., 2022).

GO enrichment analysis indicated that topping would alter the expression of genes associated with starch metabolism and other biological processes. Starch content accounts for approximately 40% of the total dry weight of mature tobacco leaves (Song et al., 2016), and thus, the synthesis and conversion of starch are important factors in determining tobacco yields. Wang et al. have reported that DEGs between topped and non-topped tobacco are enriched in processes such as starch and sucrose metabolism (Wang et al., 2018). In the present study, we found that the yield and weight per leaf of topped tobacco were significantly higher than those of non-topped tobacco, regardless of whether the plants grafted ($p < .05$, Supplementary Table S3). Having removed tobacco inflorescence, nutrients and other resources destined for reproductive purposes are diverted to the leaves(Henry et al., 2019), and we accordingly found that the proportions of medium- and high-grade leaves in the topped treatments were also significantly higher than those in the non-topped treatments ($p < .05$).

In the absence of topping, the main pathways enriched with DEGs between plants with eggplant and tobacco rootstocks include those associated with stress responses. In this regard, it has previously been demonstrated that grafting can enhance the tolerance of plants to abiotic stress and resistance to soil-borne diseases(Li et al., 2014). For example, compared with non-grafted tobacco, control of the symptoms of mosaic virus infection has been achieved using grafted plants (Bazzini et al., 2007). Furthermore, the grafting process may also promote changes in genetic signaling and gene drift (Park et al., 2007). In the present study, we found that DEGs between topped grafted and non-grafted tobacco are also involved in certain stress and stimulus responses, whereas KEGG enrichment analysis revealed that DEGs between groups are mainly involved in pathways associated with the biosynthesis and metabolism of secondary metabolites.

On the basis of GSEA, we established that inositol phosphate metabolic and biosynthetic processes were among the main enriched pathways of downregulated DEGs between non-topped and topped tobacco plants grafted onto eggplant rootstocks. Tobacco plants that have been topped are characterized by a higher biomass and a prolonged vegetative growth stage (Kurt & Kinay, 2021), and during vegetative growth, excess phosphate can be converted into a relatively fixed form (IP3–IP6) in cells (Yip et al., 2003). Among the grafting-related DEGs using eggplant as a rootstock (topped grafted tobacco vs. topped tobacco and non-topped grafted tobacco vs. topped tobacco), we detected metabolic shifts in those associated with sesquiterpene biosynthesis. In this regard, it has been found that the biosynthesis and metabolism of the sesquiterpenoid capsidiol are modified in tobacco plants subjected to biotic stress, along with the upregulation of a variety of related genes (Song et al., 2019).

The World Health Organization recommends the development of cigarettes with ultralow (.04%) nicotine contents, which would necessitate major changes in the currently used tobacco growing techniques (World Health Organization, 2015). Notably in this regard, conventional tobacco cultivation requires topping to eliminate apical dominance, which leads to further increases in nicotine content. As a means of overcoming this undesirable effect, we used eggplant as a rootstock to cultivate tobacco with an ultralow nicotine content. We found that in response to applying the same topping treatment, the nicotine content in grafted tobacco (.119%) was only 4% of that in non-grafted plants (2.920%). These findings represent an improvement on the previously reported 90% reduction in studies using tomato as a rootstock (Ruiz et al., 2005). In addition to grafting, increasing nitrogen content and planting density have also been demonstrated to contribute to reductions in nicotine levels. To date, however, it has yet to be established whether these measures would adversely affect the yield or quality of tobacco (Krishna et al., 2004). On the basis of our grafting experiments, we found that compared with using tobacco as a rootstock, we detected no significant reductions in the yield or quality of tobacco grafted onto an eggplant rootstock.

In summary, in this study, we compared changes in the alkaloid contents and transcriptomes of tobacco subjected to different grafting and topping treatments. Our findings indicate that whereas topping can promote increases in the alkaloid contents of tobacco leaves, grafting can contribute to reductions in the levels of these alkaloids. Enrichment analysis revealed that topping influences biological processes such as starch metabolism and stress responses, whereas grafting leads to changes in pathways associated with the biosynthesis and metabolism of secondary metabolites. Collectively, the findings of this study provide insights into the molecular mechanisms that underlie the effects of topping and grafting on tobacco plants.

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None.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHORS' CONTRIBUTIONS

Conception and design of the research: HS; acquisition of data: MZ and YZ; analysis and interpretation of data: MZ and YZ; statistical analysis: HS; drafting of the manuscript: MZ; revision of the manuscript for important intellectual content: HS. All authors have read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from Hongzhi Shi.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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