Cloning, Localization, and Promoter Analysis of the NDH Dehydrogenase Gene in Sandalwood (Santalum album) Postprint

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Abstract

To investigate the function and regulatory mechanism of sandalwood NDH dehydrogenase genes, the full-length sequence of the SaNDH6 gene was cloned from sandalwood heartwood using RACE technology, its expression patterns in various tissues and after hormone treatments were analyzed using real-time quantitative PCR (RT-qPCR), its subcellular localization was observed in Arabidopsis protoplasts, the promoter sequence 2 kb upstream of the SaNDH6 start codon ATG was analyzed using PlantCARE, and transcription factors potentially binding to it were predicted using PlantRegMap. The results showed that: (1) SaNDH6 encodes 303 amino acids, is a hydrophobic protein, and is localized to the chloroplast. (2) Phylogenetic tree analysis indicated that sandalwood SaNDH6 is closely related to NDH6 from woody plants. (3) PlantCARE analysis revealed that the SaNDH6 promoter contains numerous light-responsive elements such as ACE, AE-box, Box 4, G-Box, and GT1-motif, as well as methyl jasmonate (MeJA) response elements CGTCA-motif and TGACG-motif, gibberellin (GA) response element P-box, and defense and stress response element TC-rich repeats. (4) PlantRegMap analysis identified 76 transcription factors potentially binding to the SaNDH6 promoter, with the ERF family being the most abundant (40 members). (5) SaNDH6 was expressed in the roots, heartwood, leaves, and callus tissues of sandalwood, with relatively high expression in leaves; after treatment of sandalwood callus with 1$\times10^{-4}$ mol·L^{-1} MeJA and GA3, SaNDH6 expression was significantly upregulated. In summary, these results indicate that sandalwood SaNDH6 is a nuclear-encoded protein, induced by light and hormones, and may be involved in sandalwood stress responses.

Full Text

Preamble

Molecular Cloning, Subcellular Localization, and Promoter Analysis of NDH Dehydrogenase Gene from *Santalum album*

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Abstract: To investigate the function and regulatory mechanism of NDH dehydrogenase genes in sandalwood, we cloned the full-length sequence of *SaNDH6* from sandalwood heartwood using RACE technology, analyzed its expression patterns in different tissues and after hormone treatments using real-time fluorescent quantitative PCR (RT-qPCR), observed its subcellular localization in *Arabidopsis* protoplasts, analyzed the promoter sequence 2 kb upstream of the *SaNDH6* start codon ATG using PlantCARE, and predicted potential transcription factors that might bind to it using PlantRegMap. The results showed that: (1) *SaNDH6* encodes 303 amino acids, is a hydrophobic protein, and is localized in chloroplasts. (2) Phylogenetic analysis revealed that sandalwood SaNDH6 has a close evolutionary relationship with NDH6 from woody plants. (3) PlantCARE analysis identified numerous light-responsive elements in the *SaNDH6* promoter, including ACE, AE-box, Box 4, G-Box, and GT1-motif, as well as methyl jasmonate (MeJA) response elements CGTCA-motif and TGACG-motif, gibberellin (GA) response element P-box, and defense and stress response element TC-rich repeats. (4) PlantRegMap analysis predicted 76 transcription factors that might bind to the *SaNDH6* promoter, with the ERF family being the most abundant (40 TFs). (5) *SaNDH6* was expressed in roots, heartwood, leaves, and callus tissues of sandalwood, with relatively high expression in leaves. After treatment of sandalwood callus with 1$×10^{-4}$ mol · L^{-1} MeJA and GA_3, *SaNDH6* expression was significantly upregulated. These results indicate that sandalwood *SaNDH6* is a nuclear-encoded protein whose expression is induced by light and hormones, and may be involved in sandalwood stress response processes.

Keywords: *Santalum album*, chloroplast, NDH dehydrogenase, subcellular localization, expression regulation

Introduction

In photosynthesis, linear electron transport from H_2O to $NADP^+$ simultaneously produces ATP and NADPH, but the resulting ATP/NADPH ratio of less than 1.5 cannot meet the demands of the Calvin cycle. This ATP deficit is compensated by cyclic electron transport around Photosystem I (PSI) [Yamori & Shikanai, 2016]. In angiosperms, NDH complex-dependent electron transport represents one pathway for PSI cyclic electron transfer, playing roles in photosynthesis, respiration, plant growth, and protection against high-light damage and low-temperature stress [Tsuyoshi Endoa, 1999; Yamori et al., 2011; Yamori & Shikanai, 2016]. Consequently, research on the NDH complex has attracted increasing attention. Shinozaki et al. [1986] and Ohyama [1996] discovered 11 chloroplast-encoded NDH genes through chloroplast genome sequencing of tobacco (*Nicotiana tabacum*) and liverwort (*Marchantia polymorpha*). Although these genes are homologous to mitochondrial NDH genes, chloroplast NDH primarily accepts electrons from ferredoxin (Fd) [Ifuku et al., 2011; Yamamoto et al., 2011; Shikanai, 2016]. Further studies have revealed that many chloroplast NDH complex genes are encoded by the nuclear genome [Sirpio et al., 2009; Yamori et al., 2011; Shikanai, 2016]. To date, over 30 NDH complex genes have been identified in *Arabidopsis*, which can be divided into five categories [Armbruster et al., 2013; Fan et al., 2015; Peltier et al., 2016]. SubA comprises seven genes, four encoded by the chloroplast genome (*NdhH-NdhK*) and three by the nuclear genome (*NdhM-NdhO*), all involved in electron transfer to coenzyme Q [He et al., 2015]. SubM includes six members (*NdhA-NdhG*) encoded by the chloroplast genome, forming the complex arm in the membrane and participating in electron transfer across the membrane. SubB (*PnsB1-PnsB5*) and SubL (*PnsL1-PnsL5*) members are all nuclear-encoded and represent unique components of the chloroplast NDH complex. SubB may be involved in maintaining NDH complex stability [Peng et al., 2009; Takabayashi et al., 2009], while SubL helps maintain NDH-PSI supercomplex stability [Peltier et al., 2016]. SubED (*NdhS*, *NdhV*, *NdhT*, and *NdhU*) is also nuclear-encoded and interacts with SubA to form the Fd binding site [Yamamoto et al., 2011; Peltier et al., 2016].

During evolution, NDH has formed a supercomplex with PSI in higher plants, enhancing electron transfer efficiency and stabilizing NDH complex structure under stress conditions. In *Arabidopsis*, Lhca5 and Lhca6 serve as connecting components in this supercomplex formation, with Lhca6 also stabilizing NDH complex structure [Peng et al., 2009]. Recently, Otani et al. [2018] found that additional light-harvesting complex I proteins, including Lhca1, 2, 3, and 4, participate in NDH-PSI supercomplex formation through different combinations that connect NDH and PSI. While these findings demonstrate substantial progress in understanding NDH complex structure, some constituent subunits —particularly those not tightly associated with the NDH-PSI supercomplex— remain poorly characterized, and their functions and regulatory mechanisms require further investigation [Fan et al., 2015].

Santalum album is a semi-parasitic precious timber tree distributed in tropical

and subtropical regions. Its wood is not only tough and excellent in quality but also contains aromatic essential oils widely used in perfumes, aromatherapy, carving, and medicine, giving it high economic value [Baldovini et al., 2011]. Current research on sandalwood has primarily focused on essential oil synthesis and regulation, with very limited studies on its photosynthetic characteristics. The NDH complex represents an important component for electron transport during sandalwood photosynthesis, yet the composition of its genes and their functions and regulation remain unclear. Using sandalwood xylem as material, this study employs molecular biology techniques—including gene cloning, phylogenetic analysis, subcellular localization, tissue expression patterns, promoter cis-acting element analysis, and transcription factor binding prediction—to explore the specific localization and potential role of SaNDH6 in the sandalwood NDH complex, analyze its expression regulation patterns, and investigate its potential functions in stress responses, thereby laying a foundation for functional studies of the sandalwood NDH complex in photosynthesis and stress tolerance.

Materials and Methods

1.1 Plant Materials and Treatments

Sandalwood leaves, roots, and heartwood were collected from 7-year-old sandalwood trees grown at the South China Botanical Garden, Chinese Academy of Sciences (at least three normally growing trees were selected for sampling). Samples were snap-frozen in liquid nitrogen and stored at -80°C in the laboratory. Sandalwood callus was induced from tender shoots as explants following the methods of Singh et al. [2015] and Yan et al. [2018]. Equal amounts of sandalwood callus were weighed and placed in MS liquid medium, cultured in darkness at 25°C with shaking at 100 $\mathrm{r \cdot min^{-1}}$ for 24 h, then treated with methyl jasmonate (MeJA) and gibberellin (GA_3) solutions at a final concentration of 1$\times10^{-4}$ $\mathrm{mol \cdot L^{-1}}$. Samples were collected at 0, 3, and 6 h, snap-frozen in liquid nitrogen, and stored at -80°C for RNA extraction. Each treatment included three replicates.

1.2 Cloning of SaNDH6

Total RNA was extracted from sandalwood heartwood using the method for woody plants [Kolosova et al., 2004]. RNA quality and integrity were assessed using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, NC, USA) and 1.5% agarose gel electrophoresis. The full-length sequence of *SaNDH6* was amplified using the SMARTer RACE cDNA Amplification Kit (Clontech Laboratories Inc., CA, USA) with nested PCR. Amplification products were detected by 1.5% agarose gel electrophoresis, recovered, ligated into the PMD18-T vector, transformed into *E. coli* DH-5α, and positive clones were sequenced by Beijing Genomics Institute (Shenzhen). Primers for full-length amplification (3′ RACE, 5′ RACE, and ORF) are listed in Table 1 .

1.3 Bioinformatics Analysis of SaNDH6

Physicochemical properties of SaNDH6 were predicted using ExPASy ProtParam (https://web.expasy.org/cgi-bin/protparam.html), and subcellular localization was predicted using Plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/). Amino acid sequences of NDH subunits from different plants were aligned using DNAMAN software, and a phylogenetic tree was constructed using the neighbor-joining (N-J) method in MEGA 6.0.

1.4 Subcellular Localization of SaNDH6

The sandalwood genome sequence was downloaded from NCBI (accession number GCA_{002925775}.1) [Mahesh et al., 2018], annotated with reference to maize, rice, and *Arabidopsis* genome data, and the *SaNDH6* promoter sequence was extracted. The promoter was analyzed using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), and transcription factor binding sites were predicted using PlantRegMap: Plant Regulation Data and Analysis Platform @ CBI, PKU (http://plantregmap.cbi.pku.edu.cn/binding_{{{site}}_{{prediction

The *SaNDH6* ORF sequence (without the stop codon) was amplified and fused to the 35S promoter using In-Fusion technology to construct the 35S:*SaNDH6*:pSAT6-EYFP-N1 subcellular localization vector. After sequence confirmation, *Arabidopsis* protoplasts were transformed following the method of Yoo et al. [2007], cultured at 22°C under weak light for 12 h, and observed using a laser confocal scanning microscope (Zeiss, Jena, Germany).

1.5 Real-Time Fluorescent Quantitative PCR Analysis

Total RNA was extracted from sandalwood leaves, heartwood, roots, and callus tissues as described in section 1.1, treated with RNase-free DNase I (TaKaRa, Japan) to ensure no DNA contamination, and reverse-transcribed using 1 μg of RNA with A260/A280 ratio of 1.9–2.1, A260/A230 ratio >2.0, and intact bands on electrophoresis. The resulting cDNA was diluted 10-fold with nuclease-free water and stored at -20°C.

RT-qPCR was performed using an ABI 7500 Real-time system (ABI, Alameda, CA, USA) with SoAdvanced™ Universal SYBR® Green Supermix detection system (Bio-Rad, Hercules, CA, USA). The reaction mixture contained 5 μL SYBR® Green Supermix, 0.5 μL each of forward and reverse primers (1$×10^{-5}$ mol · L^{-1}), 1 μL cDNA, and ddH_2O to a final volume of 10 μL. Cycling conditions were: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Reference genes were selected according to Yan et al. [2018]: *SaFAB1A+SaPP2C* for different tissues, *SaCSA+SaFbp3* for MeJA treatment, and *SaPP2C+SaFbp2* for GA treatment. The arithmetic mean of the two corresponding reference genes was used for normalization. Each sample had three replicates, and quantitative data were analyzed using the 2^(-ΔΔCq) method. RT-qPCR primers are listed in Table 1 .

1.6 Statistical Analysis

Data were statistically analyzed using SPSS 19.0 (IBM Corp., Armonk, NY, USA). Multiple comparisons were performed using Duncan's new multiple range test ($P<0.05$).

Results

2.1 Cloning of SaNDH6

Based on the NDH dehydrogenase Unigene annotated in the sandalwood transcriptome, primers were designed. A specific band of 528 bp was obtained by 3 RACE amplification [Figure 1: see original paper]A, and a 317 bp band by 5 RACE amplification [Figure 1: see original paper]B. Both sequences correctly assembled with the existing sequence and contained a Poly A tail at the 3 end, confirming successful acquisition of the 3 and 5 ends. After analysis with NCBI ORF Finder and assembly, a 912 bp target band was amplified by RT-PCR [Figure 1: see original paper]C. Sequencing confirmed the target sequence, which was designated as *SaNDH6*.

2.2 Bioinformatics Analysis of SaNDH6

SaNDH6 encodes 303 amino acids [Figure 2: see original paper] with a molecular weight of 33.75 kDa and a theoretical isoelectric point of 9.31. The protein contains 28 acidic amino acids, 45 basic amino acids (76 charged residues total), 79 polar uncharged amino acids, and 160 hydrophobic amino acids, indicating it is a hydrophobic protein. Subcellular localization prediction suggested chloroplast targeting.

Amino acid sequences of NDH subunits from various plants were downloaded from NCBI and aligned using DNAMAN. As shown in [Figure 3: see original paper], sandalwood SaNDH6 shares 53.46% sequence similarity with peach (*Prunus persica*) PpNdh6, 52.6% with sesame (*Sesamum indicum*) SiNdh6, 51.84% with both cassava (*Manihot esculenta*) MeNdh6 and white mesquite (*Prosopis alba*) PaNdh6, and 51.3% with grape (*Vitis vinifera*) VvNdh6, confirming successful cloning of the sandalwood NDH complex subunit 6 gene.

A phylogenetic tree was constructed using MEGA 6.0 based on amino acid sequences of NDH subunits from different plants. As shown in [Figure 4: see original paper], sandalwood SaNDH6 clustered with SiNdh6 and VvNdh6, consistent with the multiple sequence alignment results. Notably, SaNDH6 showed close evolutionary relationships with NDH6 from woody plants including grape, sweet cherry (*Prunus avium*), mulberry (*Morus notabilis*), poplar (*Populus trichocarpa*), physic nut (*Jatropha curcas*), and rubber tree (*Hevea brasiliensis*).

2.3 Subcellular Localization of SaNDH6

The sandalwood SaNDH6 subcellular localization vector was transiently transformed into *Arabidopsis* protoplasts, with YFP empty vector as a control. Yellow fluorescent protein was observed in both 35S:*SaNDH6*:pSAT6-EYFP-N1 and YFP empty vector transformations, confirming reliable transformation. The yellow fluorescence of the 35S:*SaNDH6*:pSAT6-EYFP-N1 fusion protein was primarily distributed in chloroplasts, demonstrating that SaNDH6 localizes to chloroplasts [Figure 5: see original paper], consistent with the prediction results.

2.4 Tissue Expression of SaNDH6

Expression analysis in different tissues revealed that *SaNDH6* was expressed in roots, heartwood, leaves, and callus tissues of sandalwood, with the highest expression in leaves, followed by callus, and relatively low expression in the other two tissues [Figure 6: see original paper].

2.5 Promoter Analysis of SaNDH6

After annotating the sandalwood genome data, we extracted a 2,000 bp promoter sequence upstream of the *SaNDH6* start codon ATG. PlantCARE analysis identified numerous light-responsive elements including ACE, AE-box, Box 4, G-Box, GT1-motif, LAMP-element, MRE, TCT-motif, and chs-CMA1a, indicating that *SaNDH6* expression is primarily induced by light. Hormone-responsive elements were also identified, including abscisic acid (ABA) response element ABRE, MeJA response elements CGTCA-motif and TGACG-motif, and gibberellin response element P-box, suggesting hormonal regulation of *SaNDH6* expression. Additionally, stress-responsive elements were found, including anaerobic induction element ARE and defense and stress response element TC-rich repeats, indicating that *SaNDH6* may also participate in sandalwood stress response processes [Figure 7: see original paper].

2.6 Analysis of Transcription Factors Potentially Binding to SaNDH6

PlantRegMap analysis identified 76 transcription factors that may bind to the *SaNDH6* promoter [Figure 8: see original paper]. The ERF family was most abundant with 40 TFs, followed by B3 family with 13 TFs. MIKC_{MADS} and AP2 families had 5 and 4 TFs respectively, while FAR1 and MYB families had only one each. These results suggest that *SaNDH6* expression is primarily regulated directly by ERF and B3 family transcription factors.

2.7 Expression Analysis of SaNDH6 After Different Hormone Treatments

The presence of MeJA and GA response elements in the *SaNDH6* promoter suggested these hormones might induce its expression. To test this hypothesis, sandalwood callus was treated with 1$×10^{-4}$ $\mathrm{mol \cdot L^{-1}}$ MeJA and GA_3.

Expression results showed that compared with 0 h, *SaNDH6* expression was significantly elevated after 3 h of both MeJA and GA_3 treatments [Figure 9: see original paper], demonstrating that both MeJA and GA_3 can positively induce *SaNDH6* expression.

Discussion

3.1 Localization of SaNDH6 in the Sandalwood NDH Complex

Chloroplast NDH dehydrogenase is a thylakoid membrane protein complex composed of multiple subunits, including 11 chloroplast-encoded subunits and at least 19 nuclear-encoded subunits, with at least 16 genes involved in its assembly [Ifuku et al., 2011; Yamori & Shikanai, 2016]. We cloned *SaNDH6* from sandalwood and found high sequence similarity with NDH6 subunits from various plants through Blast searches, with close evolutionary relationships to NDH6 from woody plants. Sequence analysis indicated SaNDH6 is a hydrophobic protein, and subcellular localization confirmed its chloroplast targeting, suggesting SaNDH6 functions by localizing to the thylakoid membrane in chloroplasts. *SaNDH6* showed high expression in sandalwood leaves but was also expressed in heartwood, roots, and callus tissues that lack chloroplasts, leading us to speculate that SaNDH6 is a nuclear-encoded protein.

3.2 Regulation of SaNDH6 Expression

Transcriptional regulation plays a key role in promoting or inhibiting gene expression, primarily controlled by gene promoters and cis-acting elements within them [Hernandez-Garcia & Finer, 2014; Zou et al., 2011]. Currently, few studies have reported on the expression regulation of chloroplast NDH complexes. Analysis of the 2 kb promoter sequence upstream of the *SaNDH6* start codon ATG revealed numerous light-responsive elements, indicating that light is the main regulator of its expression, consistent with its primary role in photosynthesis. Additionally, *SaNDH6* expression was positively regulated by GA_3 and JA. Romanowska [1984] and Tsai [1985] demonstrated that exogenous GA_3 can increase growth rate and photosynthetic efficiency in some plants, suggesting GA may participate in sandalwood photosynthesis by regulating *SaNDH6* expression. Besides specifically regulating plant responses to insect feeding and necrotrophic pathogen infection [Wasternack, 2015; Hickman et al., 2017], JA also participates in plant growth, development, and resistance to abiotic stresses [Qiu et al., 2014; Per et al., 2018]. While few studies have examined NDH complex involvement in biotic stress responses such as pathogen infection, numerous reports have documented its role in abiotic stress tolerance [Yamori & Shikanai, 2016]. We therefore speculate that JA may primarily regulate *SaNDH6* expression to participate in sandalwood responses to abiotic stresses, though the specific mechanisms require further investigation.

3.3 Role of SaNDH6 in Stress Responses

Electron transport through NDH and the PGR5/PGRL1-dependent pathway exhibit partial functional redundancy. Under normal growth conditions, mutations in NDH complex components do not produce obvious phenotypic changes [Munekage, 2004; Yamori & Shikanai, 2016]. However, detailed studies have demonstrated that the NDH complex functions in plant resistance to various abiotic stresses. Hibino et al. [1996] found that high salt specifically induced expression of cyclic electron transfer proteins in the salt-tolerant cyanobacterium *Aphanothece halophytica*, increasing NDH-dependent electron transport and enabling adaptation to high-salt environments. Zhao et al. [2017] proved that under multiple environmental stresses, NDH-1 in *Synechocystis* could maintain PSI structural stability. Li et al. [2004] exposed tobacco to low temperature (4°C) and low light intensity (100 $\mu mol \cdot m^{-2} \cdot s^{-1}$), finding that the ndhB mutant showed significantly lower maximum photochemical efficiency (Fv/Fm) and PSII-driven electron transport efficiency than wild-type plants, suggesting that NDH-dependent electron transport protects photosynthetic organs under low temperature and weak light. Wang et al. [2006] examined reactive oxygen species accumulation differences between wild-type and NDH mutant ndhC-ndhK-ndhJ (ΔndhCKJ) tobacco plants under different temperature treatments, finding that NDH reduced high-temperature stress-induced reactive oxygen species production by enhancing CO_2 assimilation through electron transport. These studies indicate that NDH-dependent electron transport maintains photosystem structural stability, promotes CO_2 assimilation, prevents over-reduction of the thylakoid lumen, reduces H_2O_2 production, and maintains normal photosynthetic rates under various stresses including high/low light, high/low temperature, high salinity, and low humidity. In this study, we found that the sandalwood *SaNDH6* promoter contains stress-responsive elements including JA and ABA response elements, anaerobic induction elements, and defense and stress response elements. MeJA response elements were most abundant (four total: two TGACG-motif and two CGTCA-motif), followed by drought stress-related elements (three total: two ABRE and one MBS). Combined with previous research, we speculate that besides its primary role in photosynthesis, SaNDH6 may also participate in sandalwood responses to drought and other abiotic stresses.

Conclusion

This study cloned the chloroplast NDH dehydrogenase subunit gene *SaNDH6* from sandalwood, which encodes 303 amino acids, localizes to chloroplasts, and shows close evolutionary relationships with nuclear-encoded subunits of *Arabidopsis* chloroplast NDH dehydrogenase. The *SaNDH6* promoter contains numerous light-responsive elements, several hormone-responsive elements, and stress-responsive elements. ERF and B3 family transcription factors may directly bind to the gene promoter to regulate its expression. Tissue expression

analysis revealed high expression in sandalwood leaves, and its expression was significantly induced by MeJA and GA_3.

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Note: Figure translations are in progress. See original paper for figures.