BMC Plant Biology

RESEARCH Open Access

The formation of nicotine heterosis is mainly achieved by enhancing the nicotine transport capacity in hybrids

Zejun Mo[1,3], Ying Huang[1,3], Lili Duan[2,3], Kai Pi[2,3], Benshan Long[1,3], Min Xie[2,3], Shuxian Yang[1,3], Guizhi Wu[1,3] and Renxiang Liu[1,3*]

Abstract

Nicotine exhibits obvious heterosis, which can be used to create *Nicotiana tabacum* L. (tobacco) varieties with varying nicotine content. However, the reasons for the formation of nicotine heterosis and its relationship to nicotine transport and accumulation remain unknown. This study conducted a comprehensive analysis of six tobacco hybrids with varying heterosis levels and their parent materials from various aspects, such as phenotype, physiology, and transcriptomics. The results showed that the direct path coefficient of transport heterosis to nicotine heterosis was highest in hybrids, at 0.98, and a highly significant positive correlation between the two. The plant height, thick stalk circumference, large flow of tissue fluid in the stalk, and high nicotine concentration of tobacco were the underlying factors that led to the strong nicotine transport capacity of hybrids. The formation of nicotine transport heterosis in hybrids was mainly influenced by non-additive gene effects (accounting for 89.93%), with over-dominant effects playing a dominant role (accounting for 58.79%). Among non-additive expression DEGs, nicotine transporter related multi antimicrobial extrusion protein, drug/metabolite transporter, ABC family transporter, and glutathione S-transferase were significantly upregulated in hybrid strains. The RT-qPCR results indicated that these genes related nicotine transport also exhibited heterosis at the expression level. Our results revealed that the formation of nicotine heterosis is mainly achieved by enhancing the nicotine transport capacity in hybrids. The results are not only beneficial for promoting the theoretical study of nicotine heterosis in tobacco and the breeding and utilization of hybrids, but are also of great significance for guiding nicotine production and promoting its multipurpose utilization.

Keywords Heterosis, Nicotine, Transport, Transcriptomics, *Nicotiana tabacum* L.

*Correspondence:
Renxiang Liu
rxliu@gzu.edu.cn
[1]College of Tobacco, Guizhou University, Guiyang, China
[2]College of Agriculture, Guizhou University, Guiyang, China
[3]Key Laboratory of Tobacco Quality in Guizhou Province, Guiyang, China

Introduction

Heterosis is a common genetic phenomenon that refers to hybrids that often exceeding their parents in terms of growth, reproductive rate, stress resistance, yield, and quality traits, which improves the economic benefits of agricultural production [1–3]. Numerous scholars have conducted extensive research on the formation mechanism of heterosis, focusing on identifying methods to predict its strength and fixing its performance. Many hypotheses have been proposed to explain the genetic mechanisms of heterosis, with the dominance hypothesis [4, 5], overdominance hypothesis [6], and epistasis [7, 8] being the most influential and widely accepted ones. With the advancement of quantitative genetics, the "allele-specific expression" [9] and "single-parent expression complementation" [10] hypotheses have also driven the research progress in crop heterosis.

Nicotine has significant heterosis and that the overdominance effect plays a crucial role in the formation of nicotine heterosis in tobacco leaves [11]. Nicotine is a unique alkaloid found in *Nicotiana tabacum* L. (tobacco) and is the basis for its commercial value [12]. The optimal nicotine content in tobacco leaves is typically 2.5%, as levels that are too high or too low can directly affect the aroma, taste, and strength of the resulting cigarette products [13]. Essence derived from natural nicotine can significantly enhance the aroma and strength of cigarette products. Adding natural nicotine to the essence can reduce the consumption of superior tobacco leaves and the cost of cigarette production [14]. Natural nicotine and its derivatives have significant developmental and utilization value and can be used in various fields such as cosmetics and skincare production, medical drug research, and pest and disease control [15, 16]. Regulating nicotine content plays a crucial role in the growth of the tobacco industry and its multipurpose use.

Nicotine is a long-distance transport metabolite that is initially synthesized in the roots, transported through the xylem, and finally accumulated in the vacuoles of leaf cells. This process requires transport across multiple tissue membranes [17, 18]. *NUP1/2*, *JAT2*, and *NtMATE1/2* play crucial roles in the transport of nicotine in the aboveground part and nicotine deposition in the vacuoles [19, 20]. These transport proteins play a protective role in cells against toxic damage caused by alkaloids and other cations by transporting nicotine to the vacuoles for storage. Our research has found that the expression levels of genes involved in nicotine synthesis and transport were found to be significantly upregulated in the roots of hybrid plants [21]. As a result, we speculated that the efficiency of nicotine transport and accumulation in hybrids may have been improved.

Therefore, based on the long-distance transport characteristics of nicotine, we divided the tobacco plants into three parts: roots, stalks, and leaves. By using various research methods such as phenotype, physiology, and omics, comparative analyses were conducted on different hybrids and their parent materials to clarify the relationship between nicotine heterosis and nicotine transport capacity, as well as physiological and molecular mechanisms underlying the formation of nicotine heterosis in hybrids. Our research results are crucial for promoting the theoretical understanding of tobacco nicotine heterosis and the breeding and utilization of hybrids. At the same time, it can also serve as a valuable reference for guiding nicotine production and promoting its multipurpose utilization through methods of metabolic engineering and synthetic biology.

Materials and methods

Plant cultivation and sample collection

Based on a previous study [22], six hybrid combinations and their eight parents were used as plant materials, all of which were provided by the Key Laboratory of Tobacco Quality in Guizhou Province. We ensured that the collection of plant material, experimental research, and field studies, complied with relevant institutional, national, and international guidelines and legislation. The field experiment was conducted in the tobacco scientific research and experimental base of Guizhou University in 2021 and 2022 (E 106°16'; N 26°06'). A completely randomized block design with three replicates was used. Each plot was planted in two rows, with 15 plants per row, and the row spacing was 110×55 cm. All plants were topped after 50% of the center flowers had opened, and removed the bottom two leaves, resulting in an actual number of 18 leaves left. Other field cultivation and management measures should be implemented in accordance with the Guizhou Province standard high-quality tobacco production plan.

The initial sampling occurred when 50% of the central flowers of all the plants bloomed, followed by topping treatment on the same day, followed by weekly sampling. Three tobacco plants were randomly selected from each plot, and samples were taken from their roots, stems, and leaves. The sampling method was as follows: taking tender and small non-oriented root tips from the roots; selecting the stalk located between the 9th and 11th leaves, removing the pith and xylem, retaining the xylem; and selecting three leaves between the 9th and 11th leaves. Then, the partial samples were kept in different sterilized centrifugation tubes frozen with liquid nitrogen and immediately stored in a refrigerator at −80 ℃. The remaining samples were denatured at 105 ℃ for 30 min, dried at 75 ℃, ground into powder, bagged, and sealed for storage.

Method for measuring nicotine content and calculating heterosis

According to the method of Shoji et al. [23], nicotine was separated from the extract of dry leaf samples and analyzed through gas chromatography. The calculation formulas for nicotine transport coefficient (TF) and nicotine transport heterosis are as follows:

$$\text{Nicotine heterosis } (\%) = \frac{F_1 - MP}{MP} \times 100\%$$

$$\text{Nicotine transport coefficient (TF)} = \frac{\text{Leaf nicotine content}}{\text{Root nicotine content}}$$

$$\text{Nicotine transport heterosis } (\%) = \frac{F_{1(TF)} - MP_{(TF)}}{MP_{(TF)}} \times 100\%$$

Determination of agronomic traits and physiological indicators related to nicotine transport

Agronomic trait indices were measured based on the YCT 142–2010 tobacco agronomic trait survey method. The plant height, stalk circumference, leaf length, leaf width, leaf thickness, and leaf area were measured.

Bleeding fluid was collected through a natural collection method that utilizes plant root pressure (gravimetric method). On the 75th day after transplantation, starting at 7:00 am, the tobacco plants were cut off 10 cm from the base of the stalk. Then, 2 g of cotton was placed at the end of the stalk and covered with a self-sealed bag. After 3 h, the cotton was removed and weighed to determine the amount of bleeding fluid. The bleeding liquid soaked in cotton was squeezed out using a syringe for absorption and filtered through a filter membrane specification of 0.45 μm. The processed liquid was collected in a 10 mL centrifuge tube for subsequent determination of the nicotine content in the bleeding fluid.

The nicotine concentration in the bleeding fluid was determined through high-performance liquid chromatography (HPLC). The nicotine standard had a mass spectrometry purity level of 1.08 mg/mL. The chromatographic analysis conditions are as follows: Agilent 1260 high-performance liquid chromatography; chromatographic column: C18 column (250 mm × 4.6 mm, 5 μm); detector: PDA diode array detector; detection wavelength: 259 nm; flow rate: 1.0 mL/min; column temperature: 40 ℃; automatic injection volume 10 μL; mobile phase: methanol: 0.02 mol/L disodium hydrogen phosphate with 0.2% triethylamine (pH 6.0)=40:60, with isocratic elution. Nicotine migration was calculated based on these results.

On the 75th day after transplantation, we selected and cut the stalks located between the 9th to 11th leaves, cut them in half from the middle, and placed them vertically in a 1% magenta solution. After 3 h, the migration rate of the magenta solution in the stalks was measured.

RNA extraction and transcriptome sequencing

Nine RNA samples were extracted using the RNAprep Pure Plant Total RNA Extraction Kit. Nanodrop2000 was used to detect the concentration and purity of the extracted RNA, agarose gel electrophoresis was used to detect the integrity of the RNA, and Agilent 2100 was used to determine the RIN value. The Illumina-HiSeqXT0n platform was commissioned by Shanghai Meiji Biotechnology Co., Ltd. for sequencing.

Align clean reads with the tobacco genome using HISAT2 (version 2.1.0) [24]. The exon model fragment (FPKM) of each gene was calculated using Cufflinks (version 2.2.1), and reads were obtained for each gene using the HTSeq-count tool [25, 26]. Differential gene expression was analyzed using DESeq2 (version 1.10.1) [27]. $P \leq 0.05$ and foldchange ≥ 1.5 or foldchange ≤ 0.67 were used as thresholds to screen for differentially expressed genes (DEGs).

Functional annotation of the DEGs was performed using databases such as GO (http://www.gencontology.org/) and KEGG (http://www.genome.jp/kegg/). Based on the hypergeometric distribution, the R language package was used to enrich the annotation information into the corresponding GO terms and KEGG pathways to explore the biological functions of these important DEGs [28].

Determination of gene expression levels (RT-qPCR)

The RNAprep PurePlant RNA Mini Kit was used to extract total RNA, and the FastKing gDNA Dispatching RT SuperMix was used to reverse transcribe the qualified RNA into cDNA. Both kits were purchased from TIANGEN Biotechnology Co., Ltd. The operation was performed as per the manufacturer 's instructions. Six genes were designed and detected using NCBI online software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome), and the designed primers were submitted to Beijing Tsingke Biotech Co., Ltd. for synthesis. Tobacco L25 was used as the reference gene (GenBank Accession number: L1890, F: CCCCTCAC CACAGAGTCTGCG, R: AAGGGTGTTGTTGTCCT CAATCTT). The RT-qPCR analysis was performed on six genes. The specific experimental procedure was performed according to the instructions of the Baoguang Biological SYBRPRIME qPCR Kit (Fast HS) BG0014, and fluorescence product amplification was performed using an Applied Biosystems 7500 real-time PCR system (Life Technologies Corporation, Beverly, MA, USA). The relative expression level of the gene was calculated by $2^{-\Delta\Delta Ct}$ method [29].

Table 1 Nicotine content in different tissues of hybrids and parental inbred lines (%)

Number	Material name	Root	Stalk	Leaf
1	Hongda	1.72a	0.32ab	2.05a
2	H×B	1.57ab	0.35a	2.10a
3	Basma	1.38bcde	0.19cde	1.16 cd
4	V×B	1.42bcd	0.27abc	1.58b
5	Va116	1.37bcde	0.24bcd	1.43bc
6	V×G88	1.48bc	0.25abc	1.35bcd
7	GDH88	1.33cde	0.22bcd	1.16 cd
8	GDH94	1.33cde	0.18cdef	1.07 cd
9	G94×Q	1.39bcde	0.13def	1.29bcd
10	Qinggeng	1.19efg	0.11ef	1.59b
11	K×Q	1.20ef	0.09ef	1.2bcd
12	K326	1.37bcde	0.17cdef	1.55b
13	K×M	1.24de	0.09ef	1.00d
14	Meida	1.00fh	0.08f	1.03d

The lowercase letters are significantly different ($P \leq 0.05$). Significant differences at $P \leq 0.05$ was determined using the Duncan's new multiple range test, and similarly hereinafter

Table 2 The heterosis performance of nicotine content in the roots, stalks, and leaves of six hybrids (MP, %)

Number	Material name	Root	Stalk	Leaf
1	H×B	1.18	36.97	31.04
2	V×B	3.44	21.79	21.94
3	V×G88	9.91	6.84	4.26
4	G94×Q	10.68	-4.97	-3.45
5	K×Q	-6.05	-31.18	-23.70
6	K×M	4.96	-26.97	-22.30

Data statistics and analysis

Using SPSS 16.0 software, Duncan's new multiple range tests were used to analyze the significance of the test data ($P \leq 0.05$ and $P \leq 0.01$). Origin 2018 (95_64), GraphPad Prism 9, and Adobe Illustrator CS6 were used to plot images.

Results

Nicotine content and heterosis performance of different materials

The differences in materials are the foundation for trait improvement and genetic research. Our results showed a significant difference in nicotine content among different materials in the same tissue (Table 1). The hybrid H×B had the highest nicotine content in the roots, stalks, and leaves, while the nicotine content in K×M was relatively low. Nicotine content in the roots, stalks, and leaves of different materials varied from 1.00 to 1.72%, 0.08–0.35%, and 1.00–2.10%, respectively. The results showed that the nicotine content of these materials varies greatly, and further analysis can be conducted to determine the relationship between nicotine accumulation, nicotine synthesis, and transport. We then analyzed nicotine heterosis in six hybrids and found that there were certain differences in nicotine heterosis among different hybrids in the roots, stalks, and leaves, with ranges of -6.05–10.68%, -31.18–36.97%, and −23.706–31.04%, respectively (Table 2). The analysis of nicotine heterosis in leaves showed that H×B and V×B exhibits strong heterosis (31.04% and 21.94%), whereas V×G88 and G94×Q shows weak heterosis (4.26% and −3.45%), K×Q and K×M exhibits a negative strong heterosis (-23.70% and −22.30%). The results indicated that there are significant differences in nicotine heterosis among different hybrids, providing abundant material resources for theoretical research on nicotine heterosis.

To verify the reliability and accuracy of the materials screened in the early stage, we randomly selected one hybrid and its corresponding parents with positive strong heterosis (H×B), weak heterosis (V×G88), and negative strong heterosis (K×Q) in nicotine heterosis, and conducted inter-annual validation experiments the following year. In the stalk and leaf tissues, the hybrid H×B displayed strong heterosis, particularly after 69 days of transplantation, the leaf nicotine heterosis value reached 51.68%. In all four sampling stages, the heterosis of the hybrid V×G88 was less than 6%. The hybrid K×Q has consistently shown strong negative heterosis, with values of -26.91% and −27.84% at 62 and 69 days after transplantation, respectively (Fig. 1A, B and C). However, the nicotine heterosis in the roots of the three hybrid varieties was opposite to that in the leaves. The results showed nicotine heterosis of these hybrids showed consistent performance at different stages of different years, indicating that the selected hybrids and their parent materials were less affected by the environment. The above results provide a solid foundation for theoretical research on nicotine heterosis, which can be used to analyze the relationship between nicotine heterosis and the capacity of nicotine synthesis in roots, transport in stalks, and accumulation in leaves.

The relationship between nicotine heterosis and nicotine synthesis, transport, and accumulation

The analysis of the relationship between nicotine heterosis in hybrids and nicotine content in different tissues can be used to demonstrate its relationship with nicotine synthesis, transport, and accumulation. The results of correlation and path analysis between nicotine heterosis and nicotine content in roots, stalks, and leaves and transport coefficient, indicated that there was a highly significant positive correlation between the nicotine heterosis in hybrid leaves and the nicotine content in roots, stalks, and leaves and the transport coefficient, with correlations of 0.632, 0.652, 0.912, and 0.807, respectively (Fig. 2A). The direct path coefficient of the transport coefficient to nicotine heterosis was the highest, at 0.72 (Fig. 2B). The results indicated that the formation of nicotine heterosis was mainly influenced by the capacity of leaves to

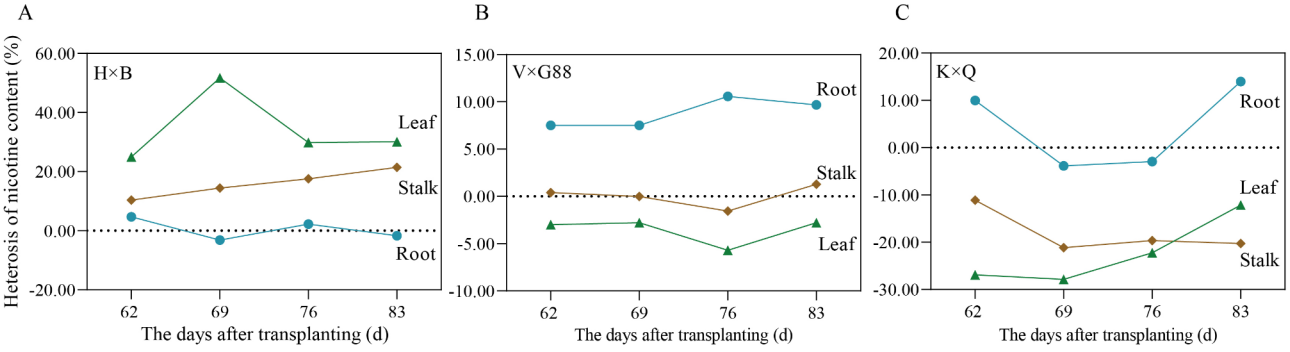


Fig. 1 The heterosis performance of nicotine content in the roots, stalks, and leaves of three hybrids at different stages. (**A**), (**B**), and (**C**) show the nicotine heterosis performance of hybrid (H×B, V×G88, and K×Q) at different stages, respectively. The blue, brown, and green lines represent the nicotine heterosis of roots, stalks, and leaves, respectively

accumulate nicotine and stalks to transport nicotine. The correlation between the nicotine heterosis in leaves and the nicotine heterosis in stalks and transport heterosis reached a highly significant level with correlations coefficients of 0.95 and 0.65, respectively (Fig. 2C). The direct path coefficient of nicotine transport heterosis to nicotine heterosis in leaves was the highest, at 0.98 (Fig. 2D). The results showed that the formation of nicotine heterosis in the leaves was mainly affected by the nicotine transport heterosis of the hybrids, and that the hybrids had stronger nicotine transport capacity than their parents.

Analysis of differences in phenotypes and physiological indicators related to nicotine transport capacity of hybrids

To understand the physiological mechanisms underlying nicotine transport heterosis, we measured the phenotypes and physiological indices related to nicotine transport. The research results showed significant significant differences in the bleeding amount among different materials, with a range of 5.59 to 8.13 g/3 h (Fig. 3A). It has been speculated that the nicotine transport in plants may be affected by root pressure. There was a significant difference in nicotine concentration in the bleeding fluid among the six materials, ranging from 0.0747 to 0.1345 mg/ mL (Fig. 3B), indicating that the nicotine concentration in the bleeding fluid can be used as an indicator of nicotine transport capacity. The nicotine migration amount in H×B was significantly higher than that in V×G88 (Fig. 3C), indicating that the strong heterosis hybrid had a stronger capacity to transport nicotine. We used the apoplast staining tracer method to analyze the water migration characteristics of tobacco xylem ductus. The results showed that the areas where the dye solution passed through the ductus were clearly stained (Fig. 3D). There were significant differences in water migration rates between different materials, ranging from 2.46 to 6.28 cm/3 h (Fig. 3E), indicating that the formation of nicotine heterosis was related to the water migration rate in stalks, that was, related to the transport rate of nicotine in stalks.

The relationship between nicotine transport capacity of hybrids and phenotypes and physiological indicators

The analysis results showed a significant or extremely significant correlation between plant height, stalk circumference, leaf length, and nicotine transport coefficient (Fig. 4A). The direct path coefficient of stalk circumference to the transport coefficient was the highest, followed by plant height with values of 0.4865 and 0.4529, respectively (Fig. 4B). These results indicated that the nicotine transport capacity of hybrids was mainly influenced by stalk circumference and plant height. There were significant or extremely significant correlations between the heterosis of plant height, stalk circumference, and leaf length and the nicotine transport heterosis, with correlations coefficients of -0.8313, 0.4067, and 0.6030, respectively (Fig. 4C), indicating that the nicotine transport heterosis in the hybrids may be related to the heterosis of plant height, stalk circumference, and leaf length.

Further analysis revealed that the nicotine transport coefficient was highly significant and positively correlated with the amount of bleeding, nicotine concentration in the bleeding fluid, nicotine migration amount, and water migration rate, with correlations coefficients of 0.9541, 0.8720, 0.9402, and 0.9570, respectively (Fig. 4D). The direct path coefficient of nicotine migration to the transport coefficient was the highest, reaching 3.5988 (Fig. 4E). The heterosis of these four indicators was significantly and positively correlated with nicotine transport heterosis, with correlations coefficients of 0.9323, 0.7564, 0.7952, and 0.9282, respectively (Fig. 4F). Among them, heterosis of the bleeding and nicotine migration amounts had the highest direct path coefficient to nicotine transport heterosis (Fig. 4G). The results revealed that the large flow of tissue fluid in the stalk and the high nicotine concentration were the underlying factors for the strong nicotine transport capacity of the hybrids.

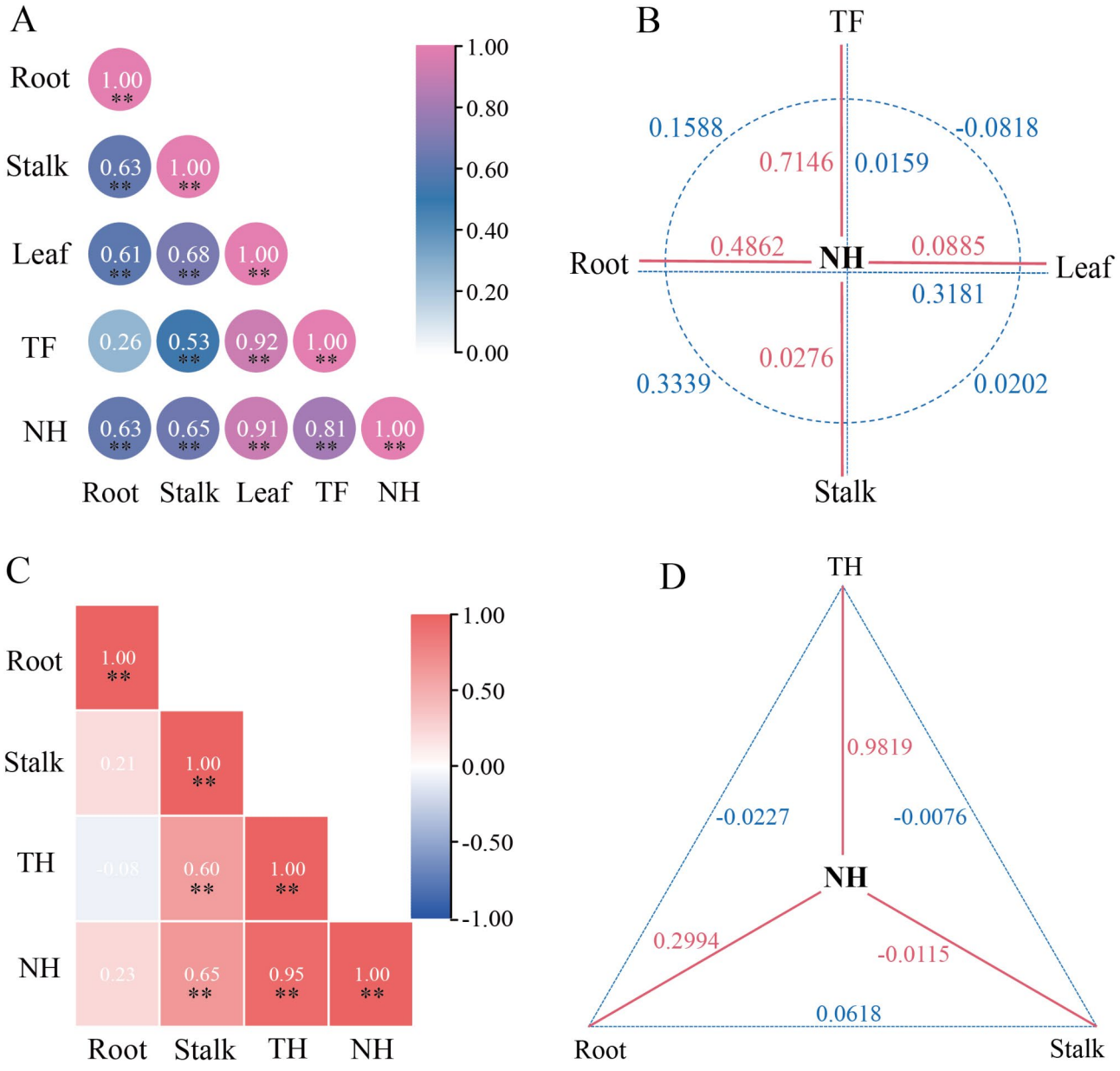


Fig. 2 Correlation and pathway analysis between nicotine heterosis and other traits. (**A**) The correlation analysis of nicotine heterosis and transport capacity and nicotine content in the roots, stalks, and leaves of hybrids. (**B**) Path analysis of the nicotine transport coefficient and nicotine content in the roots, stalks, and leaves of hybrids to nicotine heterosis. In (**A**) and (**B**), "root (stalk, leaf)" refers to the nicotine content of the root (stalk, leaf), "TF" refers to the nicotine transport coefficient, and "NH" refers to nicotine heterosis in leaves. "**" represents an extremely significant correlation, while "*" represents a significant correlation, as shown below. (**C**) The correlation analysis of nicotine heterosis in leaves and nicotine heterosis in the roots and stalks, as well as transport heterosis in hybrids. (**D**) Path analysis of transport heterosis and nicotine heterosis in the roots and stalks of hybrid to nicotine heterosis in leaves. In (**C**) and (**D**), "root (stalk)" refers to the nicotine heterosis in the root (stalk), "TH" refers to the heterosis of nicotine transport coefficient, and NH refers to the nicotine heterosis in leaves. In (**B**) and (**D**), the red solid line represents the direct path coefficient, and the blue dashed line represents the indirect path coefficient

Identification and expression pattern analysis of differential genes in hybrids and its parents

Based on the results, we used the strong heterosis combination H×B and its parents, Hongda and Basma, to analyze the causes of nicotine heterosis at the transcriptional level. Information on the quality control analysis of the sequencing data is presented in Table S1, and the results of the comparison between the original data and the reference genome after quality control are presented in Table S2. These results showed that the sequencing data were reliable and could be used for further analyzing differentially expressed genes associated with target traits. The analysis revealed that a total of 34,914 genes were detected, with 31,330 co-existing with the three

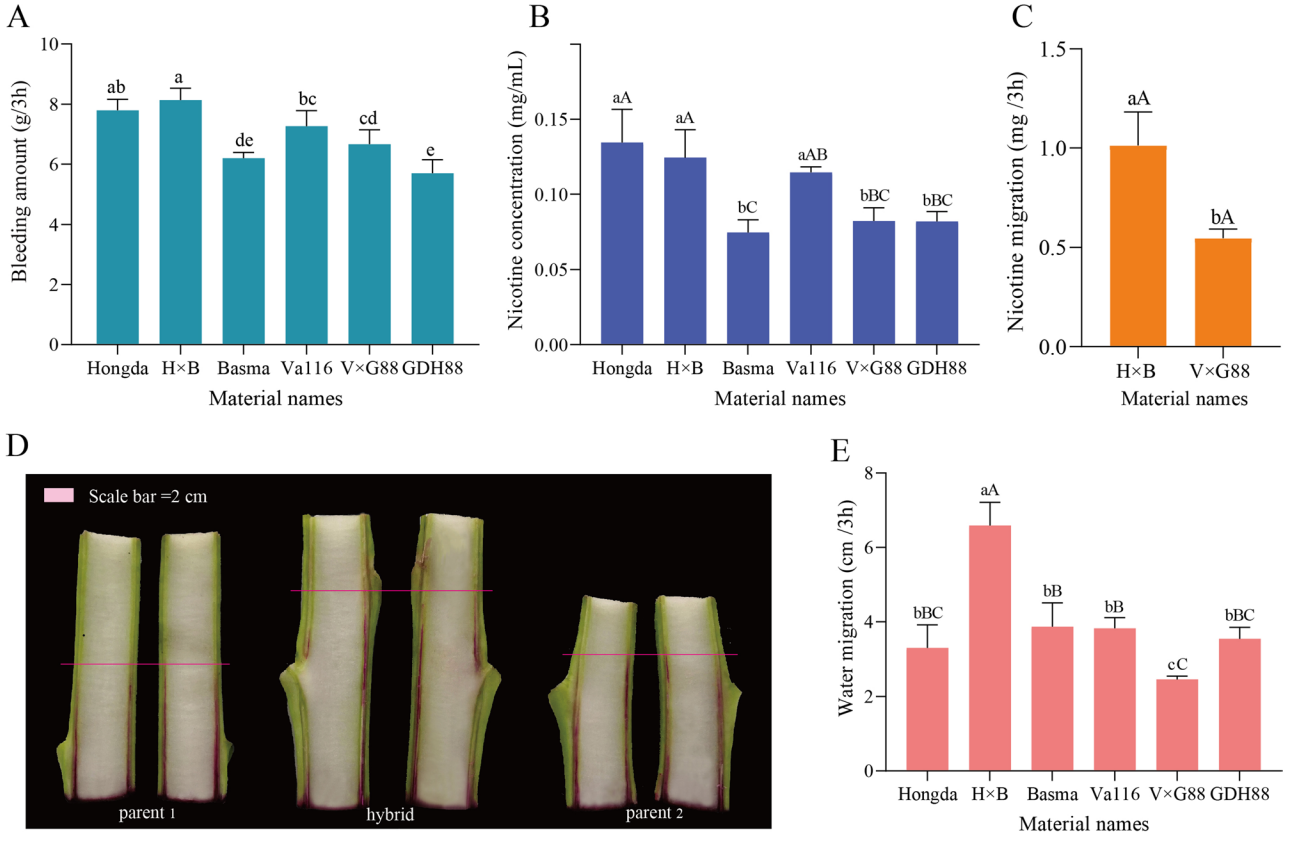


Fig. 3 Analysis of differences in physiological indicators of different materials. (**A**) Differences in the bleeding amounts of different materials. (**B**) The difference in nicotine concentration in the bleeding fluid of different materials. (**C**) Nicotine migration amounts in hybrids. (**D**) Dyeing tobacco stalks. (**E**) Comparison of water migration rates among different materials. In (**A**), (**B**), (**C**), and (**E**), different lowercase letters above the bars indicate significant differences between different materials ($P \leq 0.05$), and different uppercase letters indicate highly significant differences between different materials ($P \leq 0.01$). Significant differences at $P \leq 0.05$ and $P \leq 0.01$ were determined using the Duncan's new multiple range test

genotypes (Fig. 5A). There are numerous differentially expressed genes (DEGs) in different comparison groups (Fig. 5B), which may contribute to some trait differences between the two materials. There were 116 DEGs in the three comparison groups (Fig. 5C), and the number of DEGs shared between the comparison groups Hongda_vs_Basma and H×B_vs_Hongda was the largest. The results showed that the difference between the hybrid H×B and the male parent Basma was the largest, which was consistent with the nicotine content, indicating that these DEGs may be involved in the process of nicotine transport and accumulation, and the formation of heterosis.

To further analyze the potential effects of these DEGs on the formation of nicotine heterosis, we divided them into 12 expression patterns (P1-P12) to explore the impact of additive and non-additive gene expression patterns on nicotine heterosis [30]. The results showed that in hybrid H×B, only 10.07% (P1 and P2, 255, and 148) of the genes had an expression level between their parents, exhibiting an additive expression pattern; and the remaining 89.93% of genes exhibited non-additive expression patterns. Among the non-additive expressed genes, 31.14% (P3-P6) showed dominant expression pattern and 58.79% (P7-P12) showed over-dominant expression mode (Fig. 5D). The results indicated that nicotine heterosis was mainly affected by the non-additive effect of genes. And the overdominance effect played a major role (Fig. 5E). Therefore, we speculate that the high enrichment of DEGs in the over-dominant pattern promoted the formation of nicotine transport heterosis in hybrids.

Mining of genes related to the formation of nicotine transport heterosis of hybrids

We conducted a GO functional enrichment analysis on the overexpressed genes to gain a deeper understanding of their biological functions. The results showed that the biological processes involved in nicotine transport, such as the Jasmonic acid metabolic pathway, methyl jasmonate, protein transporter, transmembrane transporter, hormone-mediated signaling pathway, and glutathione transferase activity, were significantly enriched (Fig. 6A). As a result, we speculate that these DEGs

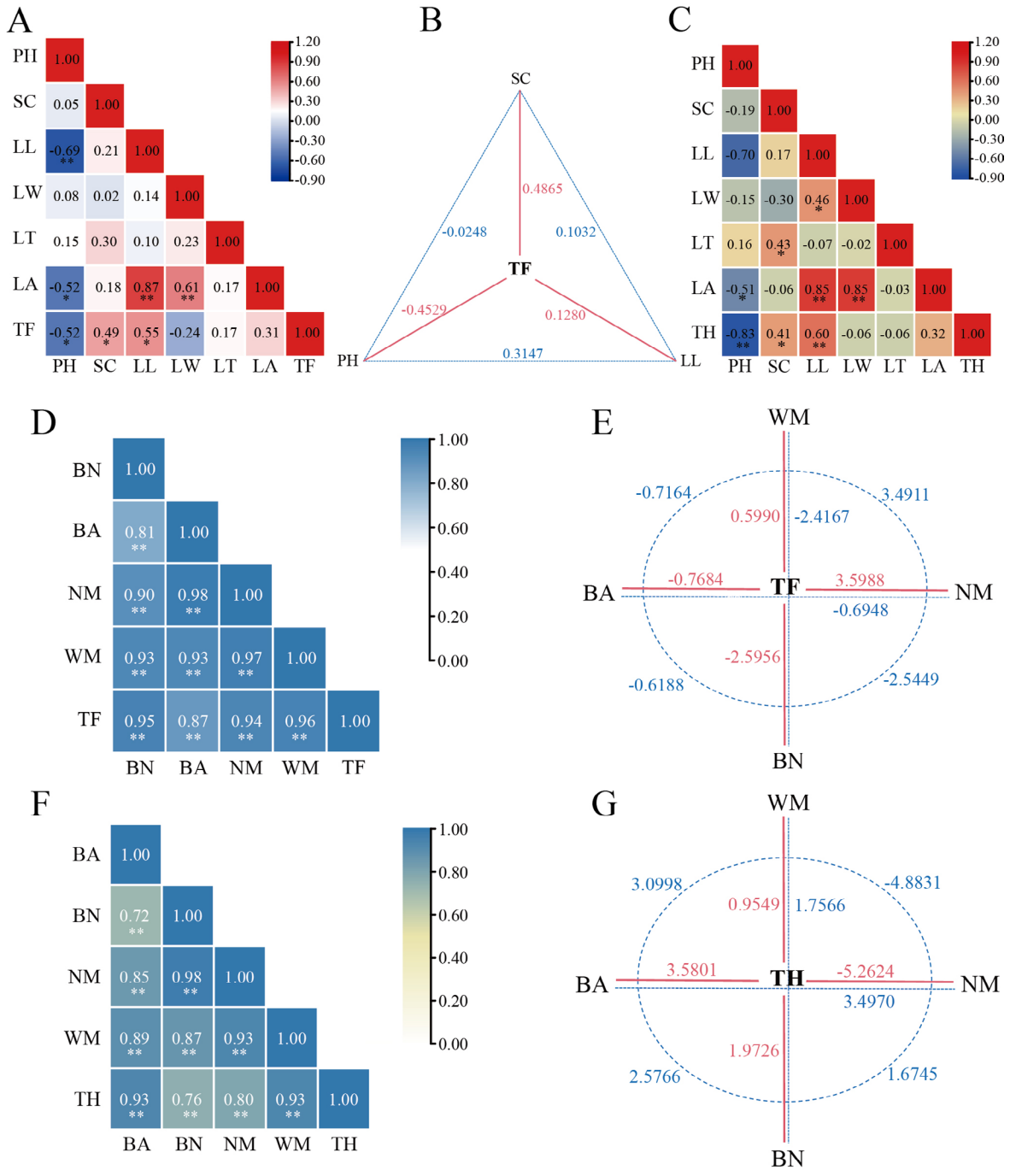
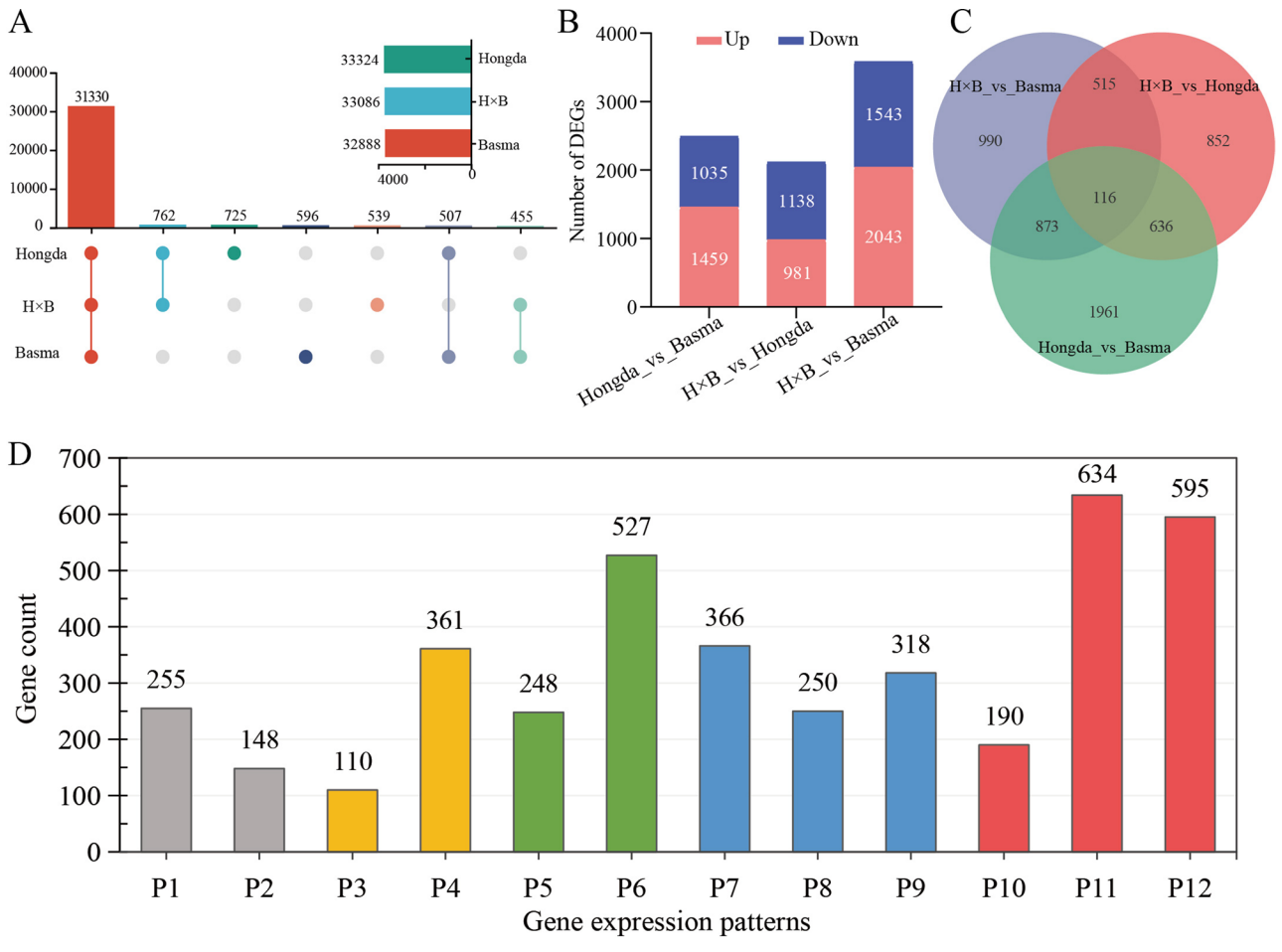


Fig. 4 The correlation and path analyses between nicotine transport capacity and phenotypes and physiological indicators. (**A**) Correlation analysis between agronomic traits and the transport coefficients. PH: plant height; SC: stalk circumference; LL: leaf length; LW: leaf width; LT: leaf thickness; LA: leaf area; TF: transport coefficient. (**B**) Path analysis of agronomic traits and transport coefficients. (**C**) Correlation analysis of agronomic traits heterosis and nicotine transport capacity heterosis in hybrids. PH: plant height heterosis; SC: stalk circumference heterosis; LL: leaf length heterosis; LW: leaf width heterosis; LT: leaf thickness heterosis; LA: leaf area heterosis; TH: transport coefficient heterosis. (**D**) and (**E**) were the correlation and path analyses of physiological indicators and transport coefficients, respectively. BN: nicotine concentration in the bleeding fluid; BA: bleeding amount; NM: nicotine migration amount; WM: water migration rate; TF: transport coefficient. (**F**) and (**G**) were the correlation and path analysis of physiological indicators heterosis and transport coefficient heterosis, respectively. BN: heterosis of nicotine concentration in bleeding fluid; BA: heterosis of bleeding amount; NM: heterosis of nicotine migration amount; WM: heterosis of water migration rate; TH: transport coefficient heterosis. In (**A**), (**C**), (**D**), and (**F**), the '*' below the numbers indicates a significant correlation between two traits, while '**' below the numbers indicates a highly significant correlation between two traits. In (**B**), (**E**), and (**G**), the red solid line represents the direct path coefficient between two indicators, while the blue dashed line represents the indirect path coefficient between two indicators



E

Additivity	Partial paternal dominant	Partial maternal dominant	Under low-parental dominant	Over high-parental dominant
403	471	775	934	1419
10.07%	11.77%	19.37%	23.34%	35.46%

Fig. 5 Transcriptome profiling analysis of hybrid and its parents. (**A**) Gene numbers comparison and analysis of the three genotype materials. (**B**) Statistical plots of DEGs for the three comparison groups. (**C**) Interactive Venn diagram of DEGs for the three comparison groups. (**D**) Identifying of the differential gene expression patterns. (**E**) Number statistics of genes for different expression patterns

regulate nicotine transport by participating in biological processes. Therefore, we identified 36 genes related to nicotine transport in hybrids based on a set of genes with dominant and over-dominant expression. The expression levels of these genes in the hybrid H×B were significantly higher than those of the two parents, and the difference with Basma was even greater. Overall, the expression level of maternal Hongda was higher than that of paternal Basma, indicating a close correlation between the expression level of transport related genes and nicotine content. We have provided a more detailed description of these transport related genes. They mainly included the drug/metabolite and extrusion transporter, cytochrome P450, transcription factor GRAS, ABC transporter, glutathione S-transferase, and some unknown genes (Fig. 6B). It has been speculated that these transport-related genes may explain the formation of nicotine transport heterosis in tobacco hybrids.

Nicotine transport related genes exhibited expression level heterosis

Nicotine synthesized by the roots was transported through the xylem of the stalk to the leaves for storage under the action of transport factors. Numerous

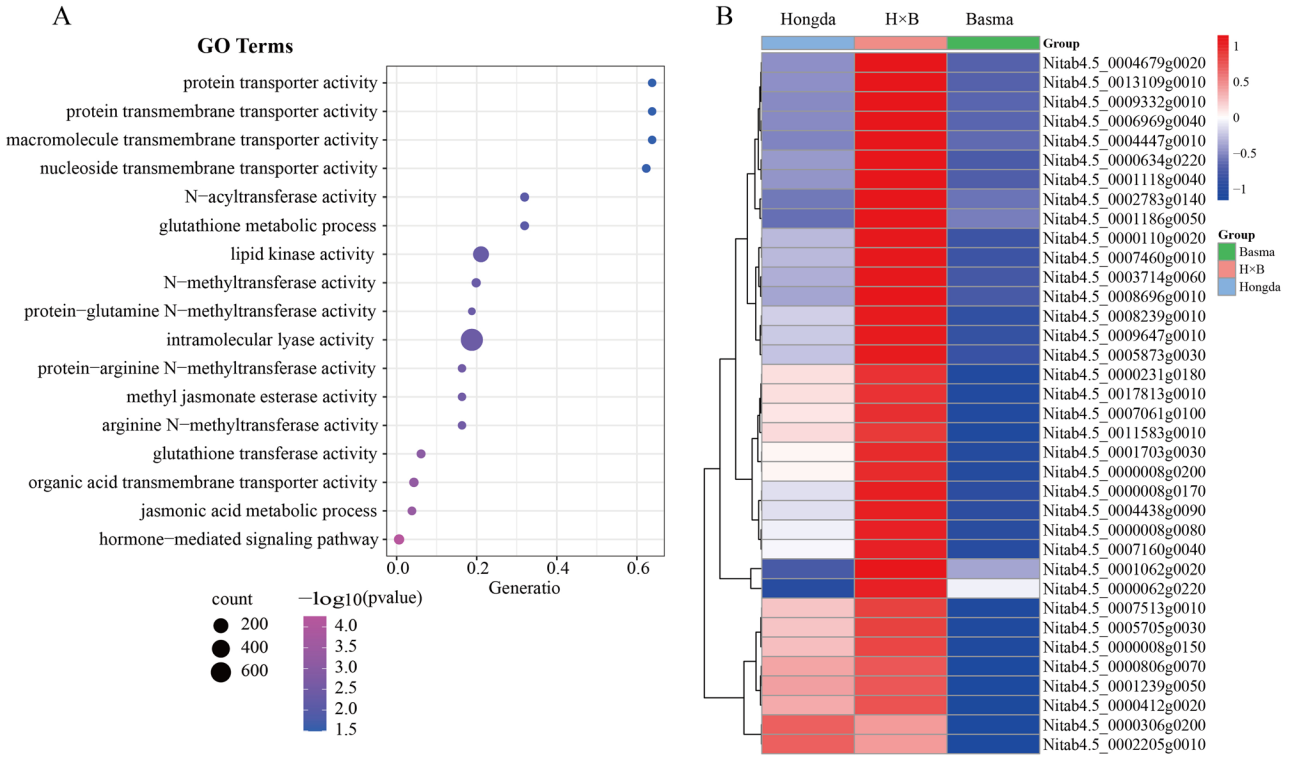


Fig. 6 The analysis of GO functional enrichment and expression level of DEGs. (**A**) The GO functional enrichment analysis of over-dominant expressed genes. (**B**) Cluster analysis of the expression of nicotine transport related genes in hybrids

studies have indicated that genes such as *NtGEF*, *NtNUP1*, *NtJAT1*, *NtJAT2*, *NtMATE1*, and *NtMATE2* play a role in regulating nicotine transport. *NtGSTU10* is a transport gene identified by the research team in the early stages that may be related to nicotine transport. Based on the transcriptome analysis results of this study, we measured the expression levels of these genes in the stalks of strong heterosis hybrid H×B and weak heterosis hybrid V×G88, as well as their parents, to explore the potential role of transport related genes in the formation of nicotine heterosis (Fig. 7). The results showed that the expression levels of these six genes in the hybrid H×B were significantly higher than those of its parents, and significantly higher than its father Basma. However, the expression level in V×G88 was between the two parents and there was no significant difference compared to the parents. These results showed that these genes related to nicotine transport also exhibited heterosis at the expression level, which may positively regulate the formation of nicotine heterosis. This result also supports the conclusion that the formation of nicotine heterosis is due to the improvement in nicotine transport capacity in hybrids.

Discussion

There are significant differences in nicotine content among different tobacco varieties, which provide valuable resources for genetic regulation of nicotine content [31]. This study found significant or extremely significant differences in nicotine content among different materials at different growth stages and tissues. Previous studies on the nicotine content in tobacco stalks are limited. We found significant differences in nicotine content in the stalks of different materials, ranging from 0.05 to 0.35%, which provides a useful reference for studying the nicotine transport mechanism.

Previous studies on nicotine heterosis have mainly focused on analyzing the genetic characteristics of nicotine content in tobacco leaves and comparing the differences in heterosis among different hybrids. The six selected hybrid combinations in this study showed extremely significant differences in nicotine content heterosis at different stages, consistent with the results of Mo [22] and Wang [32]. Furthermore, nicotine content heterosis in these combinations showed a consistent trend over the past two years, indicating that the genetic differences between the selected hybrids and their corresponding parent materials were relatively stable and less affected by environmental factors. There have been no reports on the heterosis of nicotine content in the stalks and roots. The results of this study showed that there was a heterosis phenomenon in nicotine content in the roots and stalks, indicating that there were also differences in nicotine synthesis and transport between hybrid and their parents. The concept of the nicotine transport

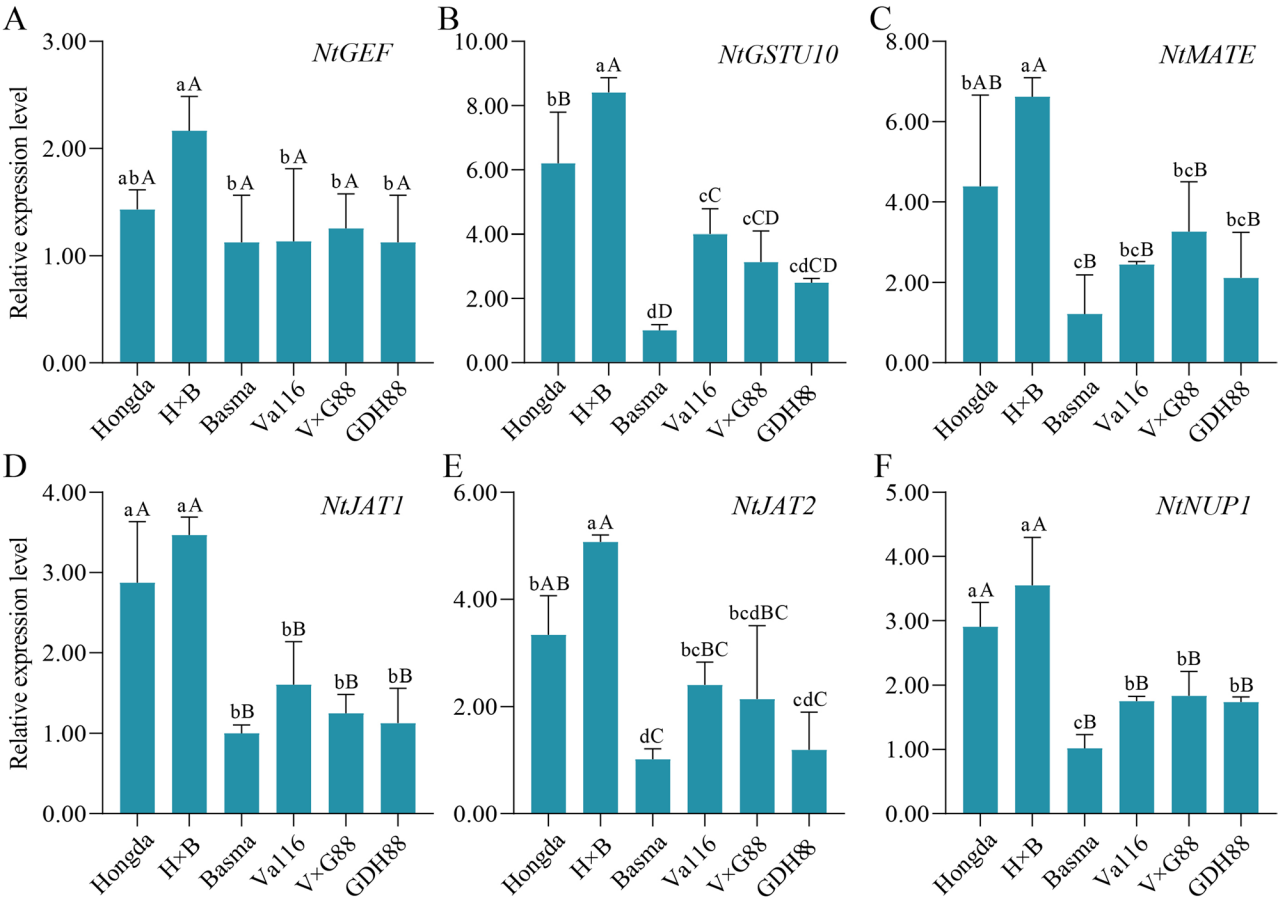


Fig. 7 The Relative expression level of transport related genes in different materials. The lowercase letters above the bars indicate significant differences in gene expression levels between different materials ($P \leq 0.05$), and uppercase letters indicate highly significant differences in gene expression levels between different materials ($P \leq 0.01$). Significant differences at $P \leq 0.05$ and $P \leq 0.01$ were determined using the Duncan's new multiple range test

coefficient was further introduced to evaluate the capacity of nicotine synthesized in roots to be transported to leaves in different materials. There was a highly significant positive correlation (P=0.0000, r=0.9474) between hybrid nicotine heterosis and transport heterosis, indicating that studies on the causes of nicotine heterosis should pay attention to the nicotine transport heterosis in hybrids. Positive regulation of the nicotine content and heterosis of hybrids can be achieved by selecting parents with strong nicotine transport.

The amount of bleeding over a certain period of time is influenced by the root pressure of the plants and affects the formation of root morphology and the growth and development of the aboveground portion [33, 34]. The bleeding fluid generated by the transverse cutting of the stalk during long-distance transportation of plants can be used as a comprehensive indicator to evaluate the horizontal upward transport capacity [35]. It reflects the upward transportation of the xylem, bidirectional transportation of the phloem, absorption of the root, and substance transportation among the three [36]. Li et al. [37] found that potassium accumulation per tobacco plant was determined by the amount of bleeding and was significantly positively correlated with potassium accumulation in the bleeding fluid. This study found significant or extremely significant differences in the amount of bleeding among different hybrids, and the hybrid H×B also showed strong heterosis in the amount of bleeding. We also measured the nicotine concentration in the bleeding fluid and found that the differences between the different hybrids were consistent with the bleeding amount, indicating that tobacco accelerates nicotine transport through water metabolism, which is a metabolic response of plants to nicotine under heterozygous conditions. In future studies, we can validate this by testing the expression of water transporter aquaporin genes in hybrid and their parents.

The xylem is a composite tissue within a plant that functions as a transduction and mechanical support, serving as the main pathway connecting the root system and canopy [38, 39]. The higher the water transport efficiency of plants, the stronger their capacity to transport minerals, amino acids, and metabolites within the plant [40, 41]. The stalk is the main organ and channel for

nicotine transport from the roots to the leaves. Nicotine is transported upward along with the bleeding fluid in the stalk, and the capacity of the stalk to transport water reflects its capacity to transport nicotine. This study found significant or extremely significant differences in the water migration rate of stalks between different materials. The hybrid H×B was significantly higher than that of the other five hybrids, and the weak heterosis hybrid V×G88 was significantly lower than that of the other five hybrids. Importantly, the correlation coefficient between the water migration rate and the nicotine transport coefficient of the hybrid was 0.9570, indicating that the formation of nicotine transport heterosis in the hybrid was influenced by the stalk water migration rate.

Nicotine is synthesized in the roots and ultimately accumulates in the leaves, during which transport plays an important role. Therefore, it is speculated that several transport genes are involved. Morita et al. [19] isolated a nicotine transporter gene from the MATE family of tobacco, which was induced by Jasmonic acid and named JAT1. JAT1 is located on the vacuolar membrane of tobacco leaf cells and transports nicotine. Two other nicotine transporter genes, *MATE1* and *MATE2*, were also isolated from tobacco, and the MATE1 and MATE2 proteins were also localized on the vacuolar membrane of tobacco leaf cells [23]. This study identified many multi-antimicrobial extrusion proteins and drug/metabolite transporters with transmembrane transport functions that exhibited significant or extremely significant over-dominant expression patterns in hybrids. The results indicate that these genes that may be related to nicotine transport may also play an important role in the formation of nicotine transport heterosis in hybrids.

ABC family transporters are a class of transmembrane superfamily proteins that are widely present in natural organisms [42]. Most ABC transporters exhibit transport activity within an organism, relying on the energy generated by ATP hydrolysis to achieve substrate transmembrane transport both inside and outside the cell. Substrates of these transport proteins include amino acids, liposomes, polysaccharides, peptides, heavy metal chelates, alkaloids, and drugs [43]. Xie [44] screened and cloned a candidate gene (*NtWBC13*) involved in nicotine transport in the ABCG subfamily, which confirms that NtWBC13 is a nicotine transport protein that may regulate nicotine accumulation in tobacco taproots. Members of the same gene family may have had similar biological functions during evolution. Therefore, we speculate that members of other ABC family subfamilies may also participate in nicotine transport. The transcriptome analysis of the stalk revealed that three members of the ABC family (ABC transporter type 2, ABC transporter type 1, and ABC transporter-like) were highly upregulated in the hybrid and its parents and had biological functions in transmembrane transport. These results indicate that upregulation of ABC transporter family members in hybrids may promote nicotine transport within the tobacco plant.

Glutathione S-transferase (GST) catalyzes the binding of substrates to glutathione, facilitating substrate transport [45]. However, glutathione S-transferase, which does not have a catalytic activity, can act as a ligand protein and bind to the substrate, transporting it to the vacuole membrane [46]. This study found that multiple glutathione S-transferase genes exhibit significant over-dominant upregulation, and previous studies have shown that both ABC and MATE transporters require the involvement of GST for the transport and accumulation of flavonoids [47]. As a result, we speculate that glutathione S-transferase (GST) may work with ABC and MATE type transporters to promote nicotine transport heterosis in hybrids.

Based on the above results, we speculate that the increase in nicotine content in hybrids may be driven by these transport proteins, which give the hybrids stronger nicotine transport capacity and promote the formation of nicotine heterosis. However, further research is needed to explore whether glutathione S-transferase, ABC transporter C and F family member enzymes can truly participate in the transportation process of nicotine. Meanwhile, the formation of nicotine heterosis is an extremely complex biological process and not the result of the action of a single gene. Therefore, Therefore, in our next research, we need to conduct more in-depth functional identification and validation of these genes.

Conclusion

This study conducted a comprehensive analysis of hybrids with varying heterosis levels and their parent materials from various aspects, such as phenotype, physiology, and transcriptomics. We observed significant differences in nicotine content among the roots, stalks, and leaves of the test materials. Through correlation and path analysis, it was found that the formation of nicotine heterosis was mainly influenced by the nicotine transport heterosis of hybrids, which have a stronger nicotine transport capacity than their parents. The plant height, thick stalk circumference, large flow of tissue fluid in the stalk, and high nicotine concentration of tobacco were the underlying factors that led to the strong nicotine transport capacity of hybrids. The gene expression analysis indicated that the formation of nicotine transport heterosis in hybrids was mainly influenced by non-additive gene effects, with over-dominant effects playing a dominant role. These over-dominant expression genes were significantly enriched in GO functional terms such as jasmonate metabolism and mediated signaling pathways, transmembrane transport activity, and glutathione

metabolism. The transcriptome analysis and RT-qPCR results showed that these genes involved in nicotine transport also exhibited heterosis at the expression level. Overall, our results revealed that the formation of nicotine heterosis is mainly achieved by enhancing the nicotine transport capacity in hybrids.

Abbreviations

NUP1/2	Nicotine uptake permeability enzyme 1
JAT2	Jasmonate induced alkaloid transporter 2
NtMATE1/2	Multidrug and toxin extrusion 1/2
ABC	ATP-binding cassette
GST	Glutathione S-transferase
TF	Transport coefficient
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
RT-qPCR	Quantitative reverse transcription polymerase chain reaction

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-024-05670-9.

Supplementary Material 1: Table S1. Statistical table of sequencing data of nine samples

Supplementary Material 2: Table S2. Comparison results of stalk sequences of nine samples

Acknowledgements
We are very grateful to the editor and reviewers for critically evaluating the manuscript and providing constructive comments for its improvement.

Author contributions
ZJM and RXL conceived and designed the experiments. ZJM, YH, LLD, MX and XYS performed the experiments. ZJM, YH, KP, BSL and GZW analyzed the data and contributed to the reagents, materials, analytical tools, and wrote the manuscript. RXL conceived the study and participated in the design and coordination. All authors contributed to the article and approved the submitted version.

Funding
This research was supported by the National Natural Science Foundation of China (No. 32060510), the Guizhou Provincial Fund Project (ZK [2023] 116), and the Major Projects of Guizhou Tobacco Company (2022XM02). The funding institution Guizhou Tobacco Company was not involved in the study design, collection, analysis, interpretation of data, the writing of this article, or the decision to submit it for publication.

Data availability
All data generated or analyzed during this study are included in this published article and its supplementary information files. The RNA sequencing datasets analyzed in this study are available in the NCBI public repository. The name of the repository and accession number can be found below: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE270039.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 12 July 2024 / Accepted: 7 October 2024
Published online: 15 October 2024

References

1. Timberlake WE, Heterosis. Brenner's Encycl Genet Second Ed. 2013;2:451–3.
2. Hochholdinger F, Baldauf JA. Heterosis in plants. Curr Biol. 2018;28:R1089–92. https://doi.org/10.1016/j.cub.2018.06.041.
3. Li D, Huang Z, Song S, Xin Y, Mao D, Lv Q, et al. Integrated analysis of phenome, genome, and transcriptome of hybrid rice uncovered multiple heterosis-related loci for yield increase. Proc Natl Acad Sci U S A. 2016;113:E6026–35.
4. Shull G. The composition of a field of maize. J Hered. 1908;1:296–301. https://doi.org/10.1093/jhered/os-4.1.296.
5. Bruce AB. The mendelian theory of heredity and the augmentation of vigor. Sci (80-). 1910;32:627–8.
6. East EM, Heterosis. Genetics. 1936;21:375–97. https://doi.org/10.1093/genetics/21.4.375.
7. Jinks JL, Jones RM. Estimation of the components of Heterosis. Genetics. 1958;43:223–34.
8. Minvielle F. Dominance is not necessary for heterosis: a two-locus model. Genet Res. 1987;49:245–7.
9. Shao L, Xing F, Xu C, Zhang Q, Che J, Wang X, et al. Patterns of genome-wide allele-specific expression in hybrid rice and the implications on the genetic basis of heterosis. Proc Natl Acad Sci U S A. 2019;116:5653–8.
10. Baldauf JA, Liu M, Vedder L, Yu P, Piepho HP, Schoof H, et al. Single-parent expression complementation contributes to phenotypic heterosis in maize hybrids. Plant Physiol. 2022;189:1625–38.
11. Tian M, Nie Q, Li Z, Zhang J, Liu Y, Long Y, et al. Transcriptomic analysis reveals overdominance playing a critical role in nicotine heterosis in Nicotiana tabacum L. BMC Plant Biol. 2018;18:1–32.
12. Yang T. Tobacco breeding. Beijing: China Agriculture; 2003.
13. Chen J, Liu J, Long H. Study on mineral nutrition and main chemical composition content characteristics of Chinese tobacco leaf. Chin Tob. 2004;10:20–7.
14. Yu J. Cigarette technology. China Agricultural; 2003.
15. Baldwin IT. Jasmonate-induced responses are costly but benefit plants under attack in native populations. Proc Natl Acad Sci U S A. 1998;95:8113–8.
16. Mukhopadhyay I, Chowdhuri DK, Bajpayee M, Dhawan A. Evaluation of in vivo genotoxicity of cypermethrin in Drosophila melanogaster using the alkaline Comet assay. Mutagenesis. 2004;19:85–90.
17. Dawson RF. Nicotine synthesis in excised Tobacco roots. Am J Bot. 1942;29:813–5.
18. Hashimoto T, Yamada Y. New genes in alkaloid metabolism and transport. Curr Opin Biotechnol. 2003;14:163–8.
19. Morita M, Shitan N, Sawada K, Van Montagu MCE, Inzé D, Rischer H, et al. Vacuolar transport of nicotine is mediated by a multidrug and toxic compound extrusion (MATE) transporter in Nicotiana tabacum. Proc Natl Acad Sci U S A. 2009;106:2447–52.
20. Shitan N, Minami S, Morita M, Hayashida M, Ito S, Takanashi K, et al. Involvement of the leaf-specific multidrug and toxic compound extrusion (mate) transporter Nt-JAT2 in vacuolar sequestration of nicotine in nicotiana tabacum. PLoS ONE. 2014;9:1–10.
21. Mo Z, Pu Y, Zhou J, Tian Z, Teng J, Chen Q, et al. Effect of the over-dominant expression of proteins on nicotine heterosis via proteomic analysis. Sci Rep. 2021;11:1–12. https://doi.org/10.1038/s41598-021-00614-x.
22. Mo Z. Differential proteomic analysis of nicotinic heterosis in tobacco. Guizhou University; 2020.
23. Shoji T, Inai K, Yazaki Y, Sato Y, Takase H, Shitan N, et al. Multidrug and toxic compound extrusion-type transporters implicated in vacuolar sequestration of nicotine in tobacco roots. Plant Physiol. 2009;149:708–18.
24. Kim D, Langmead B. Salzberg1 SL. HISAT: a fast spliced aligner with low memory requirements Daehwan HHS Public Access. Nat Methods. 2015;12:357–60.
25. Roberts A, Trapnell C, Donaghey J, Rinn JL, Pachter L. Improving RNA-Seq expression estimates by correcting for fragment bias. Genome Biol. 2011;12:114.
26. Trapnell C, Williams Ba, Pertea G, Mortazavi A, Kwan G, van Baren MJ, et al. Transcript assembly and abundance estimation from RNA-Seq reveals thousands of new transcripts and switching among isoforms. Nat Biotechnol. 2011;28:511–5. https://doi.org/10.1038/nbt.1621.Transcript.

27. Anders S, Huber W. Differential expression of RNA-Seq data at the gene level–the DESeq package. Biotechnol Bull. 2012;1–28. https://doi.org/10.13560/j.cnki.biotech.bull.1985.2018-0814.
28. Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, Robles M. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics. 2005;21:3674–6.
29. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods. 2001;25:402–8.
30. Mo Z, Luo W, Pi K, Duan L, Wang P, Ke Y, et al. Comparative transcriptome analysis between inbred lines and hybrids provides molecular insights into K + content heterosis of tobacco (Nicotiana tabacum L). Front Plant Sci. 2022;13:1–14.
31. Wang G. Studies on tobacco nicotine trait of heterosis and related gene differential expression analysis. 2015;:1–76.
32. Wang G. Studies on tobacco nicotine trait of heterosis and related gene differential expression analysis. Guizhou University; 2015.
33. Xiao JC, Wu ZH, Xu KZ, Ling F, Lou, Cui JJ, Li X. Changes of root bleeding sap weight and its correlation with flag leaf net photosynthetic rate in rice cultivars released 47 years in Jilin Province of China. Plant Physiol J. 2012;48:499–504.
34. Li H, Sun Y, Qu J, Wei C, Sun G, Zhao Y, et al. Influence of nitrogen levels on morphological and physiological characteristics of root system in japonica rice in Northeast China. Chin J Rice Sci. 2012;26:723–30.
35. Shen F, Wei Z, Huang Q, Tang X, Yin Y, Liao M, et al. Effects of the root-cutting and girding on the circulation and accumulation of the potassium, calcium and magnesium in tobacco. Chin Agric Sci Bull. 2011;27:127–31.
36. Zhang X. Study on the differences and mechanisms of potassium efficiency in tobacco with different genotypes. 2003.
37. Li F, Xiao F, Peng G. Study on the potassium content of exudation of tobacco sap. Tob Sci Technol. 2000;000:38–41.
38. Bai D, Deng X, Huang M. Water transportation and regulation in plants. Acta Bot Boreali-Occidentalia Sin. 2003;:1637–73.
39. Cruiziat P, Cochard H, Améglio T. Soil detritivore macro-invertebrate assemblages throughout a managed beech rotation. Ann Sci. 2007;64:219–28.
40. Liu X. Studies on the models of hydraulic architecture and mechanism of drought tolerance in trees species. 2003;:1–71.
41. Li J, Zhai H. Hydraulic architecture and drught resistance of woody plants. Chin J Appl Ecol. 2020;11:301–5.
42. Dean M, Moitra K, Allikmets R. The human ATP-binding cassette (ABC) transporter superfamily. Hum Mutat. 2022;43:1162–82.
43. Higgins CF. ABC transporters: from microorganisms to mHiggins, C F. 1992. „ABC transporters: from microorganisms to man. Annual review of cell biology 8: 67–113. https://doi.org/10.1146/annurev.cb.08.110192.000435.an. Annu Rev Cell Biol. 1992;8:67–113.
44. Xie X. Identification and functional characterization of nicotiana tabacum ABC transporters involved in the transport of secondary metabolites. 2014.
45. Zhao J, Dixon RA. The ins and outs of flavonoid transport. Trends Plant Sci. 2010;15:72–80.
46. Mueller LA, Goodman CD, Silady RA, Walbot V. Required for anthocyanin sequestration, is a flavonoid-binding protein 1. Society. 2000;123:1561–70.
47. Petrussa E, Braidot E, Zancani M, Peresson C, Bertolini A, Patui S, et al. Plant flavonoids-biosynthesis, transport and involvement in stress responses. Int J Mol Sci. 2013;14:14950–73.

Publisher's note