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CRISPR/Cas9-Mediated Targeted Mutagenesis of Betaine Aldehyde Dehydrogenase 2 (BADH2) in Tobacco Affects 2-Acetyl-1-pyrroline

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Abstract: 2-acetyl-1-pyrroline (2AP) is a highly effective volatile compound that gives fragrance to numerous plant species and food. Mutation(s) in the *betaine aldehyde dehydrogenase 2* (*BADH2*) gene results in the accumulation of 2AP. However, the function of *BADH* genes in tobacco (*Nicotiana tabacum* L.) remains poorly understood. In this study, we successfully obtained four *betaine aldehyde dehydrogenase* (*BADH*) genes from tobacco. Phylogenetic analysis of the protein sequences showed that two of the four BADH genes were closely related to the wolfberry (*Lycium barbarum*) *BADH* gene (*LbBADH1*), so we named them *NtBADH1a* and *NtBADH1b*, respectively. The other two *BADH* genes were orthologues of the tomato (*Solanum lycopersicum*) *aminoaldehyde dehydrogenase 2* (*SlAMADH2*) gene, and were named *NtBADH2a* and *NtBADH2b*, respectively. Expression analysis revealed that the biological functions of *NtBADH1a* and *NtBADH1b* were different from those of genes *NtBADH2a* and *NtBADH2b*. We introduced mutations into *NtBADH1a*, *NtBADH1b*, *NtBADH2a* and *NtBADH2b* in tobacco using the CRISPR/Cas9 system and identified transgenic *Ntbadh* mutant tobacco lines. Single mutants (*Ntbadh1a*, *Ntbadh1b*, *Ntbadh2a* and *Ntbadh2b*) and double mutants (*Ntbadh1a-Ntbadh1b* and *Ntbadh2a-Ntbadh2b*) harbored deletion or insertion of nucleotides, both of which led to the production of a frameshift, preventing protein accumulation. A popcorn-like scent was noticeable in tobacco leaves from the *Ntbadh2a-Ntbadh2b* double mutant, but not from any single mutant or the *Ntbadh1a-Ntbadh1b* double mutant or the wild type. Consistent with this observation, we only detected 2AP in fresh leaves from the *Ntbadh2a-Ntbadh2b* double mutant. These findings indicate that only the combined inactivation of *NtBADH2a* and *NtBADH2b* results in 2AP accumulation in tobacco, which was not related to *NtBADH1*.

Keywords: *betaine aldehyde dehydrogenase* (*BADH*); *Nicotiana tabacum*; 2-acetyl-1-pyrroline; targeted mutagenesis

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1. Introduction

Betaine aldehyde dehydrogenase (BADH) is an enzyme found in a large number of plant species; it confers the potential to accumulate glycine betaine (GB), which is a powerful osmoprotectant associated with abiotic stresses, such as salt, drought, and temperature [1]. The *BADH* gene has been cloned from higher plants and other living organisms [2], e.g., *Spinacia oleracea* [3,4], *Beta vulgaris* [5,6], barley (*Hordeum vulgaris*) [7], *Chenopodium quinoa* [8], rice (*Oryza sativa* L.) [9], and *Lycium ruthenicum* [10]. BADH plays a crucial role in enhancing tolerance to abiotic stress by facilitating the accumulation of

GB derived from betaine aldehyde (BA). In contrast, certain plant species such as tobacco (*Nicotiana tabacum* L.), tomato (*Solanum lycopersicum* L.), and rice are unable to accumulate GB owing to insufficient BA [11]. In addition to abiotic stress mechanisms, multifunctional BADH is involved in fragrance production through the polyamine oxidation pathway [12]. It has been observed that the majority of plants exhibit two BADH isozymes (BADH1 and BADH2). According to He et al., 2015 [11], there are two members of the BADH family in the rice genome: BADH1 is closely correlated with salt tolerance, while BADH2 is responsible for conferring fragrance to rice.

The 2-acetyl-1-pyrroline (2AP) is a volatile compound widespread in nature. It is the key flavor-related compound in diverse cereal products and vegetable-derived products [13–15]. A reduction in BADH2 activity or the presence of nonfunctional BADH2 enzymes results in the production of 2AP and the release of fragrances in rice [16], maize, sorghum (*Sorghum bicolor*) [17], soy-bean (*Glycine max*) [18], foxtail millet (*Setaria italica*) [19], and mung bean (*Vigna radiata*) [20]. The 2AP biosynthesis pathway comprises two main path-ways (Figure S1): glutamate-proline and ornithine metabolism, and polyamine metabolism. Glutamate, proline, and ornithine are converted to 1-pyrroline-5-carboxylate (P5C) via Δ1-pyrroline-5-carboxylate synthase (P5CS), proline dehydrogenase (ProDH), and ornithine aminotransferase (OAT) enzymes; P5C is then immediately converted to Δ1-pyrroline, which is the immediate precursor of 2AP and an important factor for regulating the 2AP biosynthesis rate; polyamines (organic compounds with more than two amino groups) are converted to GABald (the immediate precursor of γ-aminobutyric acid) [21]. In the presence of a nonfunctional BADH2 enzyme, GABald cannot be converted to GABA, resulting in the spontaneous conversion of GABald to Δ1-pyrroline, which leads to GABald accumulation and 2AP formation. Conversely, GABald is converted to GABA by a functional BADH2, which ultimately inhibits 2AP biosynthesis [22].

Tobacco is extensively cultivated as a non-food crop all over the world, and is additionally a crucial model plant species for fundamental biological investigation [23]. However, no genes in the 2AP biosynthetic pathway have been characterized in tobacco. Understanding the 2AP biosynthetic pathway in tobacco will help elucidate the function of 2AP in tobacco metabolism and biosynthesis. Over the past few years, genome-editing technology, such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the CRISPR/Cas9 system, which is associated with the clustered regularly interspaced short palindromic repeats (CRISPR) protein 9 (Cas9) system, has enabled scientists to make changes in the DNA of model organisms to generate biotechnologically important products [24–27]. Among these tools, CRISPR/Cas9 is widely used in plants because of its ease of use, great effectiveness, and low development cost [28]. In this study, we cloned four *NtBADH* genes from tobacco and characterized their roles in 2AP biosynthesis via targeted knockout using the CRISPR/Cas9 system.

2. Materials and Methods

2.1. Plant Materials

Seeds of the tobacco (*Nicotiana tabacum* L.) cultivar 'Honghuadajinyuan' and homozygous transgenic *NtBADH* knockout lines were surface-sterilized and grown on Murashige and Skoog (MS) Basal Medium (Duchefa Biochemie, Haarlem, The Netherlands) containing 30 g/L sucrose and 6 g/L agar. One-month-old seedlings were transferred to pots containing a peat moss to perlite ratio of 3:1 and kept under greenhouse conditions with a photoperiod of 16 h light at 25 °C. Roots, stems, leaves, and flowers of 'Honghuadajinyuan' seedlings were sampled at the anthesis flower stage and immediately frozen in liquid nitrogen for RNA purification.

2.2. Cloning of Tobacco NtBADH2 Genes

We acquired four NtBADH-like translated nucleotide sequences from the Sol Genomics Network (SGN) *Nicotiana tabacum* BX genome database (http://solgenomics.net/organism/Nicotiana_tabacum/genome, accessed on 4 January 2024) by performing a basic local

alignment search tool (tBLASTn; available online: https://solgenomics.net/tools/blast/, accessed on 4 January 2024) using the rice OsBADH2 protein sequences as the query. Four full-length *NtBADH* coding sequences were amplified from tobacco leaf tissue cDNA by PCR using specific primers (Table S1) and 2× Phanta Max Master Mix (Vazyme, Nanjing, China). The amplified PCR products were effectively cloned into the pEASY-Blunt Zero Cloning Vector (TransGene, Beijing, China) and subjected to sequencing analysis.

2.3. Phylogenetic Analysis

Protein sequence alignments of multiple BADH orthologs from *Arabidopsis thaliana*, *Triticum urartu*, *Hordeum vulgare*, *Oryza sativa*, *Zea mays*, *Sorghum bicolor*, *Cocos nucifera*, *Solanum lycopersicum*, *Solanum tuberosum*, *Lycium barbarum*, *Glycine max*, *Vigna radiata* (Mung bean), *Cucumis sativus*, *Cucumis melo*, and *Nicotiana tabacum* were performed using the MUSCLE method in MEGA (version 7.0.26; https://www.megasoftware.net/, accessed on 4 January 2024) with default settings. A phylogenetic tree was reconstructed with 1000 bootstrap replicates using the neighbor-joining method of MEGA version 7.0.26.

2.4. RNA Extraction and Gene Expression Analysis

Root, stem, leaf, flower and seed tissues were collected from 60-day-old tobacco plants after transplanting to the field, immediately frozen in liquid nitrogen, and kept at 80 °C until RNA extraction. An RNAprep Pure Plant Plus Kit (Tiangen Biotech, Beijing, China) was used to isolate total RNA. A NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to quantify the total RNA concentration. First-strand cDNA was synthesized using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) in a 20 µL reaction using 500 ng of starting total RNA. Quantitative reverse transcriptase PCR (qRT-PCR) was performed using SYBR Premix Ex Taq (TaKaRa Bio Inc., Otsu, Japan) and gene specific primers (Table S2) in a LightCycler® 96 instrument (Roche, Basel, Switzerland). Expression levels of the actin gene were used as an internal control. Each analysis was conducted with a minimum of three biological and technical replicates. Relative fold-change of target genes was determined by the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen 2001 [29].

2.5. Construction of CRISPR/Cas Gene Editing Vectors

To facilitate *NtBADH* genomic DNA modifications, we synthesized oligonucleotides with a specific target of two 24 base pairs in length. The CRISPOR 5.01 software (http://crispor.tefor.net/, accessed on 4 January 2024) was used to assess their efficiency. Subsequently, these oligos were annealed and inserted into the BsaI site of the enhanced pOREU3TR vector, which was optimized to enhance the expression of sgRNA cassettes and integrate a Ros1 expression cassette for efficient screening of transgene-free genome edited plants [30].

2.6. Plant Transformation and Mutant Analysis

The pOREU3TR vectors, which carried the gRNA and Cas9 expression cassettes, were introduced into *Agrobacterium tumefaciens* EHA105 through the freeze–thaw technique. Afterwards, positive clones were utilized in order to produce *NtBADH* mutant tobacco plants through the leaf discs method [31]. Seedlings resistant to kanamycin were successfully obtained and the presence of mutants was confirmed. The extracting of DNA from T0 transgenic lines was conducted using the DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany). In order to detect mutations, PCR was performed using the specific primers to amplify genomic regions including the Cas9/gRNA target sites (Table S3). The products underwent sanger sequencing directly, and the resulting reads were compared with wild-type sequences to identify potential mutant lines. To investigate the heritability of CRISPR/Cas9-mediated targeted *NtBADH* modifications in subsequent generations, T0 lines harboring homozygous or biallelic mutations were subjected to self-pollination. Then, the T1 offspring were transferred to soil and grown until maturity for further analysis.

2.7. 2AP Measurements

2AP was quantified as in a previously described protocol [32] with slight modifications. Freeze-dried tobacco leaves were ground thoroughly into 200 mesh powder using a grinder. Aliquots (0.2 g) of ground powder were weighed into 5 mL glass bottles and extracted at 80 °C for 3 h in 2 mL of extraction buffer consisting of a 1:1 (v/v) mixture of anhydrous ethanol and dichloromethane. 2,4,6-Trimethyl pyridine (TMP) was added to the mixture at a final concentration of 50 ng/mL as an internal standard. The reaction solution was cooled to room temperature and centrifuged for 5 min at 12,000 r/min. The supernatant was transferred to an injection vial with a 200 µL internal diameter tube and left to stand for 0.5 h before measurement.

The 2AP was measured using a gas chromatograph-triple quadrupole mass spectrometer (Agilent 7890A/7000 GC-QQQ, Waldbronn, Germany) equipped with an electrospray ionization source and in separation mode using a gas chromatographic column HP-5MS (30 m × 250 µm × 0.25 µm, Agilent, Santa Clara, CA, USA). The initial column chamber temperature was set to 50 °C and held for 2 min. Then the temperature was increased to 120 °C at 10 °C min^{-1}; finally, the temperature was increased by 30 °C min^{-1} to 250 °C and held for 3 min. The temperature of the injector was set to 170 °C with research grade helium (99.999%) as the carrier gas under a constant flow. The transfer line was held at 280 °C. Ions were generated using an electron impact ion source (−70 eV, 230 °C) and analyzed using a triple quadrupole. Product ion scans were acquired from 35 to 500 m/z using MRM scanning mode.

3. Results

3.1. Identification of BADH Genes in Tobacco Genome

To identify tobacco *BADH* gene homologues, we aligned amino acid sequences of OsBADH2 (Os08g424500) as the query against the Sol Genomics Network (SGN) tobacco genome database (http://solgenomics.net/organism/Nicotiana_tabacum/genome, accessed on 4 January 2024) using a tBLASTn search. Three putative coding sequences (mRNA_63486_cds, mRNA_31370_cds, and mRNA_66183_cds) of tobacco *BADH* genes were identified from the tBLASTn search. These nucleotide sequences were utilized for the purpose of designing primers that are specific to the coding sequences of *BADH*. These primers were then employed to amplify the full-length *BADH* coding sequence from tobacco leaf tissues. The tobacco *BADH* genes comprised four open reading frames (ORFs) that were 1515 bp, 1515 bp, 1515 bp, and 1500 bp in length and encoded proteins of 504, 504, 504, and 499 amino acids, respectively. The four *BADH* genes in tobacco were all predicted to consist of 15 exons and 14 introns (Figure 1).

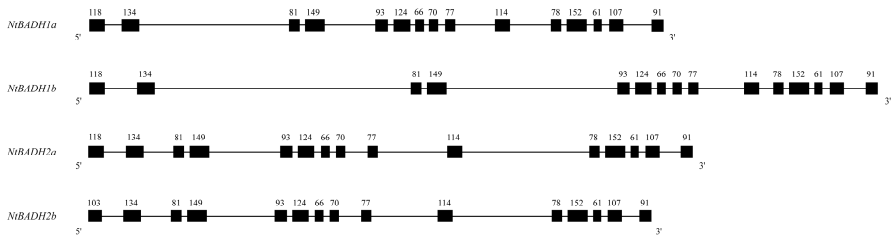

Figure 1. Putative exon/intron structure of *NtBADH1a*, *NtBADH1b*, *NtBADH2a* and *NtBADH2b* genes.

The four tobacco BADH amino acid sequences showed high similarity at the protein level with those of other species, with NtBADH1a showing 95.24%, 92.46%, and 91.98% amino acid sequence identity with NtBADH1b, NtBADH2a, and NtBADH2b, respectively. Moreover, the tobacco BADH 1 proteins exhibited a similarity of 89.88–90.84% to LbBADH1, and the BADH 2 proteins displayed a similarity of 82.54–83.53% to LbBADH1. Meanwhile, BADH2 proteins from tobacco were 91.68–92.87% similar to SlAMADH2 and 92.28–93.47% similar to PGSC0003DMT400063025. All the encoded proteins had a highly conserved decapeptide (VTLELGGKSP) at amino acids 256–265 and a cysteine residue (C) conserved in aldehyde dehydrogenase (ALDHs) at 295 (Figure 2A).

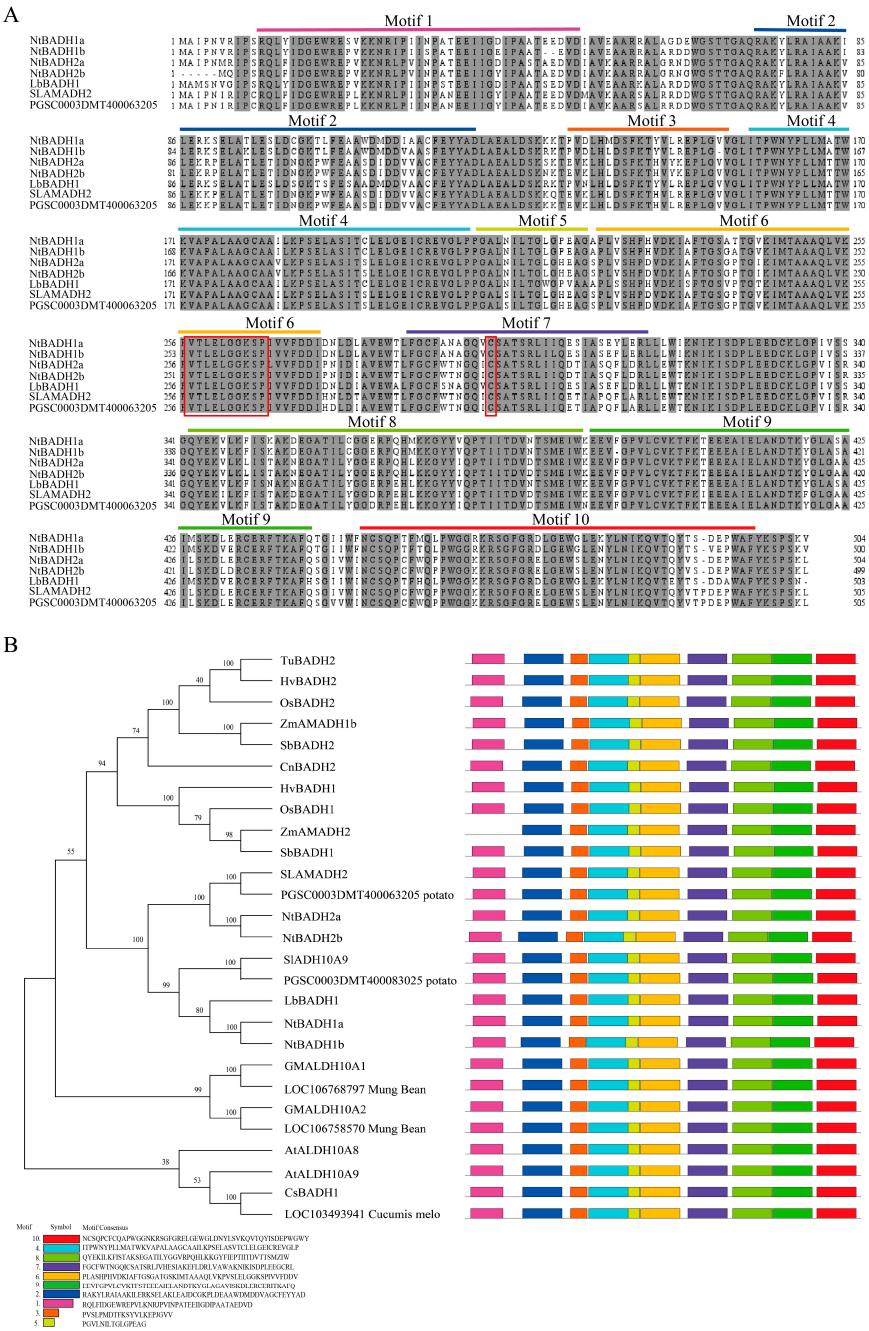

Figure 2. Characterization of NtBADH2 protein in tobacco (*Nicotiana tabacum*). (**A**) Alignment of the putative NtBADH1a, NtBADH1b, NtBADH2a and NtBADH2b amino acid sequences with Lb-BADH1, SlAMADH2 and PGSC0003DMT400063250 sequences. (**B**) Phylogenetic tree of NtBADH1a, NtBADH1b, NtBADH2a and NtBADH2b amino acid sequences with known BADH proteins from other plant species. Nt, *Nicotiana tabacum*; At, *Arabidopsis thaliana*; Tu, *Triticum urartu*; Hv, *Hordeum vulgare*; Os, *Oryza sativa*; Zm, *Zea mays*; Sb, *Sorghum bicolor*; Cn, *Cocos nucifera*; Sl, *Solanum lycopersicum*; St, *Solanum tuberosum*; Lb, *Lycium barbarum*; Gm, *Glycine max*; Cs, *Cucumis sativus*. The colored lines represent motifs and the red boxes represent the conserved decapaptide (VTLELGGKSP) and a cysteine residue (C).

A neighbor-joining phylogenetic tree of BADH proteins from tobacco with BADH protein from *Arabidopsis thaliana*, *Triticum urartu*, *Hordeum vulgare*, *Oryza sativa*, *Zea mays*, *Sorghum bicolor*, *Cocos nucifera*, *Solanum lycopersicum*, *Solanum tuberosum*, *Lycium barbarum*, *Glycine max*, *Vigna radiata* (Mung bean), *Cucumis sativus*, and *Cucumis melo* revealed a clear

evolutionary separation between NtBADH and its homologues (Figure 2B). Two of the four BADH sequences were most closely related to *LbBADH1* from *Lycium barbarum* and were named NtBADH1a and NtBADH1b, respectively. The other two BADH sequences had a close relationship with PGSC0003DMT400063250 from *Solanum tuberosum* and SlA-MADH2 from *Solanum lycopersicum*, and were hence named NtBADH2a and NtBADH2b, respectively. Tobacco is a natural allotetraploid generated by the hybridization of *Nicotiana sylvestris* and *Nicotiana tomentosiformis*. Phylogenetic analysis showed that *NtBADH1a* and *NtBADH2a* originated from *N. tomentosiformis*, while *NtBADH1b* and *NtBADH2b* originated from *N. sylvestris* (Figure S2).

3.2. Expression Patterns of NtBADH Genes in Different Tobacco Tissues

To characterize the expression patterns of the four *NtBADH* genes in wild type tobacco plants, we used qRT-PCR to analyze RNA transcript levels in various tissues. We detected the four *NtBADH* genes transcripts in all organs. Notably, *NtBADH1a* and *NtBADH1b* had similar expression patterns, with the highest transcript levels in leaves (Figure 3A). *NtBADH2a* and *NtBADH2b* also displayed similar expression patterns and were highly expressed in seeds (Figure 3B). These results indicated that *NtBADH1a* and *NtBADH1b* might have similar biological functions in tobacco, but regulation of *NtBADH1a* and *NtBADH1b* was different from that of *NtBADH2a* and *NtBADH2b*.

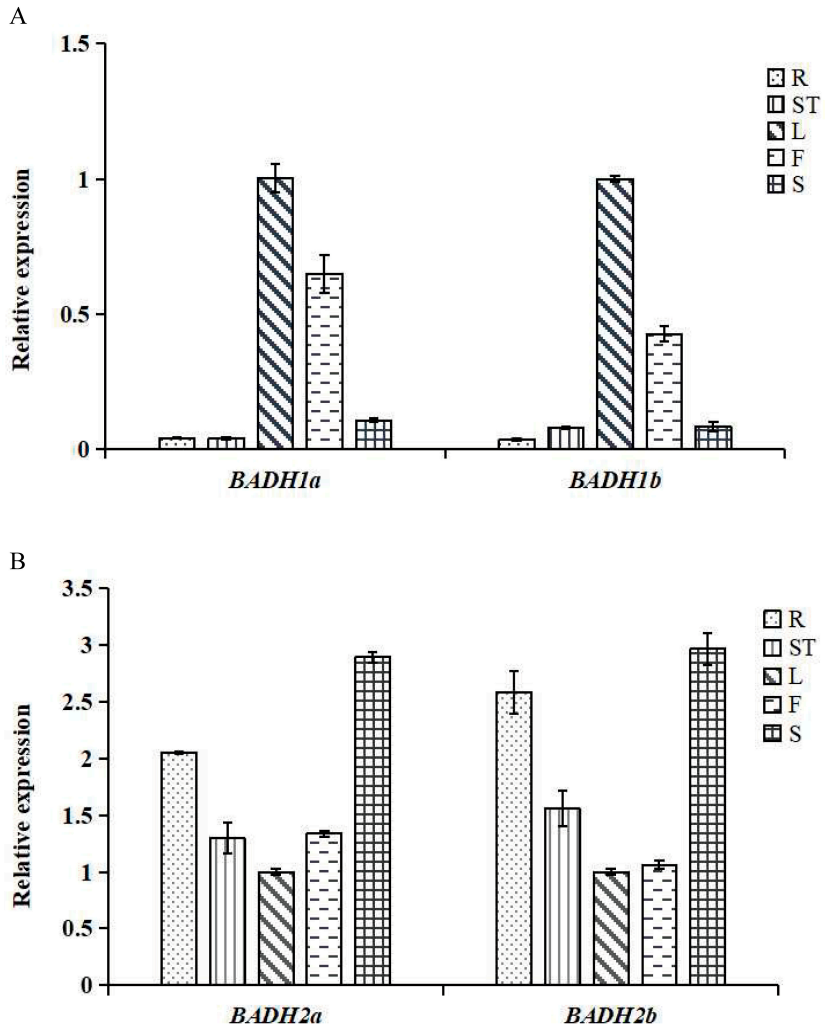

Figure 3. NtBADH expression patterns in different tissues. (**A**) Relative gene expression of NtBADH1a and NtBADH1b in different tissues of 'Honghuadajinyuan' tobacco plants. (**B**) Relative gene expression of NtBADH2a and NtBADH2b in different tissues of tobacco plants. R, root; ST, stem; L, leaf; F, flower; S, seed.

3.3. Targeted NtBADH Mutations Using the CRISPR/Cas9 System

To determine the biological function of NtBADH proteins in tobacco, we decided to simultaneously knock out *NtBADH1a*, *NtBADH1b*, *NtBADH2a*, and *NtBADH2b* by genome editing with the CRISPR/Cas9 system and subsequently investigate the effect of the combinatorial mutations on the content of 2AP in tobacco. We designed three Cas9 guide RNAs, referred to as SgRNA1, SgRNA2, and SgRNA3 (20 nucleotides), and introduced these into the binary expression vector pOREU3TR. SgRNA1 and SgRNA2 independently targeted *NtBADH1a* and *NtBADH1b*, respectively. SgRNA3 had a common target site in *NtBADH2a* and *NtBADH2b*, targeting the first exon of the coding sequence for generating Ntbadh2a and Ntbadh2b single mutants and the Ntbadh2a-Ntbadh2b double mutant (Figure 4A). To generate *Ntbadh1a-Ntbadh1b* double mutants, we created a CRISPR/Cas9 construct harboring the SgRNA2 and SgRNA3 expression cassettes for *Agrobacterium tumefaciens*-mediated tobacco transformation (Figure 4B).

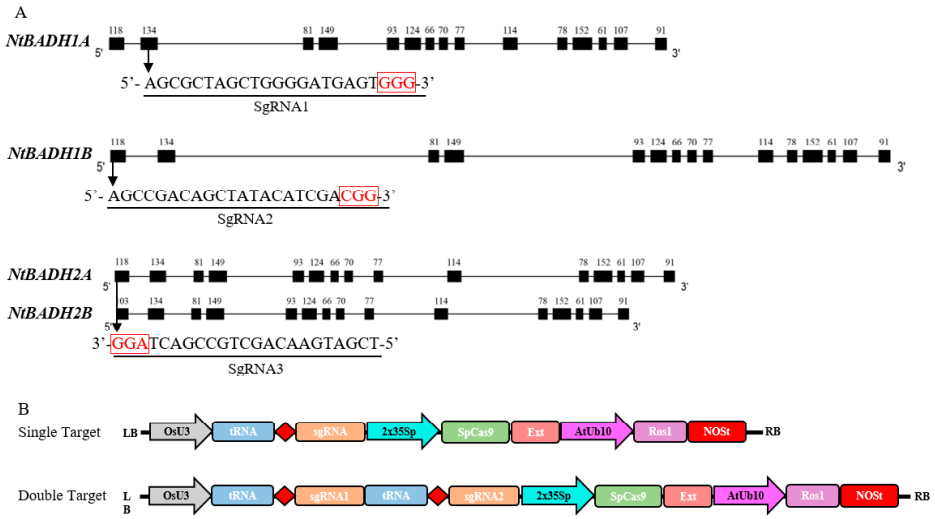

Figure 4. CRISPR/Cas9-mediated *NtBADH* knockout in tobacco. (**A**) *NtBADH* gene structure and target site selected for targeted *NtBADH1a*, *NtBADH1b*, *NtBADH2a* and *NtBADH2b* mutations. Black rectangles represent exons, black lines show introns, black arrow indicate target sites, and nucleotides in red are followed by the PAM (proto-spacer adjacent motif). (**B**) CRISPR/Cas9 vector structure.

For *NtBADH1a* (sgRNA1), we identified 28 T0 positive transgenic plants through PCR amplification. Further sequencing analysis revealed that three of the plants exhibited genomic editing of the target gene with a homozygous genotype, while six plants were identified as heterozygous. Therefore, we selected the three homozygous individuals (badh1a#11, badh1a#12 and badh1a#23) for further investigation. Comparing DNA sequences revealed that badh1a#23 possessed a 22-bp deletion, badh1a#11 possessed a 1-bp deletion, and badh1a#12 exhibited 1-bp insertion at the target site, which we refer to as *NtBADH1a* (Figure 5A). For *NtBADH1b* (sgRNA2), we identified 29 T0 positive transgenic plants edited at the target site, comprising four homozygous mutants (badh1b#5, badh1b#15, badh1b#23, and badh1b#29), thirteen heterozygous mutants, and seven biallelic (two distinct variants) mutants. Individuals badh1b#5 and badh1b#15 exhibited a 1-bp insertion, and badh1b#23 and badh1b#29 possessed 1-bp and 2-bp deletions, respectively (Figure 5B).

Using the double CRISPR/Cas9 construct harboring the sgRNA2 and sgRNA3 expression cassettes, we isolated heterozygous and biallelic (two distinct variants) Ntbadh1a-Ntbadh1b double mutants at the T0 generation. To obtain edited homozygous lines, we focused on the biallelic mutations, sequencing the *BADH1* target regions of 10 individual T1 transformants. We identified two types of *Ntbadh1a-Ntbadh1b* double mutant: a 1-bp insertion in the *NtBADH1a* and *NtBADH1b* target regions, respectively, and a 2-bp deletion in *NtBADH1a* and a 1-bp insertion in *NtBADH1b* (Figure 5C).

A

	NtBADH1A (SgRNA1)
WT	AGCTGCTCGGAGAGCGCTAGCTGGGGATGAGTGGGGTTCAACAACAG
badh1a#23	AGCTGCTCGGAG- - - - - - - - - - - - - - - - - AGTGGGGTTCAACAACAG
badh1a#11/ badh1a#12	AGCTGCTCGGAGAGCGCTAGCTGGGGATG- - TGGGGTTCAACAACAG

B

	NtBADH1B (SgRNA2)
WT	GTACGTATTCCCAGCCGACAGCTATACATCGACGGTGAATGGAGAGA
badh1b#5	GTACGTATTCCCAGCCGACAGCTATACAT*T*CGACGGTGAATGGAGAGA
badh1b#15	GTACGTATTCCCAGCCGACAGCTATACAT*A*CGACGGTGAATGGAGAGA
badh1b#23	GTACGTATTCCCAGCCGACAGCTATAC- TCGACGGTGAATGGAGAGA
badh1b#29	GTACGTATTCCCAGCCGACAGCTATA- - TCGACGGTGAATGGAGAGA

C

	NtBADH1A (SgRNA1)	*NtBADH1B* (SgRNA2)
WT	TCGGAGAGCGCTAGCTGGGGATGAGTGGGGTTCAA	ATTCCCAGCCGACAGCTATACATCGACGGTGAATG
T1-badh1-1	TCGGAGAGCGCTAGCTGGGGATG*T*AGTGGGGTTCAA	ATTCCCAGCCGACAGCTATACAT*T*CGACGGTGAATG
T1-badh1-2	TCGGAGAGCGCTAGCTGGGGA- - AGTGGGGTTCAA	ATTCCCAGCCGACAGCTATACAT*T*CGACGGTGAATG

D

	NtBADH2A (SgRNA3)	*NtBADH2B* (SgRNA3)
WT	CGGATCCCTAGTCGGCAGCTGTTCATCGA CGGTGA	CAGATCCCTAGTCGGCAGCTGTTCATCGACGGTGA
badh2a#17/badh2a#18	CGGATCCCTAGT- - GCAGCTGTTCATCGACGGTGA	CAGATCCCTAGTCGGCAGCTGTTCATCGACGGTGA
badh2a#6	CGGATCCCTAGT- GGCAGCTGTTCATCGACGGTGA	CAGATCCCTAGTCGGCAGCTGTTCATCGACGGTGA
badh2b#14/badh2b#62	CGGATCCCTAGTCGGCAGCTGTTCATCGACGGTGA	CAGATCCCTAGT- GGCAGCTGTTCATCGACGGTGA
badh2#04/badh2#35	CGGATCCCTAGT- GGCAGCTGTTCATCGACGGTGA	CAGATCCCTAGT- - GCAGCTGTTCATCGACGGTGA
badh2#16/badh2#30/ badh2#43/Badh2#45/badh2#50	CGGATCCCTAGT- GGCAGCTGTTCATCGACGGTGA	CAGATCCCTAGT- GGCAGCTGTTCATCGACGGTGA
badh2#63	CGGATCCCTAGT*T*CGGCAGCTGTTCATCGACGGTGA	CAGATCCCTAGT- GGCAGCTGTTCATCGACGGTGA

Figure 5. Sequences of the wild type and gene-edited mutants of *NtBADH* in the 'Honghuadajinyuan' background. (**A**) *Ntbadh1a* single mutants. (**B**) *Ntbadh1b* single mutants. (**C**) *Ntbadh1a-Ntbadh1b* double mutants. (**D**) Sequences of *NtBADH2a* and *NtBADH2b* in *Ntbadh2a* and *Ntbadh2b* single mutants and *Ntbadh2a-Ntbadh2b* double mutants. Guide RNA sequences are indicated in green; PAM sequences are indicated in red; italics indicates an insertion mutation; and a short transverse line indicates a deletion mutation.

For *NtBADH2a* and *NtBADH2b* (sgRNA3), we screened 60 T0 transgenic lines from 63 lines using kanamycin selection. Sequencing analysis revealed 56 edited plants. Twelve edited plants showed specific editing of *NtBADH2a*, three of which were identified as homozygous mutant lines (badh2a#6, badh2a#17, and badh2a#18), and the remaining plants were regarded as biallelic or heterozygous mutations. Five plants showed specific editing of *NtBADH2b*; two of these were identified as homozygous mutant lines (badh2b#14 and badh2b#62), one possessed biallelic mutations, and the other two plants carried heterozygous mutations. Meanwhile, we identified eight homozygous plants mutated for both *NtBADH2a* and *NtBADH2b* (badh2#04, badh2#16, badh2#30, badh2#35, badh2#43, badh2#45, badh2#50, and badh2#63). The badh2a#6 line harbored a 1-bp deletion mutation, while badh2a#17 and badh2a#18 lines harbored 2-bp deletion mutations in the *NtBADH2a* gene only. The badh2b#14 and badh2b#62 lines carried a 1-bp deletion mutation in the *NtBADH2b* gene only. The double-mutant lines badh2#04 and badh2#35 contained 1-bp deletions in the *NtBADH2a* gene and a 2-bp deletion in the *NtBADH2b* gene. In the five double-mutant lines badh2#16, badh2#30, badh2#43, badh2#45, and badh2#50, the Cas9-induced mutations disrupted both the *NtBADH2a* and *NtBADH2b* genes with

a 1-bp deletion mutation. The badh2#63 line contained a 1-bp insertion mutation in *NtBADH2a* and a 1-bp deletion mutation in *NtBADH2b* (Figure 5D). Importantly, all the above mutations led to frameshift mutations of *NtBADH2a* or *NtBADH-2B*.

3.4. Targeted NtBADH2a-NtBADH2b Double Mutations Affect 2AP Content

To verify the fragrance of the *BADH* gene-edited line, we conducted a fragrance test on fresh leaves of T0 transgenic plants with homozygous or biallelic mutations by exposing the fresh leaves to 1.7% (*w*/*v*) KOH solution for 15 min. Leaves of *Ntbadh2a-Ntbadh2b* double mutants had a distinctly popcorn-like fragrance compared with those of the wild type, as determined by organoleptic testing. To quantify the fragrance, we further characterized 2AP levels in leaves at the flowering stage for homozygous single or double mutants using GC-MS, with 2,4,6-trimethyl pyridine (TMP) as an internal control. As expected, we detected 2AP only in *Ntbadh2a-Ntbadh2b* double mutant lines, with 2AP content varying from 0.53 to 0.90 ng/mg (Figure 6A). However, levels of 2AP in any of the single mutants, the *Nntbadh1a-Ntbadh1b* double mutant or the wild type only varied from 0.01 to 0.05 ng/mg (Figure 6A). In addition, we further created T1 generation plants for *Ntbadh2a-Ntbadh2b* double mutant line badh2#16, and measured 2AP content in several tissues including the root, stem, leaf, flowers, and mature seeds. The results showed that the 2AP content in leaves was the highest with 0.71 ng/mg and was followed by the stem and flower with 0.43 ng/mg and 0.26 ng/mg 2AP content, respectively. Interestingly, the content of 2AP in seeds of double mutants was relatively low (only 0.19 ng/mg) but was still significantly higher than in the tobacco wild type. These findings indicate that only the *NtBADH2* gene, and not the *NtBADH1* gene, was responsible for conferring fragrance in tobacco. Furthermore, both *NtBADH2a* and *NtBADH2b* were involved in 2AP biosynthesis, and only their combined inactivation results in 2AP accumulation in tobacco.

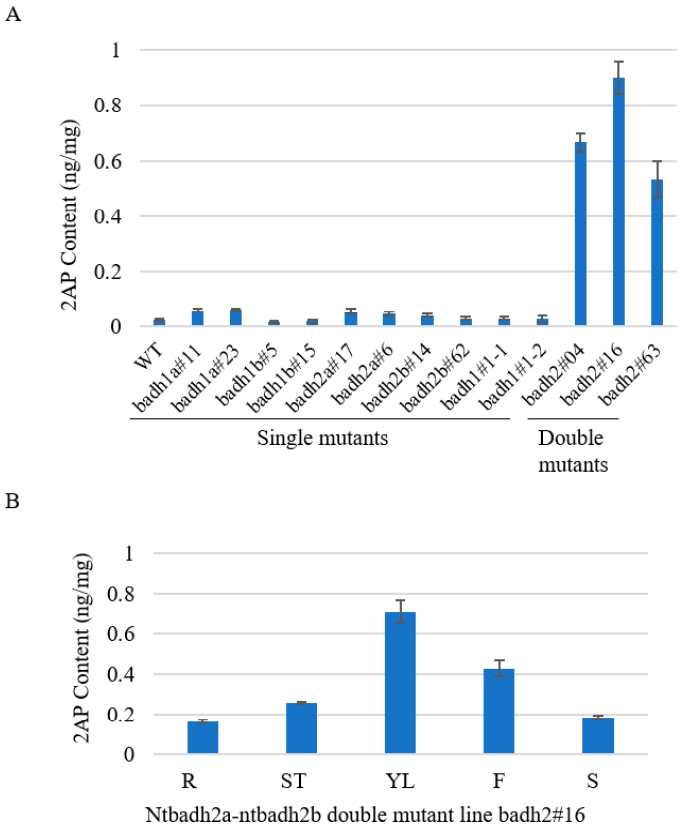

Figure 6. (**A**) Relative contents of 2AP in the leaves of the single or double mutants and the wild type 'Honghuadajinyuan'. (**B**) The 2AP content in different tissues at the flowering stage for the double mutant Ntbadh2a-Ntbadh2b line badh2#16. R, roots; ST, stems; YL, young leaves; F, flowers; S, seeds.

4. Discussion

The first BADH in higher plants was isolated from *Spinacia oleracea* [3]. BADH contains two isoenzymes, BADH I and BADH II [33]. Previous studies have classified BADH proteins into two major branches. The members of the first subfamily are mainly from monocotyledonous plants, while the members of the other subfamily are mainly from dicotyledonous plants [34], which is consistent with our present results. We identified four *BADH* genes in the tobacco genome, with *NtBADH1a* and *NtBADH1b* distributed in one cluster, and *NtBADH2a* and *NtBADH2b* distributed in another cluster (Figure 2). Structural analysis of betaine aldehyde dehydrogenase in rice showed that OsBADH contains three domains: a nicotinamide adenine dinucleotide (NAD) binding domain, a substrate-binding domain, and an oligomerization domain. BADH is localized in the chloroplasts and catalyzes the oxidation of betaine aldehyde, 4-aminobutyraldehyde, and 3-aminopropanal [2]. Li et al. reported that in most plants, BADH had a highly conserved decapeptide (VTLELGGKSP) motif, which bound to cysteine (Cys) residues to determine the catalytic activity of betaine aldehyde dehydrogenase [35]. However, the decapeptides in GmBADH, ZmBADH, OsBADH, HvBADH, and SbBADH15 are VSLELGGKSP, with the second threonine residues replaced by serine [7,36–40]. We determined that tobacco BADH proteins contain a conserved ten-peptide motif with the sequence VTLELGGKSP, a cysteine residue (C) highly conserved in aldehyde dehydrogenases (ALDHs) that is related to enzymatic function, and an SKL motif at the C terminus, which is reported to ensure precursor protein location at peroxisomes in plants [41]. Therefore, we believe that NtBADH has similar functions to other BADH proteins and may be located within peroxisomes.

Common tobacco (*N. tabacum*) is an allotetraploid (2n = 48 resulting from the chromosomes sets of both parents being present in the gametes) that resulted from a *Nicotiana sylvestris* (2n = 24) and *Nicotiana tomentosiformis* (2n = 24) hybridization [42]. We determined that the tobacco genome harbors four functional BADH homologues in the present study. The phylogenetic tree revealed that two of these proteins fall within the BADH1 cluster. In contrast, the other two protein sequences were more similar to proteins in the BADH2 cluster. Although *NtBADH1* and *NtBADH2* were expressed in all tissues, *NtBADH1* transcripts were most abundant in leaves and flowers, while *NtBADH2* transcripts were most abundant in roots and seeds (Figure 3). This indicate that the function of BADH was divided in plant species. Previous studies have demonstrated that genetic mutations in *BADH2* were responsible for 2AP accumulation in crops, including rice [16], soybean [43], sorghum [17] and cucumber [44]. Dysfunction of a single *BADH2* copy is sufficient to confer 2AP accumulation in plant species other than maize, whose genome harbors two redundant genes, *ZmBADH2a* and *ZmBADH2b*, controlling 2AP biosynthesis [45]. In this study, we created tobacco lines by inactivating the four tobacco *BADH* genes by genome editing. The *ntbadh2a-ntbadh2b* double mutants accumulated 2AP in their leaves, flowers and kernels (Figure 6B). This is the first report to our knowledge of *BADH2* genes function in tobacco. It is worth noting that *NtBADH2a* and *NtBADH2b* were highly expressed in roots and seeds, while their expression was relatively low in leaves. These expression patterns are inconsistent with previous reports in rice, sorghum and foxtail millet, where the *BADH2* gene was highly expressed in leaves and panicles but showed poor expression in roots [13,19,46]. These differences suggest that the *NtBADH2* gene contributes to the regulation of 2AP accumulation in a species-specific manner in tobacco.

Betaine aldehyde dehydrogenase 1 (*BADH1*), a homologous gene to *BADH2*, has been found to play a role in salt stress by facilitating the accumulation of biosynthesized glycine betaine (GB), which is known to participate in the response to abiotic stresses [47,48]. However, there are divergent findings from various studies in rice regarding the correlation between BADH1 and salt stress tolerance. Studies by Bradbury et al., 2008 showed that rice BADH1 has very low activity on BA, but the function and mechanism of BADH1 action were uncertain [49]. Meanwhile, Singh et al., 2010 argued that BADH1 is associated with rice fragrance [9]. The function of BADH1, therefore, needs further study. BADH1

in tobacco was not associated with 2AP accumulation because the content of 2AP did not change in either single *Ntbadh1a* or *Ntbadh1b* lines or double *Ntbadh1a-Ntbadh1b* mutant plants (Figure 6A). Whether *BADH1* is a candidate gene associated with abiotic stresses in tobacco, such as salt, drought, and temperature, will be the focus of our research in the future.

The accumulation of 2AP can also be affected by growth conditions, which can regulate the expression of genes or the enzymes activity involved in 2AP biosynthesis. Conditions of alternating wetting and moderate drying [50] or low light treatment [51] significantly increase 2AP concentration. Additionally, 2AP contents of aromatic rice grains were increased under foliar application of zinc via promotion of the activity of P5CS, proline de-hydrogenase, and diamine oxidase enzymes [52]. Molybdenum can also enhance 2AP contents in rice grains through the promotion of nitrogen utilization and assimilation, stimulation of glutamate synthase activity, and elevation of proline content [53]. Conversely, drought stress [51] and cadmium stress [53] cause various inhibitory effects in rice, such as reducing 2AP contents. In addition, Maize lines carrying similar presumed loss-of-function alleles, was lower in the XCW175 inbred line than in Zheng58 and LN005M, suggesting that even genotypes carrying loss-of-function or weak alleles of the *BADH2* gene may still accumulate different levels of 2AP [45]. Likewise, the same *badh2* mutant could potentially accumulate varying levels of 2AP under diverse circumstances. The same may hold true in tobacco. As illustrated in Figure 6, changes in 2AP content observed in *Ntbadh2a-Ntbadh2b* double mutants over different generations may reflect different growth conditions. Even more importantly, 2AP content in tobacco leaves was significantly higher than in other tissues (Figure 6B); tobacco (*Nicotiana tabacum*) demonstrates adaptability, effective manipulation of genetic material, regeneration ability, and the capability to generate substantial quantities of leaf biomass. These characteristics contribute to achieving high levels of desired proteins, thereby simplifying the process of protein extraction and purification. Consequently, tobacco is regarded as an excellent candidate for plant-based protein production [54]. In the future, tobacco leaves may be utilized as a bioreactor under optimal conditions to enhance the accumulation of 2AP by editing additional genes involved in the 2AP biosynthetic pathway.

5. Conclusions

In summary, we successfully identified four *NtBADH* genes (*NtBADH1a*, *NtBADH1b*, *NtBADH2a* and *NtBADH2b*) from tobacco cultivar 'Honghuadajinyuan'. To the best of our knowledge, this study is the first to report 2AP contents in tobacco. Our results demonstrate that *NtBADH2* genes, but not *NtBADH1* genes, are required for 2AP accumulation in tobacco, while *NtBADH2a* and *NtBADH2b* are redundant genes controlling 2AP biosynthesis in tobacco. In addition, we generated tobacco lines with stable and high 2AP content using CRISPR-Cas9-mediated gene knockout. The mutants in this study could be used for advanced engineering to produce unusual 2AP phenotypes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14020321/s1. Figure S1: The hypothetical mechanism of the synthesis of 2AP by BADH. Figure S2: Phylogenetic tree of NtBADH1a, NtBADH1b, NtBADH2a and NtBADH2b amino acid sequences with BADH proteins from *N. tomentosiformis* and *N. sylvestris*. Table S1: Specific primers used for amplifying the four full-length *NtBADH* gene in tobacco. Table S2: Gene-specific primers used for analysis of expression levels of four *NtBADH* genes. Table S3: Specific primers for mutant analysis of CRISPR/Cas9-mediated mutant lines.

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