LIÈGE université
Gembloux
Agro-Bio Tech

Identification and Characterization of the Role of Acetolactate Synthase 1 and Acylsugar Acyltransferases 1 and 2 in Acylsugar Biosynthesis in *Nicotiana tabacum*

Aixia CHANG

Promoters: Prof. Hervé Vanderschuren
Prof. Yuanying Wang

2022

COMMUNAUTÉ FRANÇAISE DE BELGIQUE
UNIVERSITÉ DE LIÈGE – GEMBLOUX AGRO-BIO TECH

IDENTIFICATION AND CHARACTERIZATION OF THE ROLE OF ACETOLACTATE SYNTHASE 1 AND ACYLSUGAR ACYLTRANSFERASES 1 AND 2 IN ACYLSUGAR BIOSYNTHESIS IN NICOTIANA TABACUM

Aixia CHANG

Dissertation originale présentée en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

Promoteurs: Prof. Hervé Vanderschuren & Prof. Yuanying Wang

Année civile: 2022

Résumé

Aixia Chang (2022) IDENTIFICATION ET CARACTÉRISATION DU RÔLE DE L'ACETOLACTATE SYNTHASE 1 ET DES ACYLSUGAR ACYLTRANSFERASES 1 ET 2 DANS LA BIOSYNTHÈSE DES SUCRES ACYLÉS DANS LE NICOTIANA TABACUM (thèse de doctorat). Gembloux, Belgique, Gembloux Agro-Bio Tech, Université de Liège.

Le tabac est l'une des cultures commerciales importantes dans le monde, et il est également considéré comme un bioréacteur vert efficace grâce à sa capacité de transformation génétique transitoire et stable ainsi qu'à sa biomasse foliaire élevée. Dans le tabac, les sucres acylés sont spécifiquement synthétisés dans les trichomes glandulaires et ils sont principalement des tétra- acylsaccharoses, qui représentent la deuxième grande classe de produits chimiques de surface et leurs structures diffèrent souvent de celles des autres espèces de la famille des solanacées. Il a été démontré que les sucres acylés de tabac ont des propriétés anti-oviposition, anti-appétissantes, antimicrobiennes, anticancéreuses et ainsi que des propriétés toxiques qui leur permet d'être mis en une ample application commerciale potentielle dans les pesticides, les additifs alimentaires et cosmétiques, etc. En outre, ils sont d'importants précurseurs de composés aromatiques et leurs produits de dégradation contribuent à la qualité aromatique des feuilles de tabac. Par conséquent, la recherche des gènes clés de la biosynthèse des sucres acylés du tabac et l'analyse de leurs mécanismes fonctionnels sont non seulement d'une grande importance pour la résistance au tabac et la sélection de qualité, mais peuvent également établir les bases de développement et d'utilisation des sucres acylés du tabac. Cependant, bien que de grands progrès aient été réalisés dans la recherche des gènes clés concernant la biosynthèse des sucres acylés dans *Solanum*, *Pétunia* et *Salpiglossis*, les gènes et le mécanisme de biosynthèse des sucres acylés sont restés insaisissables dans le tabac.

Des études antérieures sur des espèces de Solanaceae ont montré que la voie métabolique des acides aminés à chaîne ramifiée (BCAA) est la principale source des chaînes d'acyle des sucres acylés, et les enzymes appartenant à la famille d'acyltransférases (BAHD) catalysent l'attachement des chaînes d'acyle aux noyaux de sucre pour former différents types de sucres acylés. Basé sur les progrès de recherche de la biosynthèse des sucres acylés, via comparaison d'homologie génique et détection du niveau d'expression transcriptionnelle, notre groupe de recherche a préalablement obtenu trois gènes à forte expression dans les trichomes glandulaires à partir du tabac, y compris un gène de l'acétolactate synthase (NtALS1, l'un des gènes clés impliqués dans la voie métabolique des BCAA) et deux gènes l'acyltransférase des sucres acylés (NtASAT1 et NtASAT2, appartenant à la famille BAHD). Dans cette étude de thèse, notre objectif de recherche est d'identifier et de caractériser si ces trois gènes sont impliqués dans la biosynthèse des sucres acylés de *N. tabacum*. Les principaux résultats sont les suivants:

Pour NtALS1, les analyses de distribution tissulaire ont montré que NtALS1 était fortement exprimé dans les extrémités des trichomes glandulaires. L'analyse de

localisation subcellulaire a montré que NtALS1 était localisé dans le chloroplaste. De plus, dans le contexte de la variété K326 de type sauvage, nous avons généré deux mutants perte-de-fonction NtALS1 à l'aide du système CRISPR-Cas9. Les teneurs en sucres acylés des deux mutants NtALS1 étaient significativement inférieures à celles du type sauvage. Grâce à l'analyse de l'arbre phylogénétique, nous avons découvert que chaque espèce de *Solanaceae* contenant des sucres acylés possédait un homologue ALS qui se regroupait avec NtALS1, alors que l'homologue ALS de *N. sylvestris* (un ancêtre de *N. tabacum*), une espèce qui ne contient pas des sucres acylés, ne se regroupe pas avec NtALS1. En conclusion, ces résultats indiquent un rôle fonctionnel de NtALS1 dans la biosynthèse des sucres acylés dans le tabac et indiquent également que NtALS1 provient probablement de l'ancêtre *N. tomentosiformis*.

Pour NtASAT1 et NtASAT2, les analyses de localisation tissulaire et subcellulaire ont montré que les deux gènes étaient spécifiques au trichome et fonctionnaient dans le réticulum endoplasmique. In vitro, NtASAT1 pourrait utiliser du saccharose et des substrats C2-, iC4- et iC5-CoA pour produire des mono-acylsaccharoses S1:2 (acétyl-CoA), S1:4 (iC4-CoA) et S1:5 (iC5-CoA), respectivement, avec l'activité la plus élevée lorsque iC5 a été utilisé comme donneur d'acyle. L'analyse RMN du mono-acylsaccharose purifié (S1:5) a révélé que le groupe acyle était attaché à la position R2 du saccharose. NtASAT2 catalyse l'attachement du donneur d'acyle iC5 au mono-acylsaccharose iC5 produisant le bi-acylsaccharose (S2: 10). Ces résultats étaient correspondants au fait que iC5 est plus fréquemment trouvé sous forme de chaîne latérale d'acyle dans le tabac cultivé. Knocking-out de NtASAT1 ou NtASAT2 a entraîné une carence en acylsaccharose dans le tabac, cependant, aucun effet sur l'accumulation des sucres acylés n'a été observé dans les plantes en surexpression de NtASAT1 ou de NtASAT2. L'analyse du profil génomique et des sucres acylés a révélé que NtASAT1 et NtASAT2 sont probablement issus de l'ancêtre diploïde *N. tomentosiformis* produisant des sucres acylés. Ces résultats ont révélé que NtASAT1 et NtASAT2 sont des enzymes clés impliquées dans l'assemblage des sucres acylés dans le tabac.

Ce étude a élargi notre compréhension de la biosynthèse de l'acylsaccharose dans les plants de tabac et a également fourni de nouveaux gènes pour une étude plus approfondie des mécanismes de régulation moléculaire de la biosynthèse des sucres acylés, la sélection moléculaire de la qualité aromatique du tabac et l'ingénierie des cultures résistantes aux agents pathogènes et aux insectes.

Mots-clés: Nicotiana tabacum, trichomes glandulaires, biosynthèse des sucres acylés, identification de la fonction des gènes, acétolactate synthase (ALS), acylsaccharose acyltransférases (ASAT)

Abstract

Aixia Chang (2022) IDENTIFICATION AND CHARACTERIZATION OF THE ROLE OF ACETOLACTATE SYNTHASE 1 AND ACYLSUGAR ACYLTRANSFERASES 1 AND 2 IN ACYLSUGAR BIOSYNTHESIS IN NICOTIANA TABACUM (PhD thesis). Gembloux Agro-Bio Tech, University of Liège, Belgium.

Tobacco is one of the important commercial crops in the world, and it is also considered an efficient green bioreactor because of its amenability to transient and stable genetic transformations, as well as its high leaf biomass. In tobacco, acylsugars are specifically synthesized in glandular trichomes and are mainly tetra-acylsucroses, which represent the second major class of surface chemicals and their structures often differ from those in other species of the *Solanaceae* family. Tobacco acylsugars have been shown to have antioviposition, antifeedant, antimicrobial, antitumor, and other toxic properties which make them a wide range of potential commercial applications in pesticides, food and cosmetic additives etc. In addition, they are important precursors of flavor compounds and their degradation products contribute to the aroma quality of tobacco leaves. Therefore, the exploration of key genes of tobacco acylsugar biosynthesis and the analysis of their functional mechanisms are not only of great significance for tobacco resistance and quality breeding, but also can lay a certain foundation for the development and utilization of tobacco acylsugars. However, although great progress has been made in mining key genes involved in acylsugar biosynthesis in *Solanum, Petunia* and *Salpiglossis*, the genes and biosynthesis mechanisms of acylsugars have remained elusive in tobacco.

Previous studies in *Solanaceae* species have shown that the branched chain amino acid (BCAA) metabolic pathway is the main source of acylsugar acyl chains, and enzymes belonging to the acyltransferase family (BAHD) catalyze the attachment of acyl chains to sugar cores to form different types of acylsugars. Based on the research progress of acylsugar biosynthesis, through gene homology comparison and transcriptional expression level detection, our research group previously obtained three genes with high expression in glandular trichomes from tobacco, including one acetolactate synthase gene (*NtALS1*, one of the key gene involved in BCAA metabolic pathway) and two acylsugar acyltransferase gene (*NtASAT1* and *NtASAT2*, belonging to BAHD family). In this thesis study, our research objective is to identify and characterize if these three genes are involved in the acylsugar biosynthesis of *N. tabacum*. The main results are as follows:

For *NtALS1*, tissue distribution analyses suggested that *NtALS1* was highly expressed in the tips of glandular trichomes. Subcellular localization analysis showed that the NtALS1 localized to the chloroplast. Moreover, in the wild-type K326 variety background, we generated two NtALS1 loss-of-function mutants using the CRISPR-Cas9 system. Acylsugars contents in the two NtALS1 mutants were signifcantly lower than those in the wild type. Through phylogenetic tree analysis, we found that each acylsugar-containing *Solanaceae* species possessed an ALS

homolog that clustered together with NtALS1, whereas the ALS homolog of *N. sylvestris* (one ancestor of *N. tabacum*), a species that does not contain acylsugars, did not cluster with NtALS1. Taken together, these findings indicate a functional role for NtALS1 in acylsugar biosynthesis in tobacco and also indicate that NtALS1 is likely from the ancestor *N. tomentosiformis*.

For *NtASAT1* and *NtASAT2*, Tissue and subcellular localization analyses showed that both genes were trichome-specific and functioned in the endoplasmic reticulum. In vitro, NtASAT1 could utilize sucrose and C2-, iC4-, and iC5-CoA substrates to produce monoacylsucroses S1:2 (acetyl-CoA), S1:4 (iC4-CoA) and S1:5 (iC5-CoA), respectively, with the highest activity when iC5 was used as the acyl-donor. NMR analysis of the purified mono-acylsucrose (S1:5) revealed that the acyl group attached to position R2 of sucrose. NtASAT2 catalyzes the attachment of iC5 acyl-donor to iC5 mono-acylsucrose producing bi-acylsucrose (S2:10). These results were consistent with the fact that iC5 is more frequently found as an acyl side-chain in cultivated tobacco. Knocking-out of *NtASAT1* or *NtASAT2* led to the deficiency of acylsucrose in tobacco, however, no effect on the acylsugar accumulation was observed in *NtASAT1* or *NtASAT2* overexpression plants. Genomic and acylsugar profile analysis revealed that *NtASAT1* and *NtASAT2* are likely originated from diploid ancestor *N. tomentosiformis* producing acylsugars. These results revealed that NtASAT1 and NtASAT2 are key enzymes involved in the acylsugar assembly in tobacco.

This work extends our understanding of acylsucrose biosynthesis in tobacco plants, and also provides new genes for further study on molecular regulatory mechanisms of acylsugar biosynthesis, for the molecular breeding of tobacco aroma quality and engineering of pathogen- and insect-resistant crops.

Keywords: *Nicotiana tabacum*, glandular trichomes, acylsugar biosynthesis, gene function identification, acetolactate synthase (ALS), acylsucrose acyltransferases (ASATs)

Acknowledgments

With the opportunity provided by the GSCAAS-Ulg Joint PhD Program and the passion for scientific research, I started the life mode of studying for a doctorate while working seven years ago. For me, it has been a long and fruitful academic journey, which has not only brought me many unforgettable moments and academic progress, but also enabled me to make many excellent colleagues and friends in scientific research. On this occasion, I would like to thank all those who contributed to the final completion of my doctoral thesis. Especially to:

My supervisors, Prof. Hervé Vanderschuren and Prof. Sébastien Massart from Belgium, and Prof. Yuanying Wang and Prof. Yongfeng Guo from China. All of them provided me with this valuable learning opportunity, which enabled me to cooperate with international researchers and broadened my horizon during this period. Their profound knowledge, rigorous scientific research attitude, guidance and constructive suggestions to my scientific research work have benefited me all my life.

I would also like to extend my thanks to the colleagues and master students I worked with in China. The list was put into alphabetical order, Aiguo Yang, Biao Chen, Caihong Jiang, Changqing Yang, Chenggang Luo, Chenying Li, Dan Liu, Huiqing Sun, Jianmin Cao, Lili Shen, Min Ren, Ming Chen, Quanfu Feng, Ruimei Geng, Shuai Chen, Weisong Yu, Xuhao Pan, Yafang Qu, Yanan Cao, Yiting Li, Yu Zhang, Zhiqiang Chen and Zhongyi Hu. It is all of your selfless help and support that enabled me to finish the work smoothly, as well as my doctoral study and thesis writing. Especially Prof. Chenggang Luo, you are my guide and staunch supporter in my work. Without your support and help, I could not have started my journey of doctoral study. Biao Chen, Ming Chen, Yafang Qu, Chenying Li, Yanan Cao and Jianmin Cao, Without your help and support in laboratory work, my doctoral research could not have been completed smoothly; There are also Prof. Aiguo Yang and Prof. Changqing Yang. Due to your guidance and help, the papers related to my doctoral research were successfully published. Thank you all for your help. What you have done for me will always be in my heart.

Many thanks to my colleagues in the Plant Genetics Lab of Gembloux Agro Bio-Tech, University of Liège. The list was put into alphabetical order, Briue Lecart, Emanoella Lima Soares, Ivan Jauregui, Izargi Vega Mas, Kumar Vasudevan, Leonard Shumbe, Sara Shakir, Shan e Ali Zaidi, Sok Lay Him Mercier. When I went to Belgium, my spoken and listening English was very poor. I was very upset when I first came to the lab. It was your kindness that made me feel the warmth of home. During the nine months of my stay in Belgium, no matter what difficulties I encountered in the lab, you were always there for me, giving unselfish help. There are too many unforgettable moments to list. The days I worked and played with you will be fond memories of my life.

For my Chinese partners who studied together in Belgium, Fengbo Li, Hejun Lu and Xiaoming Gao, I would like to thank you for guiding me how to apply for the ID card, train card, insurance and other related matters, and for helping me get familiar with supermarkets and the surrounding environment of the city when I first

arrived in Belgium. It eliminated my discomfort in a strange city. Wanying Hou and Xiaoxu Feng, Your coming brought us a lot of joy. I still remembered cooking and chatting with you in your rented house for many times. Thank you all, my dear friends. It was because of your company and encouragement that my work and study life in Belgium was full of motivation, and I no longer felt lonely.

Finally, I would like to give thanks to my husband, my son, my brothers and sisters, and all my relatives and friends. Your continuous love, understanding and unconditional support made me feel very happy and gave me the courage to face those difficult moments. Thank you for being there for me all the time. I love you all.

Sincere thanks and best wishes to all of you!

Aixia Chang

Tables of Contents

List of Figures

List of Tables

List of Abbreviations

Full Name	Abbreviate
Acyl-sugar acyl-COA synthase	AACS
Acyl-CoA synthetase	ACS
Acylsugar enoyl COA hydratase	AECH
Acylglucose acyltransferase	AGAT
Anthocyanin-O-hydroxycinnamoyltransferase	AHCT
Acetolactate synthase	ALS
Acylsucrose acyltransferases	ASATs
Acylsucrose fructofuranosidase	ASFF
Abbreviation for four types of acyltransferases (BEAT, AHCT, HCBT, DAT)	BAHD
Branched-chain amino acid	BCAA
Branched-chain fatty acids	BCFAs
Branched-chain ketoacid dehydrogenase	BCKD
Benzylalcohol-O-acetyltransferse	BEAT
Beinhart1000-1	BH
Bovine serum albumin	BSA
Bis(trimethylsilyl)trifluoroacetamide	BSTFA
Complementary DNA	cDAN
Coding sequence	CDS
Co-immunoprecipitation	Co-IP
Deacetylvindoline-4-O-acetyltransferase	DAT
7,12-dimethylbenz(a)anthracene	DMBA
N,N-Dimethylformamide	DMF
Endoplasmic reticulum	ER
Fatty acid synthase	FAS
Gas Chromatography-Mass Spectrometer	GC/MS
Glucose esters	GEs
Green fluorescent protein	GFP
Greenhouse whitefly	GHWF
Guide RNAs	gRNAs
Glandular trichomes	GTs
Anthranilate-N-hydroxycinnamoyl/benzoyltransferase	HCBT
Hongda	HD
Hydroxyethylthiamine pyrophosphate	HETPP
Heteronuclear Single Quantum Coherence	HSQC
Inositol esters	IEs

Isoleucine	ILEU
Isopropylmalate dehydrase	IPDS
Isopropylmalate dehydrogenase	IPMD
Isopropylmalate synthase	IPMS
Isopropyl *β*-D-1- thiogalactopyranoside	IPTG
β-Ketoacyl-ACP synthase	KAS
Ketoacid dehydrogenase	KD
Liquid Chromatography/Mass Spectrometer	LC/MS
Liquid Chromatography Quadrupole Time-of-flight Mass Spectrometry	LC-Q-TOF-MS
Leucine	LEU
Methyl	Me
2-(4-Morpholino)ethanesulfonic acid	MES
Nuclear Magnetic Resonance	NMR
Overexpression	OE
Open Reading Frame	ORF
Polymerase Chain Reaction	PCR
Real-time quantitative PCR	RT-qPCR
Straight-chain fatty acid	SCFA
Serinecarboxypeptidase-like	SCPL
Sucrose esters	SEs
Solanaceae Genomics Network	SGN
Small guide RNA	sgRNA
Sweet potato whitefly	SPWF
Triacylinositol acyltransferase	TAIAT
Threonine deaminase	TD
Threonine	THR
12-O-tetradecanoylphorbol-13-acetate	TPA
Ultra High Performance Liquid Chromatography/Mass Spectrometer	UHPLC/MS
Ultra-high-performance liquid chromatography-triple/time-of-flight mass spectrometry	UHPLC/TOF MS
Ultra High Performance Liquid Chromatography-High Resolution Mass Spectrometer	UHPLC-HR-MS
Valine	VAL
Wild type	WT
α-keto acid extension	α-KAE

1

Objective and Thesis Structure

Chapter One Objective and Thesis Structure

Glandular trichomes (GTs) are epidermal structures on the surface of many plants that synthesize and store or secrete a diverse set of specialized metabolites. Trichomes of some plants, especially within the *Solanaceae* family, secrete compounds called acylsugars (sugar esters) that are reported to have defense-related activity such as antifungal, insecticidal and antioxidant activities. In addition, they are considered to be the precursors of many important flavour components in tobacco, and their volatile breakdown products are believed to contribute to the aromatic and flavor properties of cured leaves of tobacco. Moreover, acylsugars have also a great potential as a new type of pesticides that degrade rapidly, as anti-inflammatory compounds and as antibiotics for cosmetic and food additives. So a more complete understanding of the mechanism of acylsugar synthesis and its regulation is necessary to increase their potential for further exploitation in plants, and which has long prompted interests in mining the genes involved in acylsugar synthesis and clarifying the metabolic pathways these genes belong to and how they regulate the biosynthesis of acylsugars.

Studies in *S. pennellii* and *N. benthamiana* suggested that the branched-chain amino acid (BCAA) metabolism pathway and α-keto acid elongation (α-KAE) or fatty acid synthase (FAS) route are the main sources of acyl chains of acylsugars. However, the above pathways involved in primary metabolites are present in various tissues throughout the plant in *Solanaceae* family, whereas, acylsugars are secondary metabolites that are synthesized only in GTs on the surface. Moreover, through transcriptomic comparison of trichomes and leaves without trichomes in *Nicotiana benthamiana* and tomato, multiple genes involved in BCAA metabolism and fatty acid chain elongation were specifically or highly expressed in GTs. Therefore, although the acylsugar acyl chain biosynthetic genes exert their functions through the primary metabolism pathways in GTs, these genes are distinct from those in other tissues. Aside from several genes such as SlIPMS3, E1-β subunit of BCKD, β-Ketoacyl-ACP synthase I/II/III (which are trichome-specific and involved in acylsugar acyl-chain biosynthesis), our current understanding of the molecular mechanisms and other genes involved in fatty acid chain biosynthesis of acylsugars remains poor.

Researches in *Solanum*, *Petunia,* and *Salpiglossis* showed that a group of enzymes known as acylsucrose acyltransferases (ASATs)，which belong to the acyltransferase family (BAHD), were responsible for acyl chains attaching to sugars. Although two acyltransferases (NbASAT1 and NbASAT2) have been found to contribute to the acylsucrose biosynthesis in *N. benthamiana* recently, their catalytic activities remain elucidative. In tobacco, the acylsugar structures are different from those in other *Solanaceae* species, but enzymes catalyzing the assembly of side-chains remain unknown.

Based the above research progresses, our research group have been working on mining the genes involved in tobacco acylsugar biosynthesis. Through transcriptome analysis of tobacco GTs (The results of this part were not present in this thesis.) and

gene homology comparison, we obtained several trichome-sepcific genes, including one acetolactate synthase (ALS) and two ASATs, which were predicted to be involved in acylsugar biosynthesis. Therefore, the objective of this thesis is to focus on the functional identification of the ALS (Chapter Three) and these two ASATs (Chapter Four).

This dissertation begins with a general introduction on acylsugars, further reviews the chemical structures, the biosynthesis and the bioactivity of *Solanaceous* acylsugars (Chapter Two). Then the function of NtASL1 was confirmed by using several methods, including real-time quantitative PCR (RT-qPCR) analysis, tissue and subcellular localization, and CRISPR-Cas9 technology. In addition, the source of NtALS1 and whether other acylsugar-containing *Solanaceae* crop species have NtALS1 homologs were further explored (Chapter Three). Following, two NtASATs were characterized *in vitro* and *in vivo* using enzyme activity tests, tissue and subcellular localization, gene knockout, and gene overexpression experiments. Further, the evolutionary origin of them were also investigated (Chapter Four). Finally, a conclusion and future prospects for the results above are proposed (Chapter Five).

2

General Introduction

Chapter Two General Introduction
Chemical Structure, Biosynthesis and Bioactivity of Sugar Esters in *Solanaceae* Crops

Abstract

Acylsugars (sugar esters) synthesized in the glandular trichomes (GTs) of *Solanaceae* plants are an important class of defensive secondary metabolites, and have anti-insect, antibacterial, anti-oxidation, and anti-dehydration properties. They also have many potential commercial uses. Different types and structures of acylsugars can result in significant differences in their biological activities. To date, the acylsugars identified from *Solanaceae* are primarily sucrose esters (SEs), glucose esters (GEs), and inositol esters (IEs). Although there are only three types, they have great structural diversity in Solanaceae due to differences in their sugar cores, acyl chain numbers , structures, and attachment positions on the sugar backbone. The structural diversity of *Solanaceae* acylsugars is primarily due to differences in their synthetic genes and molecular mechanisms. In this part, we briefly review the research progresses on the structural identification, biosynthesis and bioactivity of *Solanaceae* acylsugars, summarize the identified acylsugar structures, synthesis pathways and key genes identified in *Solanaceae* plants, and the identified bioactivities of *Solanaceae* acylsugars. This information will help promote the development, utilization, and future researches of *Solanaceae* acylsugars in crop breeding and commercialization.

Keywords

Solanaceae family; glandular trichomes; acylsugars; chemical structures; biosynthesis.

Introduction

Sugar esters (i.e. acylsugars) refer to the acyl glycosides formed by esterification of one or more hydroxyl groups on the sugar backbone (such as sucrose, glucose, and inositol) with acyl chains of different lengths and numbers (Arrendale et al., 1990; Ghosh et al., 2014; Kroumova et al., 2016; Fan et al., 2019). In plants, acylsugars were first discovered in *Solanaceae* glandular trichomes (GTs), and are typically considered defensive substances unique to *Solanaceae* plants (Huchelmann et al., 2017). However, in recent years, they have also been detected in several plant families, such as *Rosaceae* and *Caryophyllaceae*. (Fan et al., 2019; Liu et al., 2019). Acylsugars have insect resistance (Severson et al., 1984; Neal et al., 1994; Liu et al., 1996; Puterka et al., 2003; Simonovska et al., 2006; Jose Rodriguez-Lopez et al., 2012; Smeda et al., 2018), bactericidal (Cutler et al., 1986; Menetrez et al., 1990; Kennedy et al., 1992; Van Thi et al., 2017), antioxidant (Dufour et al., 2002; Niggeweg et al., 2004; Hoffmann et al., 2003), anti-dehydration (Fobes et al., 1985; Matsuzaki et al., 1989; Feng et al., 2021) and plant growth regulation (Severson et al., 1985; Y 1991) properties. Therefore, increasing acylsugar content has become an important breeding goal in tomatoes, potatoes, and other *Solanaceae* crops (Bonierbale et al., 1994; Lawson et al., 1997; Leckie et al., 2014; Leckie et al., 2012; Smeda et al., 2018). In tobacco, acylsugars are considered the precursors of many important flavor components, and their volatile breakdown products are believed to contribute to the aromatic and flavor properties of cured leaves of tobacco (Danehower 1987; Jackson et al., 1998; Ashraf-Khorassani et al., 2005; Gang et al., 2002; Arrendale et al., 1990). Additionally, acylsugars have a wide range of potential commercial uses in food additives, preservatives, emulsifiers, plasticizers, cosmetic ingredients, and pharmaceutical formulation ingredients (Perez et al., 2017; Lalot et al., 2004; Bazito and El Seoud 2001; Okabe et al., 1999; RicoLattes and Lattes 1997; Maugard et al., 1997). Differences in the type and structure of acylsugars in different *Solanaceae* plants can result in significant differences in their functions, such as insect resistance and bactericidal properties, making them promising prospects for the development and utilization of *Solanaceae* acylsugars (Puterka et al., 2003; Van Thi et al., 2017; Smeda et al., 2018). As such, a clear understanding of the structure of *Solanaceae* acylsugars and the molecular mechanism behind their synthesis and metabolism will allow for the breeding of *Solanaceae* crops with more useful acylsugars. Little was known about the synthesis and regulation of acylsugars over the last century due to technical limitations hindering the identification of their genes and chemical components. In recent years, the rapid development of chromatographic detection technology, molecular biology, and bioinformatics have facilitated breakthroughs in the identification of the structure and analysis of the synthesis mechanism of *Solanaceae* acylsugars (Pratap Singh et al., 2003; Schilmiller et al., 2015; Fan et al., 2016; Fan et al., 2017; Moghe et al., 2017; Lou et al., 2021). In recent years, Ghosh and Jones have reviewed various methods of acylsugar detection and structural identification, and Fan et al. have reviewed the evolution of the metabolic diversity of acylsugars in *Solanaceae*

(Ghosh et al., 2014; Fan et al., 2019). In this review, we summarize recent research progress on acylsugars in *Solanaceae* crops, including their structural identification, biosynthetic pathways, and key genes related to synthesis. This will provide researchers with a more comprehensive understanding of current research and future research directions on acylsugars of *Solanaceae*.

1 Diversity of Acylsugar Chemical Structures in *Solanaceae*

While the *Solanaceae* family includes more than 2700 species in 98 genera (Olmstead and Bohs, 2006), a limited number of *Solanaceae* genera and species have been tested for acylsugars. In these *Solanaceae* plants, the precise structures of some acylsugars have only been identified recently. Known acylsugars in *Solanaceae* plants generally consist of a sucrose, glucose, or inositol sugar backbone and acyl chains attached to different positions of the sugar core (Figure 2-1). They often contain 2 to 6 short to medium straight- or branched-chain aliphatic acyl groups, which are typically 2 to 12 carbon chains in length.

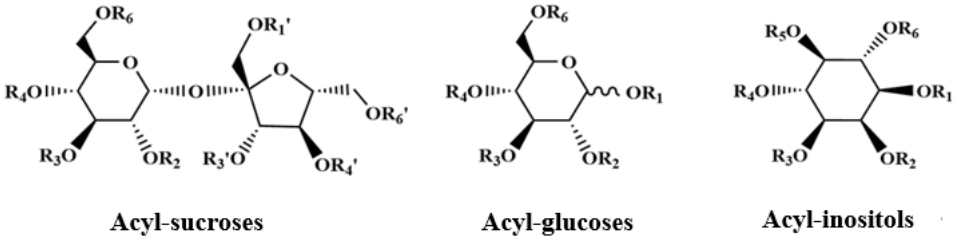


Figure 2-1 Structures of common acylsugars (Rn = H or acyl) in *Solanaceae* family

1.1 Acylsugars in Solanum

In *Solanum* genus, relatively in-depth studies on acylsugar structural identification have been conducted in *S. lycopersicum, S. pennellii, S. habrochaites*, *S. nigrum*, and *S. quitoense.* It has been reported that the primary acylsugars in wild *S. pennellii* species are triacylglucose with acyl chain lengths of C4 to C12 and some triacylsucroses. These acylsugars contain at least 13 different acyl chain structures. Recently, Lybrand et al. (2020) performed an untargeted metabolomic analysis of glandular trichomes (GTs) extracts from 16 *S. pennellii* cultivars from northern and southern Peru using UHPLC–HR–MS and NMR techniques and identified a total of 39 acylsugars, including 19 triacylglucoses, 18 triacylsucroses , and 2 newly identified tetraacylglucoses (Table 2-1). The acyl chains of the triacylsucroses, which are primarily composed of medium and short chains, are connected to the R2, R3, and R4 positions of the pyranose ring (the acylsugars whose acyl chains are all on the pyranose ring are called P-type acylsugars). The acyl chains of the Tetra-acylsugars are mainly short chains, and the specific connection positions of the acyl groups have not yet been identified.

Using UHPLC/MS technology, Ghosh et al. detected a total of 82

Tetraacylsucroses, 34 Triacylsucrosess, and 13 Pentaacylsucrosess in two *S. habrochaites* accessions (LA1777 and LA1392) and tomato (*S. lycopersicum* M82). As many as 11 isomers exist in each kind of acylsugar with the same molecular formula. These isomers are mainly determined by the length of the acyl chains, the position of the acyl chain branch, and the difference in the connection positions of the acyl chains on the sugar ring. No acylglucose were detected in the three varieties. 24 kinds of acylsugars were separated and purified and their structures were identified using NMR technology, including 15 tetraacylsucrosess, 7 triacylsucrosess, and 2 pentaacylsucrose. The acyl chain lengths of these acylsugars are between C2~C12 and are connected at different positions on the sucrose backbone (Table 2-2). Most acylsugars have one or two acyl chains on the furanose ring (these acylsucroses with acyl chains on the furanose ring are called F-type acylsugars) (Ghosh et al., 2014).

Lou et al. (2021) identified at least 45 acylsugars from *S. nigrum* using LC/MS, GC/MS, and 2D NMR techniques. These acylsugars are primarily acylhexoses with 39 acylglucoses and 6 acylinositols. These acylsugars are primarily diacylglucose, triacylglucose and triacylinositols (Table 2-3). In triacylglucosess, a C8-C10 acyl chain is attached to the R4 position, a C4-C5 acyl chain is attached to the R3 position, and an acetyl group is attached to the R2 position. In diacylglucoses, a medium-length acyl chain is attached to R4 and a shorter acyl chain is attached to R3. In triacylinositols, a short acyl chain is attached to each of the R2 and R3 positions, and a medium-length acyl chain is attached to the R4 position.

Leong et al.(2020) assessed the acylsugar types and structures of *S. quitoense* and demonstrated that this species contains abundant acyl-inositol monosaccharides and a small amount of acyl-inositol disaccharides. Of them, the acyl-inositol monosaccharides are primarily triacylinositols and tetraacylinositolss (Table 2-3), whichcontain 1 or 2 acetyl groups and 2 C10 or C12 acyl chains. However, the precise positions of C10 and C12 on the inositol ring need to be determined by further research.

Table 2-1 Annotations of acylsugars in *S. pennellii.*

Triacylsucroses	Triacylglucoses	Tetraacylglucoses
S3:12(4,4,4)	G3:12(4,4,4)	G4:14(2,4,4,4)
S3:13(4,4,5)	G3:13(4,4,5)	G4:15(2,4,4,5)
S3:14(4,5,5)	G3:14(4,5,5)	
S3:15(5,5,5)	G3:15(5,5,5)	
S3:16(5,5,6)	G3:16(5,5,6)	
S3:16(4,4,8)	G3:16(4,4,8)-1	
S3:17(4,5,8)	G3:16(4,4,8)-2	
S3:17(4,4,9)	G3:17(4,5,8)-1	
S3:18(4,4,10)-1	G3:17(4,5,8)-2	
S3:18(4,4,10)-2	G3:18(4,4,10)-1	
S3:19(4,5,10)-1	G3:18(4,4,10)-2	
S3:19(4,5,10)-2	G3:19(4,5,10)-1	
S3:20(5,5,10)	G3:19(4,5,10)-2	
S3:20(4,4,12)	G3:20(5,5,10)	
S3:21(5,5,11)	G3:20(4,4,12)	
S3:21(4,5,12)	G3:21(5,5,11)	
S3:22(5,5,12)	G3:21(4,5,12)	
S3:23(5,6,12)	G3:22(5,5,12)	
	G3:23(5,6,12)	

Acylsugar nomenclature is as follows: the first letter indicates the sugar core (“S” for sucrose, “G” for glucose); the number before the colon indicates the number of acyl chains; the number after the colon indicates the sum of carbons in all acyl chains; the numbers in parentheses indicate the number of carbons in individual acyl chains. (Lybrand et al., 2020).

Table 2-2 NMR-elucidated structures of acylsugars purified from leaf dip extracts of *S. habrochaites* and *S. lycopersicum* M82

Acylsugar[a]	Acyl group at position						Metabolite abundances		
	R_2	R_3	R_4	R_1'	R_3'	R_6'	*S. lycopersicum* M82	*S. habrochaites* LA1392	*S.habrochaites* LA1777
Triacylsucroses									
S3:19	H	iC4	iC5	H	iC10	H	+	++	+++
S3:19	iC4	iC10	iC5	H	H	H	ND	++	ND
S3:20	H	aiC5	iC5	H	iC10	H	+	+++	ND
S3:21	H	aiC5	iC5	H	aiC11	H	ND	++	+
S3:21	iC5	aiC11	iC5	H	H	H	+	++	++
S3:22	H	nC12	iC5	H	iC5	H	++	+++	++
S3:22	aiC5	nC12	iC5	H	H	H	ND	+++	+
Tetraacylsucroses									
S4:16	C2	iC4	iC5	H	iC5	H	++	++	+++
S4:17	C2	aiC5	iC5	H	iC5	H	+++	+++	+++
S4:19	iC4	iC5	iC5	H	H	iC5	ND	++	++
S4:20	iC5	iC5	iC5	H	H	iC5	ND	+++	+
S4:20	C2	iC4	iC4	H	iC10	H	++	++	+++
S4:21	C2	iC4	iC5	H	iC10	H	++	+++	+++
S4:22	C2	aiC5	iC5	H	iC10	H	+	+++	+++
S4:22	C2	iC4	iC5	H	aiC11	H	ND	++	++
S4:22	C2	iC4	iC4	H	nC12	H	+	+	+++

S4:23	C2	aiC5	iC5	H	aiC11	H	ND	++	++
S4:23	C2	iC4	iC5	H	iC12	H	+	++	++
S4:23	C2	iC4	iC5	H	nC12	H	+	++	+++
S4:24	C2	aiC5	iC5	H	iC12	H	+	++	++
S4:24	C2	aiC5	iC5	H	nC12	H	+	++	++
S4:24	C2	nC12	iC5	H	iC5	H	++	++	++
Pentaacylsucroses									
S5:24	aiC5	iC4	iC5	iC5	H	iC5	ND	+++	++
S5:25	iC5	iC5	iC5	iC5	H	iC5	ND	+++	++

Acylsugar nomenclature is as follows: the first letter indicates the sugar core ("S" for sucrose, "G" for glucose); the number before the colon indicates the number of acyl chains; the number after the colon indicates the sum of carbons in all acyl chains. Relative abundance indicators were generated by using the formula Relative Abundance (X) = (peak area * 100/dry weight). Indicators +++, ++, + and ND were used when X > 1000, 1000 > X > 100, 100 > X > 1 and X < 1 respectively. ND = not detected

[a] C2 = acetate, iC4 = $(CH_3)_2CHCO$, iC5 = $(CH_3)_2CHCH_2CO$, aiC5 = $CH_3CH_2(CH_3)CHCO$, iC10 = $(CH_3)_2CH(CH_2)_6CO$, aiC11 = $CH_3CH_2(CH_3)CH(CH_2)_6CO$, iC12 = $(CH_3)_2CH\ (CH_2)_8CO$, nC12 = $CH_3(CH_2)_9CO$.(Ghosh et al., 2014).

Table 2-3 Acylsugars annotated with high confidence in S. nigrum and *S. quitoense*.

S. nigrum			***S. quitoense***	
Diacylglucoses	**Triacylglucoses**	**Triacylinositols**	**Triacylinositols**	**Tetraacylinositols**
G2:12(4,8)	G3:14(2,4,8)	I3:16(4,4,8)	I3:22 (2,10,10)	I4:24 (2,2,10,10)
G2:13(5,8)	G3:15(2,5,8)	I3:17(4,5,8)	I3:24 (2,10,12)	I4:26 (2,2,10,12)
G2:13(4,9)	G3:15(2,4,9)	I3:17(4,4,9)		
G2:14(5,9)	G3:16(2,5,9)			
G2:14(4,10)	G3:16(2,4,10)			
G2:15(5,10)	G3:17(2,5,10)			
G2:18(8,10)				

Acylsugar nomenclature is same as Table 2-1 (“G” for glucose, “I” for inositol). (Leong et al., 2020; Lou et al., 2021).

1.2 Acylsugars in Petunia

In the 1990s, several studies profiling acylsugars in *Petunia* plants showed that the surface secretions of *Petunia* hybrids contained tri- and tetra-acylglucoses, and tri-, tetra- and penta-sucroses (Chortyk et al., 1997; Dieter Moser 1999). In 2016, Kroumova et al. used GC/MS technology to study the acylsugar acyl chains of five petunia hybrids and found that the main acyl chains were malonic acid and C2-C8 straight-chain fatty acids (Kroumova et al., 2016). In 2017, Liu et al. used UHPLC/TOF MS technology to detect the acylsugar phenotype of three species of *Petunia* (*P. integrifolia*, *P. exserta*, and *P. axillaris*) and identified 37 acylsugars with different molecular weights, including 2 tri-acylsucroses, 16 tetra-acylsucroses, and 19 penta-acylsucroses, exceeding a total of 100 acylsugar structures. Using separation, purification, and NMR techniques, the structures of 28 acylsugars were identified (Table 2-4), including 13 tetra-acylsucrosess and 15 penta-acylsucroses. Of these, four acyl chains in 13 tetra-acylsucroses were respectively linked to R2, R3, R4, R6 positions of the pyranose ring, and the acyl chain length ranged from C4 to C8. Of the 15 penta-acylsucroses, four C4 to C7 acyl chains are respectively attached to the R2, R3, R4, and R6 positions of the pyranose ring, and one malonate acyl chain is attached to the R1’ or R6’positions of the furanose ring. The three petunia species are rich in acylsugars with malonate acyl chains (Liu et al., 2017).

Table 2-4 NMR elucidated structures of acylsugars purified from leaf extracts of *P. integrifolia*, *P. exserta* and *P. axillaris.*

Acylsugar[a]	Acyl group at position						Metabolite abundances[b]		
	R_2	R_3	R_4	R_6	R_1'	R_6'	*P. integrifolia*	*P. exserta*	*P. axillaris*
Neutral acylsucroses									
S4:19	aiC5	iC5	aiC5	iC4	H	H	ND	ND	+
S4:20	aiC5	iC5	aiC5	aiC5	H	H	+	+	+
S4:20	aiC5	aiC6	aiC5	iC4	H	H	+	+	+
S4:21	iC6	aiC5	aiC5	aiC5	H	H	+	+	+
S4:21	aiC5	aiC6	aiC5	aiC5	H	H	+	+	+
S4:21	aiC5	iC6	aiC5	aiC5	H	H	++	++	++
S4:21	aiC5	iC6	aiC5	iC5	H	H	++	+	++
S4:22	aiC5	iC7	aiC5	aiC5	H	H	+++	+++	++
S4:22	aiC5	iC7	iC5	aiC5	H	H	++	++	ND
S4:22	aiC5	iC8	aiC5	iC4	H	H	++	++	+
S4:23	aiC5	iC8	aiC5	aiC5	H	H	+	++	++
S4:24	iC6	iC8	aiC5	aiC5	H	H	ND	+	+
S4:24	iC8	aiC6	aiC5	aiC5	H	H	++	++	+
Malonate esters									
S(m)5:23	aiC5	iC5	aiC5	aiC5	malonyl	H	++	+	++
S(m)5:23	aiC5	iC6	aiC5	iC4	malonyl	H	ND	ND	++
S(m)5:24	aiC5	iC6	aiC5	aiC5	H	malonyl	ND	+	ND
S(m)5:24	iC4	iC7	aiC5	aiC5	malonyl	H	++	+	+
S(m)5:24	aiC5	iC7	iC4	aiC5	malonyl	H	++	+	+

S(m)5:24	iC5	iC7	iC4	aiC5	malonyl	H	+	+	++
S(m)5:24	aiC5	iC6	aiC5	aiC5	malonyl	H	+	+	++
S(m)5:24	aiC5	aiC6	aiC5	aiC5	malonyl	H	+	+	++
S(m)5:25	aiC5	iC7	aiC5	aiC5	H	malonyl	ND	+++	ND
S(m)5:25	aiC5	iC5	iC7	aiC5	malonyl	H	+++	+	ND
S(m)5:25	aiC5	iC7	aiC5	aiC5	malonyl	H	+++	++	++
S(m)5:25	aiC5	iC8	aiC5	iC4	malonyl	H	+	+	++
S(m)5:26	aiC5	iC8	aiC5	aiC5	H	malonyl	ND	++	ND
S(m)5:26	aiC5	iC8	aiC5	aiC5	malonyl	H	ND	++	ND
S(m)5:27	aiC5	iC7	aiC5	iC7	malonyl	H	+	+	ND

ND not detected

[a] The nomenclature of acylsugars is as follows: "S" refers to the sucrose core, and the letter "m" in parentheses identifies compounds containing a malonate ester. The first number indicates the number of acyl groups attached to the sugar core, and the number following the colon represents the total number of carbons in acyl chains. *iC4* = $(CH_3)_2CHCO$, iC5 = $(CH_3)_2CHCH_2CO$, aiC5 = $CH_3CH_2(CH_3)CHCO$, iC6 = $(CH_3)_2CHCH_2CH_2CO$, aiC6 = $CH_3CH_2(CH_3)CHCH_2CO$, iC7 = $(CH_3)_2CHCH_2CH_2CH_2CO$, iC8 = $(CH_3)_2CHCH_2CH_2CH_2CH_2CO$.

[b]Acylsugar abundance of acylsugars in each species were calculated as relative abundance (X), similar to that of Table 1. (Liu et al., 2017).

1.3 Acylsugars in Nicotiana

The genus *Nicotiana* contains 76 species. According to previously published reports, the main types of sugar esters identified in *Nicotiana* species are acylsucroses and acylglucoses (Severson et al., 1985; Ghangas and Steffens 1993; Haliński Ł and Stepnowski 2013).

To date, only tetra-acylsucroses were detected in *N. tabacum*. These acylsugars include a series of isomers and are divided into 6 types (sucrose esters I-VI), each of which differs by several methylene groups in molecular weight. Each type of isomer is connected to an acetyl group on the R6 position, and three C3 to C8 straight- or branch-fatty acids are respectively attached to the R2, R3, and R4 positions of the pyranose ring. Sucrose ester types III-VI (i.e., 3-methylvaleryl-containing acylsugars) contain at least one 3-methylvaleryl group at the R2, R3, and R4 positions of the pyranose ring (Severson et al., 1985; Vontimitta et al., 2010). The types and content of tetra-acylsucroses in different types of tobacco differ. Studies have demonstrated that flue-cured, burley, and Maryland tobacco contain a small amount of sucrose esters I and II, and trace (or undetectable) of sucrose ester types III-VI. However, most oriental and many cigar tobaccos contain large amounts of sucrose esters III-VI types. *N. tomentosiformis*, one of the ancestors of *N. tabacum*, contains type I-VI acylsugars (Vontimitta et al., 2010), which are similar to those of *N. tabacum*. However, no sugar esters were detected in the other ancestor *N. sylvestris* (Moghe et al., 2017).

Acylsugars in *N. glutinosa* (Arrendale et al., 1990) are mainly tetra- and tri-acylsucroses. The R2, R3, and R4 positions of the pyranose ring of these acylsugars are connected with the C3 to C8 acyl chains, and the R3' position of the furanose ring of the tetra-acylsucrosess is also attached with an acetyl group. This is different from the tetra-acylsucroses in *N. tabacum*.

N. rustica acylsuagrs vary widely among species. In some varieties, no acylsugars were detected (Ghangas and Steffens 1993), but in *N. rustica* cv. Machorka Brazylijska, tri-, tetra- and penta-acylsucroses, tri-acylglucoses, and small amounts of diacylglucose were detected. In sucrose esters, three C4 to C8 acyl chains are attached to the pyranose ring, and 0, 1, or 2 acetyl groups are attached to the furanose ring. The main acyl chain length of triacylglucose is C5 to C7, and the total number of carbon atoms in the acyl chain is 16 to 19. However, the specific position of these acylsugar acyl chains has not yet been identified (Haliński Ł and Stepnowski 2013).

Table 2-5 Annotations of acylsugars in *N.alata* and *N. benthamiana*

N.alata white			***N. benthamiana***	
Tri-acylsucroses	**Tetra-acylsucroses**	**Penta-acylsucroses**	**Bi-acylsucroses**	**Tri-acylsucroses**
S3:18(5,5,8)	S4:18(2,8,8,8)	S5:23(2,2,6,6,8)	S2:15(7,8)	S3:17(2,7,8)

S3:19(5,6,8)	S4:18(2,3,5,8)	S5:23(2,2,5,6,8)	S2:16(8,8)	S3:18(2,8,8)
S3:20(6,6,8)	S4:19(2,5,5,7)	S5:24(2,2,4,8,8)		
S3:20(4,8,8)	S4:19(2,4,5,8)	S5:24(2,2,5,8,8)		
S3:21(5,8,8)	S4:20(2,5,5,8)	S5:25(2,2,6,8,8)		
S3:23(7,8,8)	S4:21(2,5,6,8)	S5:28(2,2,8,8,8)		
S3:24(8,8,8)	S4:22(2,4,8,8)			
	S4:23(2,5,8,8)			
	S4:24(2,6,8,8)			

Acylsugar nomenclature is same as Table 2-1. (Mihaylova-Kroumova et al., 2020; Feng et al., 2021).

N. benthamiana contains di- and tri-acylsucroses. Each acylsugar type contains two C7 or C8 acyl chains, and tri-acysucroses also contain an acetyl group (Feng et al., 2021). *N. alata* mainly contains tri-, tetra-, and penta-acylsucroses, as well as C2 to C8 acyl chains (Table 2-5). However, the attachment positions of different acyl chains in acylsugars of *N. benthamiana* and *N. alata* remain unknown (Mihaylova-Kroumova et al., 2020).

A recent study showed that there are over 300 unique acylsugars throughout *Nicotiana*. Of them, *N. acuminata* contained 21 and *N. forgetiana* contained 19 annotated acylsugars. Sucrose and (or) glucose cores were detected in different *Nicotiana* species, and no acyl chains longer than C8 was detected (Schenck et al., 2022).

1.4 Acylsugars in Salpiglossis, Physalis, Hyoscyamus, Iochroma, and Atropa Genera.

In 2017, Moghe et al. detected acylsugar types in *Salpiglossis sinuata*, *Physalis alkekengi*, *Physalis viscosa*, *Hyoscyamus niger*, *Iochroma cyaneum*, and *Atropa belladonna* using LC/MS-CID (Table 2-6), all of which were detected as sucrose esters. However, the types of sucrose esters vary significantly across species. *Salpiglossis sinuata* has more than 300 detected sugar ester peaks, including tri-, tetra-, penta-, and hexa-acylsucroses, with C2 to C6 acyl chains. Di-, tri-, and tetra-acylsucroses were detected in *Physalis alkekengi*, containing C4 to C6, C8, and C9 acyl chains, and abundant in C6 acyl chains. Only tri- and tetra-acylsucroses were detected in *Physalis viscosa*, with C3 to C6, C8 to C10, and C12 acyl chains, and small contents of C6 acyl chains. *Hyoscyamus niger* primarily has tri- and tetraacylsucroses, and the acyl chains are C2, C4, C5, and C8 to C12. *Iochroma cyaneum* is primarily tria- and tetraacylsucroses, and the acyl chains are C4 to C6, C8, and C10 to C12. Only triacylsucroses were detected in *Atropa belladonna*, with C3 to C5 and C8 to C12 acyl chains (Moghe et al., 2017).

Table 2-6Annotations of acylsugars in *Salpiglossis, Physalis, Hyoscyamus, Iochroma* and *Atropa genera.*

Salpiglossis	***Physalis***		***Hyoscyamus***	***Iochroma***	***Atropa***
S.sinuata	***P.alkekengi***	***P.viscosa***	***H.niger***	***I.cyaneum***	***A.belladona***
S3:16(5,6,6)	S2:12(4,8)	S3:18(4,4,10)	S3:17(4,5,8)	S3:13(4,4,5)	S3:14(4,5,5)
S3:16(2,5,5,6)	S2:12(6,6)	S3:19(4,5,10)	S3:18(5,5,8)	S3:14(4,5,5)	S3:17(3,5,9)
S3:18(6,6,6)	S2:13(5,8)	S4:20(4,4,4,8)	S3:19(4,5,10)	S3:15(5,5,5)	S3:18(5,5,8)
S4:16(2,3,5,6)	S2:14(6,8)	S4:21(4,4,5,8)	S3:19(5,5,9)	S3:16(5,5,6)	S3:18(3,5,10)
S4:17(2,5,5,5)	S2:16(8,8)	S4:21(4,4,4,9)	S3:20(4,5,11)	S3:17(4,5,8)	S3:19(5,5,9)
S4:17(2,4,5,6)	S3:16(5,5,6)	S4:21(3,4,4,10)	S3:20(5,5,10)	S3:18(5,5,8)	S3:19(3,5,11)
S4:17(2,3,6,6)	S3:17(4,5,8)	S4:22(4,4,5,9)	S3:21(4,5,12)	S3:19(4,5,10)	S3:20(4,5,11)
S4:18(2,4,6,6)	S3:17(5,6,6)	S4:22(4,4,4,10)	S3:21(5,5,11)	S3:20(5,5,10)	S3:20(5,5,10)
S4:19(2,5,6,6)	S3:18(4,6,8)	S4:23(4,10,10)	S3:22(5,5,12)	S3:21(4,5,12)	S3:20(3,5,12)
S4:20(3,5,6,6)	S3:18(6,6,6)	S4:23(4,4,5,10)	S3:23(5,5,13)	S3:21(5,5,11)	S3:21(4,5,12)
S4:20(2,6,6,6)	S3:19(5,6,8)	S4:23(4,5,5,9)	S4:15(2,4,4,5)	S3:22(5,5,12)	S3:21(5,5,11)
S4:21(4,5,6,6)	S3:20(6,6,8)	S4:24(4,5,5,10)	S4:16(2,4,5,5)	S4:24(4,5,5,10)	S3:22(5,5,12)
S4:21(3,6,6,6)	S3:21(5,8,8)	S4:25(4,5,6,10)	S4:17(2,5,5,5)	S4:25(5,5,5,10)	
S4:21(5,5,5,6)	S3:22(6,8,8)	S4:25(4,4,5,12)	S4:18(2,4,4,8)	S4:25(4,4,5,12)	
S4:22(5,5,6,6)	S3:23(6,8,9)	S4:26(4,5,5,12)	S4:19(2,4,5,8)	S4:26(4,5,5,12)	
S4:22(4,6,6,6)	S4:22(4,6,6,6)	S4:26(4,4,8,10)	S4:20(2,4,5,9)	S4:27(5,5,5,12)	
S4:23(5,6,6,6)	S4:23(4,5,6,8)	S4:27(4,4,9,10)	S4:20(2,4,4,10)		
S4:24(6,6,6,6)	S4:24(4,6,6,8)	S4:28(4,4,10,10)	S4:20(2,5,5,8)		
S5:19(2,2,4,5,6)	S4:24(4,4,8,8)		S4:21(2,5,5,9)		

S5:20(2,2,5,5,6)	S4:25(5,6,6,8)	S4:21(2,4,5,10)
S5:20(2,2,4,6,6)	S4:25(4,5,8,8)	S4:22(2,5,5,10)
S5:21(2,2,5,6,6)	S4:26(4,6,8,8)	S4:23(2,5,5,11)
S5:22(2,3,5,6,6)	S4:26(6,6,6,8)	S4:23(2,4,5,12)
S5:23(2,5,5,5,6)	S4:27(5,6,8,8)	S4:24(2,5,5,12
S5:23(2,4,5,6,6)	S4:28(6,6,8,8)	
S5:24(2,5,5,6,6)		
S5:25(2,5,6,6,6)		
S5:26(2,6,6,6,6)		
S6:25(2,2,5,5,5,6)		
S6:26(2,2,5,5,6,6)		
S6:27(2,2,5,6,6,6)		

Acylsugar nomenclature is same as Table 2-1. (Moghe et al., 2017).

2 Acylsugar Biosynthesis of *Solanaceae*

Solanaceae acylsugars are primarily synthesized in the head cells of secretory GTs, which are mainly formed by the esterification of carboxyl groups of fatty acids of different chain lengths with hydroxyl groups on sucrose, glucose, or inositol. Therefore, the biosynthesis pathway of *Solanaceae* acylsugars primarily includes two parts: one is the synthesis of the acyl chain, while the other is the assembly of the acyl chain and the sugar core to form acylsugar.

2.1 Biosynthetic Pathway and Key Genes of Acylsugar Acyl Chains

The *Solanaceae* acylsugar acyl chains are primarily branched or straight chains of medium and short-length fatty acids. Previous studies have demonstrated that the plastid-located branched-chain amino acid (BCAA, i.e. leucine, valine, and isoleucine) metabolic pathway is a major source of ketoacid precursors for the synthesis of short acyl chains of acylsuagrs (Kandra and Wagner 1988; Kandra et al., 1990; Walters and Steffens 1990; Slocombe et al., 2008; Mandal et al., 2020). These short acyl chains serve as precursors, which are further extended to generate longer acyl chains. In *Solanaceae* crops, the extension of acylsugar acyl chains (such as fatty acid chains) has two different mechanisms: the fatty acid synthase extension pathway (FAS) and α-keto acid extension pathway (α-KAE) (van der Hoeven and Steffens 2000; Kroumova and Wagner 2003; Slocombe et al., 2008). The production of longer acyl chains of acylsugars in tomato and *Datura* mainly occurs through the two-carbon elongation pathway mediated by fatty acid synthase (FAS). In several tobacco varieties and petunia, longer acyl chains are primarily produced by the α-keto acid (α-KAE)-mediated one-carbon elongation pathway, which requires isopropylmalate synthase (IPMS), isopropylmalate dehydrogenase (IPMD), and isopropylmalate dehydrase (IPDS) to catalyze cycles of elongation reactions (in primary metabolism, these elongation reactions are associated with leucine synthesis, however, the choice of substrates is significantly broader in acylsugar acyl chain biosynthesis). Threonine deaminase (TD) and acetolactate synthase (ALS) are required to provide branched-chain ketoacid precursors in both acyl chain elongation mechanisms (Ghangas and Steffens 1993; Moghe et al., 2017; Binder et al., 2007). The various branched or straight-chain ketoacids provided in the above pathways generate branched or straight-chain acyl-CoAs through branched or straight-chain ketoacid dehydrogenase (KD) (Figure 2-2). Additionally, the latest research shows that the acyl-sugar acyl-COA synthase (Sl-AACS1) and acylsugar enoyl COA hydratase (Sl-AECH1) are specifically expressed in the head cells of tomato GTs and are involved in the synthesis of medium-length acyl-COAs. These acyl-CoAs can serve as donor substrates for acylsugar synthesis (Fan et al., 2020).

Although it is generally accepted that acylsugar acyl chains are primarily derived from BCAA metabolic pathways, these pathways belong to primary metabolites that are present in tissues throughout the plant, while acylsugars belong to secondary metabolites that are only synthesized in the head cells of GTs on the surface of

Solanaceae plants. Therefore, genes involved in the synthesis of the acyl chain of acylsugars could differ from the genes involved in the primary metabolism of BCAAs in other tissues. Studies have demonstrated that *Sl-IPMS3*, which is specifically expressed in GTs of cultivated tomatoes, remains active after losing 160 amino acids at the C-terminus, but becomes insensitive to feedback inhibition by leucine, resulting the composition of acylsugar acyl chains in the cultivated tomato *S. lycopersicum* is quite different from that in the wild species *S. pennellii* (Ning et al., 2015). The transcriptome analysis of *N. benthamiana* and *S. pennellii* GTs and leaves without GTs demonstrated that multiple genes involved in BCAA metabolism and acyl chain extension were highly or specifically expressed in GTs. The E1-β subunit of branched ketoacid dehydrogenase (BCKD), which is specifically highly expressed in GTs, is silenced in *Nicotiana benthamiana* or *S. pennellii* plants by VIGS, which demonstrates that BCKD contains this E1-β subunit and is involved in the synthesis of branched-chain acyl-COA donors for acylsugar assembly. Silencing the highly expressed *KAS I* gene in the GTs of tomato plants showed that these different KAS I isoforms were involved in elongating tomato acylsugar acyl chains (Slocombe et al., 2008). In addition to KAS I, other GT-specific highly expressed KAS enzymes (such as KAS II and KAS III) were also demonstrated to be involved in straight-chain fatty acid (SCFA) synthesis (Mandal et al., 2020). Therefore, although the acylsugar acyl chain-precursor biosynthetic genes that are highly or specifically expressed in GTs exert their functions through the BCAA metabolism pathway, and these genes are distinct from those involved in BCAA biosynthesis in other tissues. Moreover, whether it is fatty acid synthase or α-KAE-mediated chain extension pathways, there are questions of substrate specificity and the need to identify the corresponding specific genes (Slocombe et al., 2008). To date, only a few genes have been identified, and most genes involved in the synthesis of aclysugar acyl chains remain poorly understood.

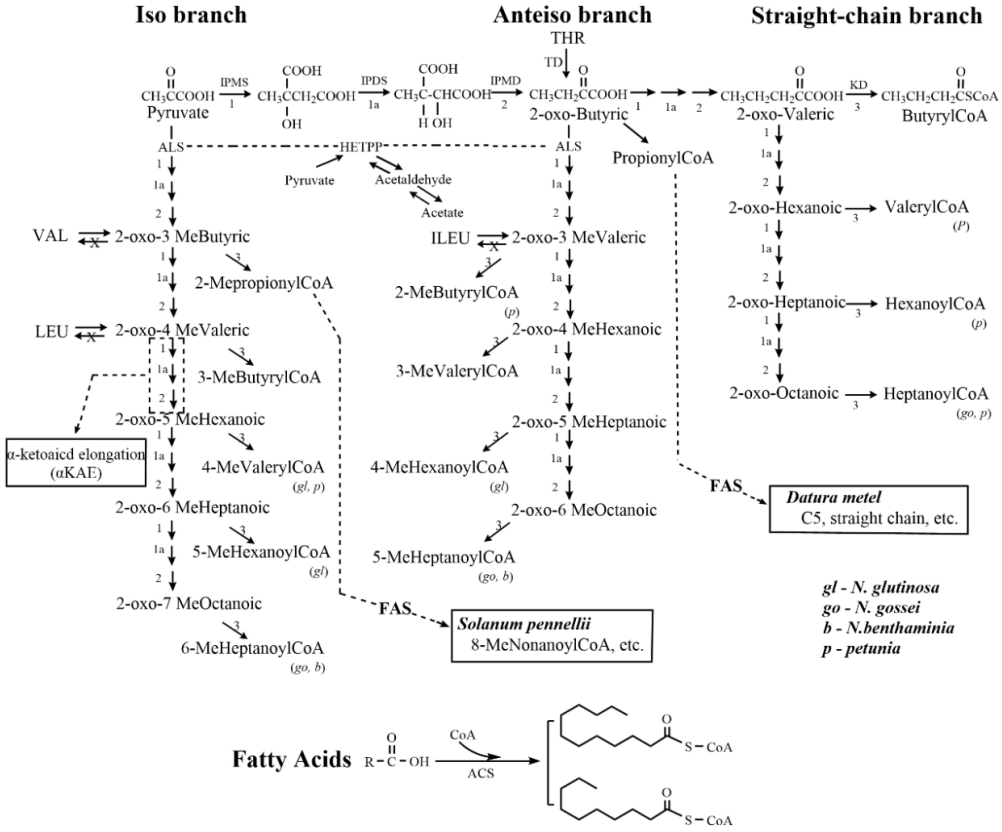


Figure 2-2 Pathways for the synthesis of valine (VAL), leucine (LEU) and isoleucine (ILEU), one-carbon elongation of 2-oxo-acid intermediates via α-KAE in *Petunia* × *hybrida* and *Nicotiana* species and two-carbon elongation via FAS in *Lycopersicon pennellii* and *Datura metel*. Enzyme reactions catalyzing reactions 1, 1a, 2 and 3 are assumed to be isopropylmalate synthase (IPMS), isopropylmalate dehydratase (IPDS), isopropylmalate dehydrogenase (IPMD) and ketoacid dehydrogenase（KD）, respectively. Letters in parentheses following various CoA products denote principal acyl groups found in SEs of different tobaccos and petunia (e.g. *b* for *N. benthamiana*, etc.). ALS Acetolactate synthase; TD Threonine dehydratase; HETPP hydroxyethylthiamine pyrophosphate; Me methyl; THR threonine; ACS Acyl-CoA synthetase (Kroumova and Wagner 2003; Fan et al., 2020).

2. 2 Assembly Genes and Pathways of Solanaceae Acylsugars

How acyl-CoAs, the active substrates produced in BCAA or acyl chain extension pathways, connect with sugar core to form acylsugars has been studied in recent years and has made great progresses in several *Solanaceae* crops. Studies have demonstrated that the trichome-specific acylsugar acyltransferases (ASATs) are the key enzymes that catalyze the connection between the acyl chains and sugar backbones. The homologous ASAT sequences of different *Solanaceae* crops are

different, and their catalytic acyl chain, sugar core and connecting position on sugar core are also different, which results in large structural differences in acylsugars across *Solanaceae* crops. Additionally, some genes derived from primary metabolism are involved in the synthesis of acylsugars after new functionalization and spatiotemporal specificity, which also contributes to the structural differences found in acylsugars. The assembly pathways of *Solanaceae* acylsugars are summarized in Figure 2-3.

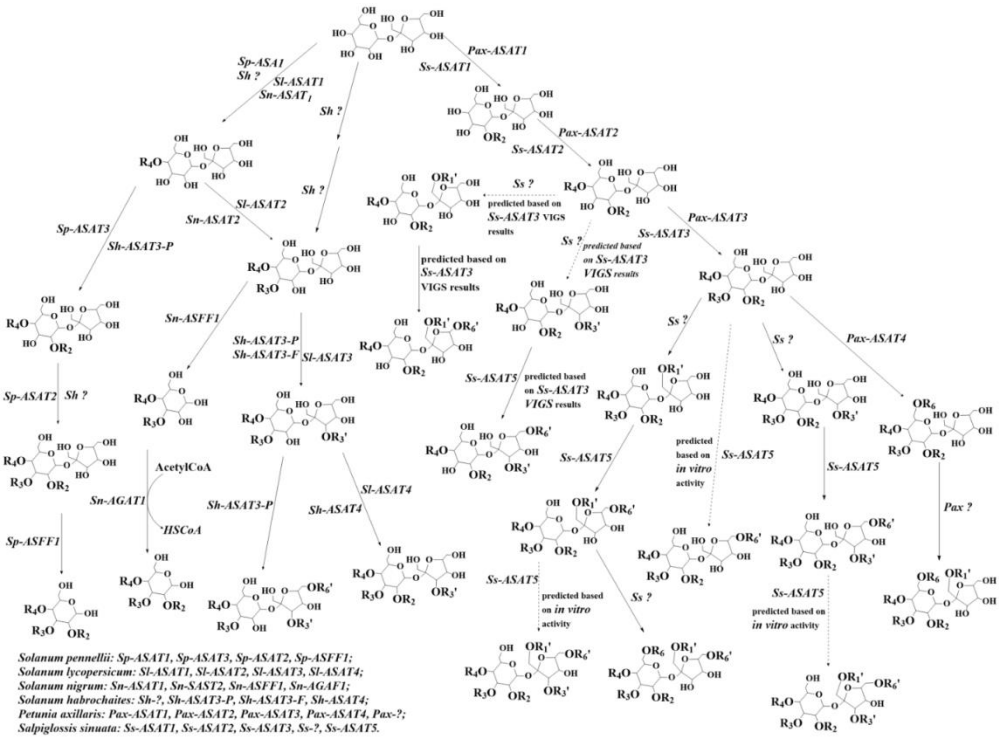


Figure 2-3 Biosynthetic pathways of *Solanaceae* acylsugars

(Schilmiller et al., 2015; Fan et al., 2016; Moghe et al., 2017; Nadakuduti et al., 2017; Fan et al., 2017; Lou et al., 2021)

2.2.1 Assembly Genes and Pathways of Solanaceae Acylsucroses

In *S. lycopersicum,* acylsugar acyltransferases SlASAT1-SlASAT4 can catalyze the esterification of acyl-CoA substrates with the R4, R3, R3', and R2 hydroxyl groups of the sucrose ring to form tetraacylsucroses in *S. lycopersicum* (Figure 2-3). Of them, SlASAT1 exhibits catalytic activity on iC4-, iC5-, aiC5-, nC10-, nC12-CoAs; SlASAT2 exhibits catalytic activity on iC4-, iC5-, aiC5-, nC10-, and nC12-CoAs (iC5-COA as a substrate has a small amount of product, indicating that the acyl substrate is not the preferred substrate of SlASAT2); SlASAT3 exhibits catalytic activity for C2-, iC4-, iC5- and aiC5-CoAs (Schilmiller et al., 2015;

Schilmiller et al., 2016; Fan et al., 2016), but cannot utilize longer nC10- and nC12-CoA acyl chains; and SlASAT4 catalyzes the formation of tetraacylsucrose from triacylsucrose and C2-CoA. Studies have demonstrated that the mutation of a few bases of tomato acyltransferase can change its catalytic substrate.

The sucrose ester type of *S. pennellii* is mainly P-type triacylsucrose, and the main reason for producing this kind of acylsugar is that the functions of SpASAT2 and SpASAT3 were reversed in the acylsugar synthesis pathway compared to their homologous genes SlASAT2 and SlASAT3 (Figure 2-3). For example, SpASAT3 can use monoacyl sucrose and iC5-CoA as substrates to synthesize diacylsucrose while the catalytic position is at the R2 position instead of using diacylsucrose and acyl-CoA as substrates like SlASAT3 to synthesize triacylsucrose while the catalytic site is at R3'. Sp-ASAT2 can catalyze the linking of acyl chains and diacyl-sucrose to form triacyl-sucrose, and its catalytic position is at the R3 position. This differs from the function of SlASAT2 in catalyzing the linking of acyl chains and monoacylsucrose to form diacyl-sucrose. Changes in individual amino acids of SlASAT2 and SlASAT3 can cause changes in their function to produce P-type triacylsucrose. (Fan et al., 2017).

In *S. habrochaites* species, significant polymorphisms in the acylation of the furanose ring of acylsugars were observed such as no acyl chain on the furanose ring, a short or long acyl chain (C10-C12) on the furanose ring, and two short acyl chains on the furanose ring. Sh-ASAT3-F can catalyze the connection of the acyl chain to the furanose ring of P-type diacylsucrose to form F-type triacylsucrose. Sh-ASAT3-P can catalyze the attachment of 1-2 acyl chains to the furanose ring of P-type diacylsucrose to form F-type tri- or tetra-acylsucroses and also catalyze the attachment of the iC5 acyl chain (cannot catalyze nC12) to the pyranose ring of P-type monoacylsucrose to generate P-type diacylsucrose. The function of Sh-ASAT4 is similar to that of SlASAT4 (Figure 2-3), which catalyzes the attachment of C2-CoA to the R2 position of F-type triacylsucrose to form tetraacylsucrose (Schilmiller et al., 2015).

In *P. axillaris*, PaxASAT1-PaxASAT4 can catalyze the sequential esterification of acyl-CoA substrates with the R2, R4, R3, and R6 hydroxyl groups of the sucrose ring to form tetraacylsucrose (Figure 2-3) (Nadakuduti et al., 2017). Of them, PaxASAT1 can catalyze the linking of C5-C8 acyl chains with sucrose to form monoacylsucroses, but it cannot utilize the iC4-CoA substrate; PaxASAT2 catalyzes the reaction of iC4-CoA, iC5-CoA, aiC5-CoA, and iC6-CoA with monoacylsucrose (S1:5(aiC5R2)) to produce diacylsucrose; PaxASAT3 can catalyze the reaction of iC6-CoA, iC7-CoA, and iC8-CoA with diacylsucrose to triacylsucrose but cannot catalyze the iC4-CoA and aiC5-CoA substrates; and PaxASAT4 can catalyze the reaction of iC4- or aiC5-CoAs with triacylsucrose to form tetraacylsucrose. *Petunia* plants are rich in pentaacylsucrose, which has a malonyl group attached to the furanose ring; however, the gene that catalyzes the assembly of the malonyl group with the sugar core is currently unknown.

In *Salpiglossis sinuata*, four acylsugar acyltransferases (SsASAT1- SsASAT3 and SsASAT5) are involved in the assembly of acylsucroses (Figure 2-3) (Moghe et al., 2017). Of them, SsASAT1 can catalyze the synthesis of monoacylsucrose from

different acyl-CoAs and sucrose. Its acyl substrates are similar to that of SlASAT1, but the catalytic position differs from SlASAT1 and is similar to PaxASAT1, which is the R2 position of sucrose. SsASAT2 can catalyze the linking of aiC5-CoA with monoacylsucrose to form diacylsucrose, and its catalytic position is the same as that of PaxASAT2, which is the R4 position of sucrose. SsASAT3 can catalyze the linking of aiC6-CoA with diacylsucrose at the R3 position to form triacylsucrose, but the enzyme cannot use aiC5-CoA as a substrate. SsASAT5 exhibits three types of catalytic activity, which can catalyze the formation of tetra- and penta-acylsucrose from the furanose ring aclyation of tri- and tetra-sucrose, and the formation of hexaacylsucrose from the pyranose or furanose ring acylation of penta-acylsucrose. The enzyme was named SsASAT5 because the acylation pattern and migration characteristics of its *in vitro* products are consistent with the most abundant pentaacyl sucroses *in vivo*. VIGS results indicatedthat tri-, tetra-, and hexa-acylsucroses with different structures in *S. sinuata* may be assembled by different ASATs.

In *Solanum nigrum*, two acyltransferases, SnASAT1 and SnASAT2, are involved in the synthesis of acylsugars (Figure 2-2), which catalyze the attachment of acyl chains to the R4 and R3 positions of the sucrose ring, respectively, to form diacylsucrose (Figure 2-3) (Lou et al., 2021).

In *N. benthamiana*, although two acyltransferases, NbASAT1 and NbASAT2, are demonstrated to be involved in the acylsucrose biosynthesis, their enzymatic activities *in vitro* and *in vivo* remain unknown (Feng et al., 2021). Recently, four BAHD acyltransferases of *N. acuminata*, NacASAT1~ NacASAT4, were verified to participate in the acylsugar aseembly *in vitro.* Of them, NacASAT1 can catalyze the connection of an acyl chain and sucrose to form monoacylsucrose, and NacASAT4 can sequentially catalyze acetyl chains attaching to the R3' and R6' positions of the furanose ring of triacylsucrose (S3:14) to produce penta-acylsucrose. NacASAT2 and NacASAT3 were demonstrated to be involved in acylsugar assembly through 'one pot' assays with NacASAT1 and NacASAT4, however, their exact catalytic activities remainl unknown, and the *in vivo* functions of NacASAT2~ NacASAT4 have not yet been clarified (Schenck et al., 2022).

2.2.2 Biosynthesis and Key Genes of Solanaceae Aclyhexoses

Currently known acyl six-carbon sugars include acylglucose and acylinositol. Regarding acylglucose synthesis, early studies on *S. Pennellii* suggested that a glucosyltransferase could synthesize 1-O-acylglucose using free acid and UDP-glucose. The activated 1-O-acylglucose molecule was shown to be substrate for a serinecarboxypeptidase-like (SCPL) acyltransferase that catalyzes a disproportionation reaction to produce free glucose and 1,2-di-O-acyl glucose (Ghangas and Steffens 1993; Li and Steffens 2000). However, the relationship between this pathway and acylsucrose synthesis was unknown. A recent study on *S. Pennellii* demonstrated that an acylsucrose fructofuranosidase (SpASFF1) derived from primary metabolism and specifically expressed in the GTs can cleave the glycosidic bond of P-type triacylsucrose to generate triacylglucose (Figure 2-3) (Leong Bryan et al., 2019). In *S. nigrum*, SnASFF1 cleaves P-type diacylsucrose to

generate diacylglucose, while an acylglucose acyltransferase, SnAGAT1, catalyzes the attachment of acetyl groups to diacylglucose to form triacylglucose (Figure 2-3) (Lou et al., 2021). Regarding the synthesis enzyme of acylinositol, only one triacylinositol acyltransferase TAIAT has been found in *S. quitoense*, which can catalyze acyl chains to link with triacylinositol to form tetraacylinositol. This gene is homologous to *SlASAT4* and has a parallel evolutionary relationship, indicating that they have a common origin (Leong et al., 2020).

3. Bioactivities of *Solanaceae* Acylsugars

Acylsugars have been identified in several genera of the *Solanaceae* family. However, studies on the biological activity of acylsugars have primarily focused on the *Nicotiana* and *Solanum* genera, and only a few records on other *Solanaceae* genera. Acylsugars from *Solanaceae* reportedly have various biological effects, including insecticidal, antimicrobial, anti-dehydration, antitumor, and anti-inflammatory activities. In particular, the insecticidal activity of *Solanaceae* acylsugars has been widely reported.

3.1 Insecticidal Activity

3.1.1 Insecticidal Activities of Nicotiana Acylsugars

In the early 1980s, researchers observed that sucrose esters of *Nicotiana tabacum* were toxic to budworm larvae and *Heliothis virescens* (Fabricius) (Johnson and Severson 1984). Rodriguez et al. (1993) observed that sucrose esters from *Nicotiana* species were effective and environmentally safe natural biocides against arthropod pests such as aphids, mites, and whiteflies. Severson et al. (1994) reported that acylglucoses and acylsucroses from *Nicotiana gossei* act as insecticides against several pests, including tobacco aphid, *Myzus nicotianae* Blackman, sweet potato whitefly (SPWF), *Bemisia tabaci* (Gennadius), and the greenhouse whitefly (GHWF), *Trialeurodes vaporariorum* (Westwood). Notably, two types of acylsugars (2,3-di-O-acyl-1'-O-acetylsucrose and 2,3-di-O-acyl-1',6'-di-O-acetylsucrose), with major acyl chains containing 5-methylhexanoyl and 5-methylheptanoyl groups, are highly toxic to GHWF. Spraying *Nicotiana gossei*-derived sugar esters emulsified in water at a concentration of 0.1% (v/v) on tomato leaves resulted in >99% mortality rate of GHWF or SPWF nymphs (Severson et al., 1994). Simonovska et al. (2006) reported that oriental tobacco Prilep P-23 (belonging to *N. tabacum*) contained acylsugars effective against *Myzus persicae* (Sulzer). Some researches showed that sugar esters and their branched fatty acid chains from *Nicotiana attenuata* have indirect and direct defenses against lepidopteran herbivores (Weinhold and Baldwin, 2011; Luu et al., 2017). Feng et al. (2021) observed that sugar esters of *Nicotiana benthamiana* were toxic to five herbivore species: *M. persicae, B. tabaci, Macrosiphum euphorbiae* (Thomas) (potato aphid), *Helicoverpa zea* (Boddie) (corn earworm), and *H. virescens* (tobacco budworm).

3.1.2 Insecticidal Activities of Solanum Acylsugars

In *Solanum* species, the wild tomato *S. pennellii* has been extensively studied for its broad-spectrum toxicity owing to the wide range and abundance of acylsugars. Studies have revealed that branched acylglucoses of *S. pennellii* have significant inhibitory activity against potato aphids (Goffreda et al., 1989). Hawthorne et al. (1992) revealed that the triacylglucoses of *S. pennellii* significantly reduced the feeding and oviposition of the leaf miner *Liriomyza trifollii* (Burgess), and a dosage of 10% acylglucoses on *S. pennellii* could reduce leaf miner damage by 91%. Rodriguez et al. (1993) observed that acylsugars in *S. pennellii* inhibited the settling and feeding of the green peach aphid *M. persicae.* Acylsugars from *S. pennellii* result in growth retardation, extended life cycle, and reduced survival rate of pests such as *H. zea* and *Spodoptera exigua* (Hubner) (Juvik et al., 1994). Mirnezhad et al. (2009) observed that sugar esters from several wild tomato species were toxic to Western flower thrips *Frankliniella occidentalis* Pergande, with the most potent toxicity exhibited by acylsugars in *S. pennellii*. Leckie et al. (2012) reported that the new tomato lines having acylsugar locus of *S. pennellii* are highly toxic to *B. tabaci*. Salad tomato varieties with a high acylsugar content similar to *S. pennellii* were resistant to two-spotted spider mites (Peixoto et al., 2020). Different accessions of *S. pennellii* produce different sugar esters, and these acylsugar components possess differential and synergistic functions in suppressing oviposition by herbivorous insects, such as the whitefly *B. tabaci*; western flower thrips, *F. occidentalis*; and tobacco thrips, *Frankliniella fusca* (Hinds) (Leckie et al., 2016). One study evaluated the resistance to *Tetranychus urticae* Koch using different genotypes, including *S. pennellii* LA-716 (wild accession rich in acylsugars and a resistance source to arthropod pests), *Solanum lycopersicum* (syn. *L. esculentum* Mill.) cv. Redencao (tomatoes with low acylsugar content and susceptible to mites), and their F1 and F2 generations. Genotypes with high sugar esters were associated with the mortality of *T. urticae* nymphs and adult females. The effects included reduced oviposition, extended egg incubation periods, and reduced viability of eggs (Lucini et al., 2015). Resistance to whiteflies (*B. tabaci*) was also evaluated using the crossed F2 generation, as well as different lines of the BC1F2 population. The results exhibited a significant negative correlation between the sugar ester contents of different genotypes and egg production and nymphal development in whiteflies (Dias et al., 2016). Association of high acylsugar with resistance to South American tomato pinworm (*Tuta absoluta* Meyrick) was also observed during the screening of BC2F2 crosses (Resende et al., 2022). Several studies on different varieties or new lines related to *S. pennellii* have suggested that increased sugar esters were associated with reduced egg production in *F. occidentalis* and *B. tabaci*, and these plants were well-adapted and exhibited resistance to viral infection (Smeda et al., 2018; Ben-Mahmoud et al., 2018,2019; Marchant et al., 2020; Dias et al., 2021). In addition, *S. pennellii*-related varieties with high acylsugar contents could suppress the psyllid (*Bactericera cockerelli* Sulc) population by inhibiting their growth and development (Li et al., 2019).

Research on sugar esters from other wild tomatoes, such as *S. galapagense*, *S. cheesmaniae*, and *S. pimpinellifolium*, has established that these acylsugars inhibit

oviposition of two-spotted spider mites (*T. urticae*) (Rakha et al., 2017). Sugar esters in the resistant tomato variety ABL 14-8, derived from *Solanum pimpinellifolium*, prevent the settling and feeding of *B. tabaci*, reducing the spread of tomato yellow leaf curl disease (Rodríguez Lo' pez et al., 2012). Kortbeek et al. (2021) reported that two acylsugars (S3:15 and S3:21) in *Solanum habrochaites* correlate with whitefly and not thrips resistance.

Neal et al. (1990) have also reported that tetra-acylsucroses secreted by the B-type glandular trichome of *Solanum berthaultii* are toxic to green peach aphids.

3.1.3 Insecticidal Activities of Acylsugars in Other Solanaceae Genera

The glandular trichomes of *Petunia* cultivars secrete various sugar esters, including tri-acylglucose, tetra-acylglucose, tri-acylsucrose, tetra-acylsucrose, and penta-acylsucrose, among which tri-acylsucrose and tetra-acylsucrose are highly toxic to SPWF, followed by penta-acylsucrose. In contrast, tri-acylglucose and tetra-acylglucose are almost non-toxic (Chortyk et al., 1997). Moser et al. (1999) observed that tetra-acylsucrose isolated from *Petunia hybrida* was 100% lethal against freshwater *Biomphalaria glabrata* Say (one of the intermediate hosts of schistosomiasis) at concentrations as low as 15 μg/mL.

Datura wrightii mainly contains straight-chain acylsugars, which are biologically active against tobacco flea beetles (*Epitrix hirtipennis* (Melsheimer)) and a weevil (*Trichobaris compacta* Casey), thereby reducing their feeding on plants but lacking significant lethal effects against both pests.

3.2 Antimicrobial Activity

In *Nicotiana*, Kennedy *et al*. (1992) isolated acylsucrose and/or acylglucose mixture fractions from seven tobacco species, including *N. acuminata, N. benthamiana, N. attenuata, N. clevelandii, N. miersii, and accessions 10 and 12 of N. bigelovii*. These fractions inhibited the germination of *Peronospora tabacina* at a concentration of 30 μg/cm^2. Chortyk *et al*. (1993) isolated and identified sugar esters from 24 tobacco species and obtained eight sucrose and two glucose esters. They treated five bacterial species [*Bacillus subtilis* (+) (destroys fermentation), *B. cereus* (+) (related to foodborne illness), *Microbacterium thermosphactum* (+), *Enterobacter cloacae* (−), and *Citrobacter freundii* (−), with the latter two having characteristics similar to *Salmonella*] with each tobacco acylsugar mixture. They observed that aclysugars containing C7 and C8 O-acyl groups had optimal inhibitory effects against various bacteria. Luu *et al*. (2017) reported that sugar esters from *Nicotiana attenuata* inhibited two pathogenic fungi (*Fusarium brachygibbosum* U4 and *Alternaria sp.* U10). However, the inhibitory concentrations for spore germination of the two pathogenic fungi were different, and higher concentrations of acylsugars were required to significantly inhibit the sporangial germination of *Alternaria* (1.5 mg/mL) than that of *Fusarium* (0.375 mg/mL).

In *Solanum*, Holley *et al*. (1987) reported that sucrose esters (3,4,6-tri-O-acylsugar and 3,3',4,6-tetra-O-acylsugar) secreted by potato *Solanum berthaultii* B-type glandular trichomes were toxic to *Phytophthora infestans* and completely inhibited the production of fungal conidia at a sucrose ester concentration of 0.2% (w/v).

Teruo *et al*. (2009) observed that secretions from type-IV glandular trichomes of wild tomato *S. pennellii* demonstrated inhibitory activity against the conidia of the tomato powdery mildew pathogen *Oidium neolycopersici*, and speculated that the active inhibitory compound might be sugar esters.

In *Petunia*, tetra-O-acyl sucrose esters obtained by isolation and purification from *Petunia* species have inhibitory activity against *Bacillus subtilis* and *Pseudomonas stutzeri* at 5 μg per spot in a direct bioautography assay (Moser et al., 1999).

3.3 Growth-regulating Activity

Matsuzaki et al. (1988) studied the effect of surface secretions from 54 tobacco species on tobacco seed germination. They demonstrated that more than half of the extracts had an inhibitory effect on seed germination, with the surface secretions of *Nicotiana glutinosa* having the most potent inhibitory effect. The two purified sugar ester components, M1 (tetra-acylsucrose) and M2 (tri-acylsucrose), obtained from *N. glutinosa* significantly inhibited the germination and growth of tobacco seeds as well as those of dicot and monocot plants. At a concentration of 5 mM, the two sugar esters caused root browning and hypocotyl bending. Furthermore, M1 and M2 severely damaged barnyard grassroots and inhibited the growth of its aerial parts. In addition, the study also demonstrated that C5 and C10 fatty acids were toxic to the germination and growth of tobacco seeds but did not cause hypocotyl bending or root damage.

Furthermore, *Nicotiana umbratica* reportedly contains three types of glucose esters (I–III) and five types of sucrose esters (IV–VIII), and treatment of barnyard grass seeds with 5 mM of type I, IV, V, and VI sugar esters caused severe root damage and inhibited the growth of the aboveground parts of barnyard grass (Shinozaki et al., 1991).

3.4 Anti-dehydration Activity

Using *N. benthamiana* mutants of knock-out acylsugar biosynthesis genes, Feng *et al*. (2021) found that reduction in the acylsugar content in *N. benthamiana* was associated with reduced leaf temperature and rapid water loss from both isolated leaves and whole plants compared to that in the wild-type control, indicating acylsugars have anti-dehydration effect.

3.5 Antitumor and Anti-inflammatory Activities

A mixture of glucose esters and sucrose esters from tobacco leaf surface inhibiting the production of tumor necrosis factor (TNF)-α as well as 7,12-dimethylbenz(a)anthracene (DMBA), which triggers skin tumor promotion in mice, can be exploited as a promising and novel cancer-preventive agent (Okabe et al., 1999).

Herrera Salgado *et al*. (2005) found that acylinositols from *Solanum lanceolatum* had *in vivo* anti-inflammatory activity against 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced mouse ear edema. In addition, a mixture of two sugar esters obtained from the surface extract of *Physalis peruviana* had anti-inflammatory and

immunomodulatory effects and was non-toxic to mice at the macroscopic, histological, molecular, and biochemical levels (Ocampo et al., 2017).

In conclusion, plant acylsugars also act as bacterial resistance modifiers that facilitate the re-introduction of therapeutically ineffective antibiotics into clinical use and help inhibit the emergence of new multidrug-resistant strains (Lira Rica´rdez et al., 2020).

4. Conclusion and Prospect

The structures of *Solanaceae* acysugars are widely diverse. There are differences in the type of sugar backbone, the structure and number of acyl chains, and the attachment positions of the acyl chains on the sugar core, which are all responsible for the diversity of *Solanaceae* acylsugar types and structures. The shorter acyl chains of *Solanaceae* acylsugars are primarily derived from the BCAA pathway, and these short acyl chains are used as primers to form longer acyl chains through the α-KAE one-carbon extension pathway or the FAS two-carbon extension pathway. Acyl-CoA is the active substrate form for the assembly of acylsugars. Different types of acylsucrose are synthesized by a series of ASATs which catalyze the assembly of acyl-CoAs and sugar core. Solanaceae aclyglucoses are primarily formed by the cleavage of P-type acylsucroses by the ASFF1 enzyme. In *S. nigrum*, SnAGAT1 can catalyze the linking of acetyl groups with diacylglucose to form triacylglucose. Only a triacylinositol acyltransferase, TAIAT, has been found in *S. quitoense*, which can catalyze the linking of acyl chain with triacylinositol to form tetraacylinositol. The differences in acyl chain pools, the diversity of acyltransferase sequences, the diversity or specificity of acyl substrates of different acyltransferases, as well as the new-functionalization of some enzymes derived from primary metabolism all contribute to the structural diversity of acylsugars across different *Solanaceae* crops. *Solanaceae* acylsugars have anti-insect, anti-microbial, anti-dehydration, and anti-tumar properties. The diversity of *Solanaceae* acylsugars also results in the diversity of their biological activities.

Although great progress has been made in identifying the structure, bioactivity and synthesis mechanism of acylsugars in several *Solanaceae* species, the acylsugar structures and biological activities of most *Solanaceae* species remain unknown. The genes and mechanisms involved in the synthesis of some acylsugars with structures identified (such as inositol esters and some pentaacylsucroses) need to be further clarified. Moreover, acylsugar acyl chains are primarily derived from the BCAA metabolic pathways and the subsequent α-KAE- or FAS extension pathways, and these primary metabolic pathways exist in various tissues of *Solanaceae* plants, however, acylsugars are only found in the head cells of the *Solanaceae* GTs, and so far, the corresponding genes that catalyze the synthesis of various acyl chains in the GTs remain poorly understood. In addition, while some acylsuagr synthesis genes have been identified, the transcription factors that regulate acylsugar content are still unknown. Therefore, future studies should focus on further identifying the structure and biological activities of *Solanaceae* acylsugars and mining functional and regulatory genes involved in acylsugar biosynthesis. This will help to identify

acylsugar molecules with high biological activities and improve acylsugar content in *Solanaceae* crops using molecular-assisted techniques, and contribute to the efficient production of active acylsugars beneficial to human beings through biosynthesis methods.

Tobacco is one of the important commercial crops in the world. As a model plant, it is also considered as efficient green bioreactor because of its amenability for transient and stable genetic transformation as well as its high leaf biomass. Tobacco acylsugars are considered to be the precursors of many important flavor components that contribute to the aroma and flavor quality of cured tobacco leaves, and have been shown to have multiple defense-related activities. Therefore, the exploration of key genes involved in tobacco acylsugar biosynthesis and the analysis of their functional mechanisms are not only of great significance for tobacco resistance and quality breeding, but also for the development and utilization of tobacco acylsugars in natural insecticides, cigarette additive and medical ingredients. Except for a few identified acylsugar structures, the specific structures of most acylsugars of *Nicotiana* remain unclear. Previous reports indicate that there are obvious differences in the structure of tobacco acylsugars and other *Solanaceae* acylsugars. However, the synthetic genes and mechanisms of tobacco acylsugars remain poorly elusidated. Previously, through transcriptome sequencing and homology comparison methods, our research group has obtained several trichome-sepcifical expression genes which were predicted to be involved in the synthesis of tobacco acysugars. In this thesis study, we will conduct functional identification of these candidate genes.

References

Arrendale RF, Severson RF, Sisson VA, Costello CE, Leary JA, Himmelsbach DS, Van Halbeek H (1990) Characterization of the sucrose ester fraction from Nicotiana glutinosa. Journal of Agricultural and Food Chemistry 38 (1):75-85. doi:10.1021/jf00091a015

Ashraf-Khorassani M, Nazem N, Taylor L, Coleman W (2005) Separation and identification of sucrose esters from Turkish tobacco using liquid chromatography-mass spectrometry. Beiträge zur Tabakforschung International/Contributions to Tobacco Research 21 (7):381-389

Bazito RC, El Seoud OA (2001) Sugar-based anionic surfactants: synthesis and micelle formation of sodium methyl 2-acylamido-2-deoxy-6-O-sulfo-D-glucopyranosides. Carbohydrate Research 332 (1):95-102. doi:10.1016/s0008-6215(01)00077-5

Ben-Mahmoud S, Anderson T, Chappell TM, Smeda JR, Mutschler MA, Kennedy GG, De Jong DM, Ullman DE (2019) A thrips vector of tomato spotted wilt virus responds to tomato acylsugar chemical diversity with reduced oviposition and virus inoculation. Scientific Reports 9. doi:10.1038/s41598-019-53473-y

Ben-Mahmoud S, Smeda JR, Chappell TM, Stafford-Banks C, Kaplinsky CH, Anderson T, Mutschler MA, Kennedy GG, Ullman DE (2018) Acylsugar amount and fatty acid profile differentially suppress oviposition by western flower thrips,

Frankliniella occidentalis, on tomato and interspecific hybrid flowers. Plos One 13 (7). doi:10.1371/journal.pone.0201583

Binder S, Knill T, Schuster J (2007) Branched-chain amino acid metabolism in higher plants. Physiologia Plantarum 129 (1):68-78. doi:10.1111/j.1399-3054.2006.00800.x

Bonierbale MW, Plaisted RL, Pineda O, Tanksley SD (1994) QTL analysis of trichome-mediated insect resistance in potato. TAG Theoretical and applied genetics Theoretische und angewandte Genetik 87 (8):973-987. doi:10.1007/bf00225792

Chortyk OT, Kays SJ, Teng Q (1997) Characterization of insecticidal sugar esters of Petunia. Journal of Agricultural and Food Chemistry 45 (1):270-275. doi:10.1021/jf960322f

Chortyk OT, Severson RF, Cutler HC, Sisson VA (1993) Antibiotic activities of sugar esters isolated from selected Nicotiana species. Bioscience, biotechnology, and biochemistry, vol 57.

Cutler HG, Severson RF, Cole PD, Arrendale RF, Sisson VA (1986) Plant growth inhibitory and antimicrobial properties of hydroxyacylnornicotines from select Nicotiana species. Proceedings of the Plant Growth Regulator Society of America Thirteenth annual meeting, St Petersburg Beach, Florida, August 1986:188-200

Danehower DA (1987) A rapid method for the isolation and quantification of the sucrose esters of tobacco. Tobacco Int 189:30-33

de Resende JTV, Dias DM, Corte LED, Constantino LV, Ventura MU, de Lima RB, de Oliveira LVB, Da-Silva PR (2022) The introgression of resistance to Tuta absoluta in tomato based on glandular trichomes. ARTHROPOD-PLANT INTERACTIONS 16 (1):87-99. doi:10.1007/s11829-021-09873-xDias

DM, Erpen-Dalla Corte L, Vilela Resende JT, Zeffa DM, Vilela Resende NC, Zanin DS, de Lima Filho RB (2021) Acylsugars in tomato varieties confer resistance to the whitefly and reduce the spread of fumagine. BRAGANTIA 80. doi:10.1590/1678-4499.20210022

Dias DM, Resende JTV, Marodin JC, Matos R, Lustosa IF, Resende NCV (2016) Acyl sugars and whitefly (Bemisia tabaci) resistance in segregating populations of tomato genotypes. Genetics and Molecular Research 15 (2). doi:10.4238/gmr.15027788

Dieter Moser IK, Bernhard Vogler and Wolfgang Kraus (1999) Molluscicidal and antibacterial compounds fromPetuniahybridat. Pesticide Science 55:336-339

Dufour C, da Silva E, Potier P, Queneau Y, Dangles O (2002) Gallic esters of sucrose as efficient radical scavengers in lipid peroxidation. Journal of Agricultural and Food Chemistry 50 (12):3425-3430. doi:10.1021/jf011683k

Fan P, Leong BJ, Last RL (2019) Tip of the trichome: evolution of acylsugar metabolic diversity in Solanaceae. Current Opinion in Plant Biology 49:8-16. doi:10.1016/j.pbi.2019.03.005

Fan P, Miller AM, Liu X, Jones AD, Last RL (2017) Evolution of a flipped pathway creates metabolic innovation in tomato trichomes through BAHD enzyme

promiscuity. Nat Commun 8 (1):2080. doi:10.1038/s41467-017-02045-7

Fan P, Miller AM, Schilmiller AL, Liu X, Ofner I, Jones AD, Zamir D, Last RL (2016) In vitro reconstruction and analysis of evolutionary variation of the tomato acylsucrose metabolic network. Proc Natl Acad Sci U S A 113 (2):E239-248. doi:10.1073/pnas.1517930113

Fan P, Wang P, Lou YR, Leong BJ, Moore BM, Schenck CA, Combs R, Cao P, Brandizzi F, Shiu SH, Last RL (2020) Evolution of a plant gene cluster in Solanaceae and emergence of metabolic diversity. Elife 9. doi:10.7554/eLife.56717

Feng H, Acosta-Gamboa L, Kruse LH, Tracy JD, Chung SH, Nava Fereira AR, Shakir S, Xu H, Sunter G, Gore MA, Casteel CL, Moghe GD, Jander G (2021) Acylsugars protect Nicotiana benthamiana against insect herbivory and desiccation. Plant Mol Biol. doi:10.1007/s11103-021-01191-3

Fobes JF, Mudd JB, Marsden MP (1985) Epicuticular Lipid Accumulation on the Leaves of Lycopersicon pennellii (Corr.) D'Arcy and Lycopersicon esculentum Mill. Plant physiology 77 (3):567-570. doi:10.1104/pp.77.3.567

Gang DR, Lavid N, Zubieta C, Chen F, Beuerle T, Lewinsohn E, Noel JP, Pichersky E (2002) Characterization of Phenylpropene O-Methyltransferases from Sweet Basil: Facile Change of Substrate Specificity and Convergent Evolution within a Plant O-Methyltransferase Family. The Plant Cell 14 (2):505-519. doi:10.1105/tpc.010327 %J The Plant Cell

Ghangas GS, Steffens JC (1993) UDPglucose: fatty acid transglucosylation and transacylation in triacylglucose biosynthesis. Proceedings of the National Academy of Sciences of the United States of America 90 (21):9911-9915. doi:10.1073/pnas.90.21.9911

Ghosh B, Westbrook TC, Jones AD (2014) Comparative structural profiling of trichome specialized metabolites in tomato (Solanumlycopersicum) and S. habrochaites: acylsugar profiles revealed by UHPLC/MS and NMR. Metabolomics 10 (3):496-507. doi:10.1007/s11306-013-0585-y

Haliński Ł P, Stepnowski P (2013) GC-MS and MALDI-TOF MS profiling of sucrose esters from Nicotiana tabacum and N. rustica. Zeitschrift fur Naturforschung C, Journal of biosciences 68 (5-6):210-222. doi:10.1515/znc-2013-5-607

Hawthorne D, Shapiro J, Tingey W, Mutschler M (1992) Trichome‐borne and artificially applied acylsugars of wild tomato deter feeding and oviposition of the leafminer Liriomyza trifolii. Entomologia experimentalis et applicata, vol 65.

Herrera-Salgado Y, Garduno-Ramirez ML, Vazquez L, Rios MY, Alvarez L (2005) Myo-inositol-derived glycolipids with anti-inflammatory activity from Solanum lanceolatum. Journal of Natural Products 68 (7):1031-1036. doi:10.1021/np050054s

Hoffmann L, Maury S, Martz F, Geoffroy P, Legrand M (2003) Purification, cloning, and properties of an acyltransferase controlling shikimate and quinate ester intermediates in phenylpropanoid metabolism. J Biol Chem 278 (1):95-103. doi:10.1074/jbc.M209362200

Holley JD, King RR, Singh RP (1987) Glandular trichomes and the resistance of Solanum berthaultii (PI 473340) to infection from Phytophthora infestans. Canadian

Journal of Plant Pathology 9 (4):291-294. doi:10.1080/07060668709501859

Huchelmann A, Boutry M, Hachez C (2017) Plant Glandular Trichomes: Natural Cell Factories of High Biotechnological Interest. Plant Physiol 175 (1):6-22. doi:10.1104/pp.17.00727

Jackson D, Chortyk O, Stephenson M, Johnson A, Harlow C, Simmons A, Sisson VJTS (1998) Potential of Nicotiana species for production of sugar esters. Tobacco Science 42 (1):1-9

Johnson AW, Severson RF (1984) Leaf surface chemistry of tobacco budworm resistant tobacco. Journal of agricultural entomology.

Jose Rodriguez-Lopez M, Garzo E, Patrick Bonani J, Fernandez-Munoz R, Moriones E, Fereres A (2012) Acylsucrose-Producing Tomato Plants Forces Bemisia tabaci to Shift Its Preferred Settling and Feeding Site. Plos One 7 (3). doi:10.1371/journal.pone.0033064

Juvik JA, Shapiro JA, Young TE, Mutschler MA (1994) Acylglucoses from wild tomatoes alter behavior and reduce growth and survival of Helicoverpa zea and Spodoptera exigua (Lepidoptera: Noctuidae). Journal of Economic Entomology, vol 87.

Kandra G, Severson R, Wagner GJ (1990) Modified branched-chain amino acid pathways give rise to acyl acids of sucrose esters exuded from tobacco leaf trichomes. European journal of biochemistry 188 (2):385-391. doi:10.1111/j.1432-1033.1990.tb15415.x

Kandra L, Wagner GJ (1988) Studies of the site and mode of biosynthesis of tobacco trichome exudate components. Archives of biochemistry and biophysics 265 (2):425-432. doi:10.1016/0003-9861(88)90145-2

Kennedy BS, Nielsen MT, Severson RF, Sisson VA, Stephenson MK, Jackson DM (1992) Leaf surface chemicals fromNicotiana affecting germination ofPeronospora tabacina (adam) sporangia. Journal of chemical ecology 18 (9):1467-1479. doi:10.1007/bf00993221

Kortbeek RWJ, Galland MD, Muras A, van der Kloet FM, Andre B, Heilijgers M, van Hijum SAFT, Haring MA, Schuurink RC, Bleeker PM (2021) Natural variation in wild tomato trichomes; selecting metabolites that contribute to insect resistance using a random forest approach. BMC PLANT BIOLOGY 21 (1). doi:10.1186/s12870-021-03070-x

Kroumova AB, Wagner GJ (2003) Different elongation pathways in the biosynthesis of acyl groups of trichome exudate sugar esters from various solanaceous plants. Planta 216 (6):1013-1021. doi:10.1007/s00425-002-0954-7

Kroumova ABM, Zaitlin D, Wagner GJ (2016) Natural variability in acyl moieties of sugar esters produced by certain tobacco and other Solanaceae species. Phytochemistry 130:218-227. doi:10.1016/j.phytochem.2016.05.008

Lalot J, Stasik I, Demailly G, Beaupere D, Gode P (2004) Synthesis and amphiphilic properties of S-alkylthiopentonolactones and their pentitol derivatives. Journal of Colloid and Interface Science 273 (2):604-610. doi:10.1016/j.jcis.2004.01.020

Lawson DM, Lunde CF, Mutschler MA (1997) Marker-assisted transfer of acylsugar-mediated pest resistance from the wild tomato, Lycopersicon pennellii, to the cultivated tomato, Lycopersicon esculentum. Molecular Breeding 3 (4):307-317. doi:10.1023/A:1009677412902

Leckie BM, D'Ambrosio DA, Chappell TM, Halitschke R, De Jong DM, Kessler A, Kennedy GG, Mutschler MA (2016) Differential and Synergistic Functionality of Acylsugars in Suppressing Oviposition by Insect Herbivores. Plos One 11 (4). doi:10.1371/journal.pone.0153345

Leckie BM, De Jong DM, Mutschler MA (2012) Quantitative trait loci increasing acylsugars in tomato breeding lines and their impacts on silverleaf whiteflies. Molecular Breeding 30 (4):1621-1634. doi:10.1007/s11032-012-9746-3

Leckie BM, Halitschke R, De Jong DM, Smeda JR, Kessler A, Mutschler MA (2014) Quantitative trait loci regulating the fatty acid profile of acylsugars in tomato. Molecular Breeding 34 (3):1201-1213. doi:10.1007/s11032-014-0110-7

Leong BJ, Hurney SM, Fiesel PD, Moghe GD, Jones AD, Last RL (2020) Specialized Metabolism in a Nonmodel Nightshade: Trichome Acylinositol Biosynthesis. Plant Physiol 183 (3):915-924. doi:10.1104/pp.20.00276

Leong Bryan J, Lybrand Daniel B, Lou Y-R, Fan P, Schilmiller Anthony L, Last Robert L (2019) Evolution of metabolic novelty: A trichome-expressed invertase creates specialized metabolic diversity in wild tomato. Science Advances 5 (4):eaaw3754. doi:10.1126/sciadv.aaw3754

Li AX, Steffens JC (2000) An acyltransferase catalyzing the formation of diacylglucose is a serine carboxypeptidase-like protein. Proceedings of the National Academy of Sciences 97 (12):6902. doi:10.1073/pnas.110154197

Li Z, Kund G, De Jong DM, Feng X, Mutschler MA, Trumble JT (2019) Effects of High-Level Acylsugar-Producing Tomato Lines on the Development of Tomato Psyllids (Hemiptera: Triozidae). Journal of Economic Entomology 112 (4):1926-1931. doi:10.1093/jee/toz070

Lira-Ricardez J, Pereda-Miranda R (2020) Reversal of multidrug resistance by amphiphilic morning glory resin glycosides in bacterial pathogens and human cancer cells. Phytochemistry Reviews 19 (5):1211-1229. doi:10.1007/s11101-019-09631-1

Liu TX, Stansly PA, Chortyk OT (1996) Insecticidal activity of natural and synthetic sugar esters against Bemisia argentifolii (Homoptera: Aleyrodidae). Journal of Economic Entomology 89 (5):1233-1239. doi:10.1093/jee/89.5.1233

Liu X, Enright M, Barry CS, Jones AD (2017) Profiling, isolation and structure elucidation of specialized acylsucrose metabolites accumulating in trichomes of Petunia species. Metabolomics 13 (7). doi:10.1007/s11306-017-1224-9

Liu Y, Jing S-X, Luo S-H, Li S-H (2019) Non-volatile natural products in plant glandular trichomes: chemistry, biological activities and biosynthesis. Natural Product Reports 36 (4):626-665. doi:10.1039/c8np00077h

Lou YR, Anthony TM, Fiesel PD, Arking RE, Christensen EM, Jones AD, Last RL (2021) It happened again: Convergent evolution of acylglucose specialized metabolism in black nightshade and wild tomato. Sci Adv 7 (46):eabj8726.

doi:10.1126/sciadv.abj8726

Lucini T, Faria MV, Rohde C, Vilela Resende JT, Freitas de Oliveira JR (2015) Acylsugar and the role of trichomes in tomato genotypes resistance to Tetranychus urticae. Arthropod-Plant Interactions 9 (1):45-53. doi:10.1007/s11829-014-9347-7

Lybrand DB, Anthony TM, Jones AD, Last RL (2020) An Integrated Analytical Approach Reveals Trichome Acylsugar Metabolite Diversity in the Wild Tomato Solanum pennellii. Metabolites 10 (10). doi:10.3390/metabo10100401

Mandal S, Ji W, McKnight TD (2020) Candidate Gene Networks for Acylsugar Metabolism and Plant Defense in Wild Tomato Solanum pennellii. Plant Cell 32 (1):81-99. doi:10.1105/tpc.19.00552

Marchant WG, Legarrea S, Smeda JR, Mutschler MA, Srinivasan R (2020) Evaluating Acylsugars-Mediated Resistance in Tomato against Bemisia tabaci and Transmission of Tomato Yellow Leaf Curl Virus. Insects 11 (12). doi:10.3390/insects11120842

Matsuzaki T, Koseki K, Koiwai A (1988) Germination and growth inhibition of surface lipids from Nicotiana species and identification of sucrose esters. Agricultural and biological chemistry, vol 52.

Matsuzaki T, Shinozaki Y, Suhara S, Shigematsu H, Koiwai A (1989) Isolation and Characterization of Tetra- and Triacylglucose from the Surface Lipids of Nicotiana miersii. Agricultural and Biological Chemistry 53 (12):3343-3345. doi:10.1080/00021369.1989.10869814

Maugard T, RemaudSimeon M, Petre D, Monsan P (1997) Enzymatic synthesis of glycamide surfactants by amidification reaction. Tetrahedron 53 (14):5185-5194. doi:10.1016/s0040-4020(97)00181-6

Menetrez ML, Spurr HW, Danehower DA, Lawson DR (1990) Influence of tobacco leaf surface chemicals on germination ofPeronospora tabacina adam sporangia. Journal of Chemical Ecology 16 (5):1565-1576. doi:10.1007/BF01014090

Mihaylova-Kroumova AB, Artiouchine I, Korenkov VD, Wagner GJ (2020) Patterns of inheritance of acylsugar acyl groups in selected interspecific hybrids of genus Nicotiana. J Plant Res 133 (4):509-523. doi:10.1007/s10265-020-01188-x

Mirnezhad M, Romero‐González RR, Leiss KA, Choi YH, Verpoorte R, Klinkhamer PG (2010) Metabolomic analysis of host plant resistance to thrips in wild and cultivated tomatoes. Phytochemical Analysis: An International Journal of Plant Chemical and Biochemical Techniques, vol 21.

Moghe GD, Leong BJ, Hurney SM, Jones AD, Last RL (2017) Evolutionary routes to biochemical innovation revealed by integrative analysis of a plant-defense related specialized metabolic pathway. Elife 6. doi:10.7554/eLife.28468

Moser D, Klaiber I, Vogler B, Kraus W (1999) Molluscicidal and antibacterial compounds from Petunia hybrida. Pesticide science, vol 55.

Nadakuduti SS, Uebler JB, Liu X, Jones AD, Barry CS (2017) Characterization of Trichome-Expressed BAHD Acyltransferases in Petunia axillaris Reveals Distinct

Acylsugar Assembly Mechanisms within the Solanaceae. Plant Physiology, vol 175.

Neal JJ, Tingey WM, Steffens JC (1990) Sucrose esters of carboxylic acids in glandular trichomes ofSolanum berthaultii deter settling and probing by green peach aphid. Journal of Chemical Ecology, vol 16.

Neal JW, Jr., Buta JG, Pittarelli GW, Lusby WR, Bentz JA (1994) Novel sucrose esters from Nicotiana gossei: effective biorationals against selected horticultural insect pests. Journal of Economic Entomology 87 (6):1600-1607. doi:10.1093/jee/87.6.1600

Niggeweg R, Michael AJ, Martin C (2004) Engineering plants with increased levels of the antioxidant chlorogenic acid. Nat Biotechnol 22 (6):746-754. doi:10.1038/nbt966

Ning J, Moghe GD, Leong B, Kim J, Ofner I, Wang Z, Adams C, Jones AD, Zamir D, Last RL (2015) A Feedback-Insensitive Isopropylmalate Synthase Affects Acylsugar Composition in Cultivated and Wild Tomato. Plant Physiol 169 (3):1821-1835. doi:10.1104/pp.15.00474

Nonomura T, Xu L, Wada M, Kawamura S, Miyajima T, Nishitomi A, Kakutani K, Takikawa Y, Matsuda Y, Toyoda H (2009) Trichome exudates of Lycopersicon pennellii form a chemical barrier to suppress leaf-surface germination of Oidium neolycopersici conidia. PLANT SCIENCE 176 (1):31-37. doi:10.1016/j.plantsci.2008.09.002

Ocampo YC, Caro DC, Rivera DE, Franco LA (2017) Safety of sucrose esters from Physalis peruviana L. in a 28-day repeated-dose study in mice. Biomedicine & Pharmacotherapy 90:850-862. doi:10.1016/j.biopha.2017.04.046

Okabe S, Suganuma M, Tada Y, Ochiai Y, Sueoka E, Kohya H, Shibata A, Takahashi M, Mizutani M, Matsuzaki T, Fujiki H (1999) Disaccharide esters screened for inhibition of tumor necrosis factor-alpha release are new anti-cancer agents. Japanese journal of cancer research : Gann 90 (6):669-676. doi:10.1111/j.1349-7006.1999.tb00799.x

Olmstead RG, Bohs L (2007) A summary of molecular systematic research in Solanaceae: 1982-2006. In: 6th International Solanaceae Conference, Madison, WI.

Peixoto JV, Ribeiro AL, Maciel GM, Oliveira CSd, Finzi RR, Moraes ERd (2020) Productivity, acylsugar concentrations and resistance to the two-spotted spider mite in genotypes of salad tomatoes. Revista Brasileira de Engenharia Agrícola e Ambiental, vol 24.

Perez B, Anankanbil S, Guo Z (2017) Synthesis of Sugar Fatty Acid Esters and Their Industrial Utilizations. Fatty Acids: Chemistry, Synthesis, and Applications. doi:10.1016/b978-0-12-809521-8.00010-6

Pratap Singh A, Singh AK, Begum AS, Sahai M (2003) Two acyl sucroses from Petunia nyctaginiflora. Phytochemistry 63 (4):485-489. doi:10.1016/s0031-9422(03)00162-6

Puterka GJ, Farone W, Palmer T, Barrington A (2003) Structure-function relationships affecting the insecticidal and miticidal activity of sugar esters. Journal of Economic Entomology 96 (3):636-644. doi:10.1603/0022-0493-96.3.636

Rakha M, Bouba N, Ramasamy S, Regnard J-L, Hanson P (2017) Evaluation of wild tomato accessions (Solanum spp.) for resistance to two-spotted spider mite (Tetranychus urticae Koch) based on trichome type and acylsugar content. Genetic Resources and Crop Evolution 64 (5):1011-1022. doi:10.1007/s10722-016-0421-0

RicoLattes I, Lattes A (1997) Synthesis of new sugar-based surfactants having biological applications: Key role of their self-association. Colloids and Surfaces a-Physicochemical and Engineering Aspects 123:37-48. doi:10.1016/s0927-7757(96)03778-8

Rodriguez AE, Tingey WM, Mutschler MA (1993) Acylsugars of Lycopersicon pennellii deter settling and feeding of the green peach aphid (Homoptera: Aphididae). Journal of Economic Entomology, vol 86.

Schenck CA, Anthony TM, Jacobs M, Jones AD, Last RL (2022) Natural Variation Meets Synthetic Biology: Promiscuous Trichome Expressed Acyltransferases from Nicotiana acuminata. bioRxiv.

Schilmiller AL, Gilgallon K, Ghosh B, Jones AD, Last RL (2016) Acylsugar Acylhydrolases: Carboxylesterase-Catalyzed Hydrolysis of Acylsugars in Tomato Trichomes. Plant Physiol 170 (3):1331-1344. doi:10.1104/pp.15.01348

Schilmiller AL, Moghe GD, Fan P, Ghosh B, Ning J, Jones AD, Last RL (2015) Functionally divergent alleles and duplicated Loci encoding an acyltransferase contribute to acylsugar metabolite diversity in Solanum trichomes. Plant Cell 27 (4):1002-1017. doi:10.1105/tpc.15.00087

Severson RF, Arrendale RF, Chortyk OT, Green CR, Thome FA, Stewart JL, Johnson AW (1985) Isolation and characterization of the sucrose esters of the cuticular waxes of green tobacco leaf. Journal of Agricultural and Food Chemistry 33 (5):870-875. doi:10.1021/jf00065a026

Severson RF, Arrendale RF, Chortyk OT, Johnson AW, Jackson DM, Gwynn GR, Chaplin JF, Stephenson MG (1984) Quantitation of the major cuticular components from green leaf of different tobacco types. Journal of Agricultural and Food Chemistry 32 (3):566-570. doi:10.1021/jf00123a037

Severson RF, Chortyk OT, Stephenson MG, Akey DH, Neal Jr JW, Pittarelli GW, Jackson DM, Sisson VA (1994) Characterization of natural pesticide from Nicotiana gossei. In. ACS Publications,

Simonovska B, Srbinoska M, Vovk I (2006) Analysis of sucrose esters - insecticides from the surface of tobacco plant leaves. Journal of Chromatography A 1127 (1-2):273-277. doi:10.1016/j.chroma.2006.05.103

Slocombe SP, Schauvinhold I, McQuinn RP, Besser K, Welsby NA, Harper A, Aziz N, Li Y, Larson TR, Giovannoni J, Dixon RA, Broun P (2008) Transcriptomic and Reverse Genetic Analyses of Branched-Chain Fatty Acid and Acyl Sugar Production in Solanum pennellii and Nicotiana benthamiana. Plant Physiology 148 (4):1830-1846. doi:10.1104/pp.108.129510

Smeda JR, Schilmiller AL, Anderson T, Ben-Mahmoud S, Ullman DE, Chappell TM, Kessler A, Mutschler MA (2018) Combination of Acylglucose QTL reveals additive and epistatic genetic interactions and impacts insect oviposition and virus

infection. Molecular Breeding 38 (1). doi:10.1007/s11032-017-0756-z

van der Hoeven RS, Steffens JC (2000) Biosynthesis and Elongation of Short- and Medium-Chain-Length Fatty Acids. Plant Physiology, vol 122.

Van Thi L, Weinhold A, Ullah C, Dressel S, Schoettner M, Gase K, Gaquerel E, Xu S, Baldwin IT (2017) O-Acyl Sugars Protect a Wild Tobacco from Both Native Fungal Pathogens and a Specialist Herbivore. Plant Physiology 174 (1):370-386. doi:10.1104/pp.16.01904

Vontimitta V, Danehower DA, Steede T, Moon HS, Lewis RS (2010) Analysis of a Nicotiana tabacum L. Genomic Region Controlling Two Leaf Surface Chemistry Traits. Journal of Agricultural and Food Chemistry 58 (1):294-300. doi:10.1021/jf903256h

Walters DS, Steffens JC (1990) Branched Chain Amino Acid Metabolism in the Biosynthesis of Lycopersicon pennellii Glucose Esters. Plant Physiol 93 (4):1544-1551. doi:10.1104/pp.93.4.1544

Y S (1991) New types of glycolipids from the surface lipids of Nicotiana umbratica. Agricultural and biological chemistry, vol 55.

3

The Trichome-specific Acetolactate Synthase *NtALS1* Gene, Is Involved in Acylsugar Biosynthesis in Tobacco (*Nicotiana tabacum L.*)

Chapter Three The Trichome-specific Acetolactate Synthase *NtALS1* Gene, Is Involved in Acylsugar Biosynthesis in Tobacco (*Nicotiana tabacum L.*)

Ai-Xia Chang[1†], Biao Chen[1†], Ai-Guo Yang[1], Ri-Sheng Hu[2], Quan-Fu Feng[1], Ming Chen[1], Xiao-Ning Yang[1], Cheng-Gang Luo[1], Yang-Yang Li[2*], Yuan-Ying Wang[1*]

[1] Tobacco Research Institute, Chinese Academy of Agricultural Sciences, Qingdao, China;
[2] Hunan Tobacco Research Institute, Changsha, China;
[†] These authors contributed equally to this study.

Adapted from the reference: Chang AX, Chen B, Yang AG, Hu RS, Feng QF, Chen M, Yang XN, Luo CG, Li YY, Wang YY. (2020). The trichome-specific acetolactate synthase NtALS1 gene, is involved in acylsugar biosynthesis in tobacco (*Nicotiana tabacum* L.). *Planta* 252: 13. (https://doi.org/10.1007/s00425-020-03418-x)

Abstract

The glandular trichomes of many species in the Solanaceae family play an important role in plant defense. These epidermal outgrowths exhibit specialized secondary metabolism, including the production of structurally diverse acylsugars that function in defense against insects and have substantial developmental potential for commercial uses. However, our current understanding of genes involved in acyl chain biosynthesis of acylsugars remains poor in tobacco. In this study, we identified three acetolactate synthase (*ALS*) genes in tobacco through homology-based gene prediction using Arabidopsis *ALS*. Quantitative real-time PCR (qRT-PCR) and tissue distribution analyses suggested that *NtALS1* was highly expressed in the tips of glandular trichomes. Subcellular localization analysis showed that the NtALS1 localized to the chloroplast. Moreover, in the wild-type K326 variety background, we generated two *ntals1* loss-of-function mutants using the CRISPR-Cas9 system. Acylsugars contents in the two *ntals1* mutants were significantly lower than those in the wild type. Through phylogenetic tree analysis, we also identified *NtALS1* orthologs that may be involved in acylsugar biosynthesis in other Solanaceae species. Taken together, these findings indicate a functional role for *NtALS1* in acylsugar biosynthesis in tobacco.

Keywords

acylsugar; *NtALS1*; tobacco; acetolactate synthase

Introduction

Acylsugars are a class of secondary metabolites that have been detected thus far in the glandular trichomes of Solanaceae, Caryophyllaceae, Martyniaceae, Rosaceae, and Geraniaceae species, as well as in the roots of Brassicaceae species (Fan et al., 2019; Liu et al., 2019). Acylsugars are formed by esterification of the hydroxyl groups on the sucrose or glucose backbone with the carboxyl groups of branched-chain or linear-chain fatty acids of varying length (C2–C20) (Arrendale et al., 1990; Ghosh et al., 2014; Kroumova et al., 2016; Ghosh and Jones 2017; Moghe et al., 2017; Fan et al., 2019). Acylsugars exhibit various functions such as their involvement in insect resistance and antibacterial activity (Johnson and Severson 1984; Rodriguez et al., 1993; Chortyk et al., 1997; Weinhold and Baldwin 2011; Rodríguez-López et al., 2012; Luu et al., 2017; Ben-Mahmoud et al., 2018). Thus, increasing acylsugar contents has been a long-standing, important breeding target of *Solanaceae* crop species such as tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum*) (Bonierbale et al., 1994; Lawson et al., 1997; Leckie et al., 2012, 2014; Smeda et al., 2016, 2018). In addition, acylsugars have considerable potential for the development of environmentally-friendly natural pesticides (Chortyk et al., 1996; Xia 1998; Puterka et al., 2003) and for commercial uses in food and cosmetic industries (Chortyk et al., 1993; Stubenrauch and Sottmann 2008). Acylsugars are important precursors of flavor compounds and their degradation products contribute to the aroma qualities of tobacco leaves (Jackson et al., 1998; Ashraf-Khorassani et al., 2016). Acylsugars in tobacco (*Nicotiana tabacum* L.) are mainly sucrose esters which have been divided into six groups (group I to group VI) and each group contains a series of isomers (Severson et al., 1985). In general, Flue-cured, Burley, and Maryland tobaccos produce low levels of group I and II acylsugars, and only trace (or undetectable) levels of group III to VI; High quantities of acylsugar groups III to VI are produced by most Oriental and many Cigar tobacco cultivars (Vijay et al., 2010). However, our current understanding of genes involved in acyl chain biosynthesis of acylsugars remains poor. Therefore, elucidating the molecular mechanism of acylsugar biosynthesis is of great significance in the breeding of Solanaceae crops, as well as in the industrial development and utilization of acylsugars.

In recent past years, studies of the acylsugar biosynthetic pathways in *Solanum pennellii, Solanum lycopersicum*, *Petunia axillaris*, and *Salpiglossis sinuata* have furthered our knowledge of the molecular mechanisms and players involved. Related studies have identified a class of trichome-specific acylsugar acyltransferase genes that encode enzymes catalyzing the esterification of acyl-COA with the hydroxyl groups on the sucrose backbone to form different types of acylsugars (Schilmiller et al., 2012, 2015; Fan et al., 2016, 2017, 2019; Moghe et al., 2017; Nadakuduti et al., 2017). The metabolic pathways of branched-chain amino acids (BCAA) are reported to be important sources of fatty acid chains (acyl chains) required for acylsugar synthesis (Kandra and Wagner 1990; Kandra et al., 1990; Walters and Steffens 1990; Slocombe et al., 2008; Mandal et al., 2020). There are two different mechanisms for

fatty acid chain elongation, namely the fatty acid synthase (FAS) elongation pathway and the α-keto acid elongation (α-KAE) pathway (Van der Hoeven and Steffens 2000; Kroumova and Wagner 2003; Slocombe et al., 2008). The long-chain fatty acids used to synthesize acylsugars in tomato and *Datura metel* are mainly produced through a two-carbon elongation pathway mediated by FAS. In tobacco and petunia, however, these long-chain fatty acids are primarily generated by one-carbon elongation mediated by the α-KAE pathway. These keto acid precursors are catalyzed by branched-chain ketoacid dehydrogenase (BCKD) to produce acyl-CoAs (Slocombe et al., 2008), which are the substrates required for the assembly of acylsugars. Although there have been studies showing that BCAA pathways are the important sources of the fatty acid chains of acylsugars (Kandra and Wagner 1990; Kandra et al., 1990; Walters and Steffens 1990; Slocombe et al., 2008; Mandal et al., 2020), However, BCAAs are primary metabolites and are present in various tissues throughout the plant, whereas, in Solanaceae species, acylsugars are secondary metabolites that are synthesized only in glandular trichomes on the leaf surface. Additionally, in tomato, the glandular trichome-specific isopropylmalate synthase (IPMS) SlIPMS3 was shown to carry a C-terminal 160 amino-acid truncation compared to the IPMSs involved in leucine synthesis in other plants and microorganisms (Ning et al., 2015). Although SlIPMS3 has maintained activity, it has lost sensitivity to leucine feedback inhibition, which also contributes to differences in the fatty acid chain composition of acylsugars between cultivated tomato and its wild relative *Solanum pennellii* (Ning et al., 2015). Moreover, through transcriptomic comparison of trichomes and leaves without trichomes in *Nicotiana benthamiana* and tomato, multiple genes involved in BCAA metabolism and fatty acid chain elongation were specifically or highly expressed in glandular trichomes (Slocombe et al., 2008). Virus-induced gene silencing of the E1-β subunit of BCKD, which is highly expressed in the glandular trichomes of *N. benthamiana* and tomato, suggested the involvement of BCKD in the synthesis of branched-chain acyl-CoA of acylsugars (Slocombe et al., 2008). Silencing of the tomato β-Ketoacyl-ACP synthase I (*KAS I*) gene, which is also highly expressed in glandular trichomes, showed that different KAS I subtypes participate in the elongation of the acylsugar fatty acid chain to varying degrees. Therefore, although the acylsugar acyl chain-precursor biosynthetic genes that are expressed highly or specifically in glandular trichomes exert their functions through the BCAA metabolism pathway, these genes are distinct from those involved in BCAA biosynthesis in other tissues. Aside from the above-mentioned genes, our current understanding of the molecular mechanisms and other genes involved in fatty acid chain biosynthesis of acylsugars remains poor. Therefore, the identification and characterization of genes involved in these metabolic processes is essential for understanding acylsugar biosynthesis, their biological production, and utilization.

Acetolactate synthase (ALS) is a key enzyme that catalyzes the common synthetic pathway of BCAA, such as valine, leucine, and isoleucine, during plant growth (Binder et al., 2007). ALS uses two pyruvate molecules as substrates to synthesize acetolactate, which is gradually converted into valine and leucine. Alternatively, ALS uses pyruvate and 2-butenoic acid as substrates to synthesize acetohydroxy

acid, which is gradually converted into isoleucine. Since animals do not contain ALS, this enzyme is a common target of many highly selective, low-toxic chemical herbicides, such as sulfonylurea and imidazolinone herbicides. Since the 1970s and 1980s, the development of herbicides targeting ALS and the characterization of how mutations in *ALS* genes affect herbicide effectiveness have been subjects of intense research efforts (Mazur et al., 1987; Lee et al., 1988; Duggleby et al., 2008; Shimizu et al., 2008; Xie et al., 2019). However, there are few reports on other aspects of *ALS*. One study incubated epidermal strips, cut from the midribs of mature tobacco leaves, with radiolabeled BCAA biosynthetic precursors and the herbicide chlorsulfuron, the latter of which specifically inhibits ALS. Results suggested that chlorsulfuron does not affect diterpene synthesis but significantly reduces the contents of acylsugar branched-chain fatty acids while increasing the contents of acylsugar linear-chain fatty acids in glandular trichomes (Kandra and Wagner 1990). It has been reported that there are two ALS genes (*SuRA* and *SuRB*) in *N. tabacum* (Lee et al., 1988; Keeler et al., 1993). Previously, expression analysis of *SuRA* and *SuRB* showed that there was a 3 to 4 fold variation in the expression levels of two genes in different organs and the expression variability of both genes correlated with the developmental stage of the samples, with the highest levels of expression found in seedlings and young leaves, intermediate levels found in stem sections and in flowers, and the lowest levels found in mature leaf samples (Keeler et al., 1993). Moreover, *SuRA* and *SuRB* were most prevalent in dividing cells of stems, roots, and floral tissue, and *SuRB* consistently expressed at higher levels than the *SuRA* (Keeler et al., 1993). However, the identity of those specifically involved in the synthesis of acylsugar acyl chain remains unclear.

In this study, using the *Arabidopsis ALS* gene as a query sequence (Binder 2010), we identified three *ALS* genes in a cultivated tobacco genome through homology-based gene prediction. We found that *NtALS1* is specifically expressed in the tips of glandular trichomes and its protein product is localized to the chloroplast. We subsequently used CRISPR-Cas9 technology to generate two *ntals1* loss-of-function mutants. Compared with the wild type, the acylsugars contents in the mutant lines were significantly reduced, indicating that *NtALS1* functions in acylsugar biosynthesis in tobacco. Through phylogenetic tree analysis, we also identified NtALS1 orthologs that may be involved in acylsugar biosynthesis in other Solanaceae species.

1 Materials and Methods

1.1 Plant Material and Growth Conditions

Tobacco (*Nicotiana tabacum* L.) seeds used in this study were obtained from the National Infrastructure for Crop Germplasm Resources (Tobacco, Qingdao). Tobacco (including flue-cured tobacco Hongda and K326), *Nicotiana tomentosiformis*, and *Nicotiana sylvestris* were germinated and cultivated in a greenhouse in Qingdao, Shandong province of China, according to the sowing and farming methods routinely used in the locality. *Nicotiana benthamiana* seeds were

germinated and planted in an artificial climate-controlled chamber under a 16-h/8-h light/dark cycle at a constant temperature of 24°C and relative humidity of 65%.

1.2 Trichome Isolation, RNA Extraction and qRT-PCR

Trichomes were isolated by a mechanical abrading method using glass beads (Amme et al., 2005). Upper leaves (approximately 15–25 cm in length, 80-d after transplantation) were cut into strips of 1–2 cm wide and 5–8 cm long, which included the main central vein, and then snap-frozen in liquid nitrogen until further use. Approximately 4 g of frozen leaf strips and 5–6 g of glass beads (average size 1.2–1.5 mm) precooled in liquid nitrogen were placed into a 50-mL plastic tube. After mechanical abrasion by shaking for 30–40 s, the mixture was filtered through a sieve (with a pore size of 0.25 mm) to separate trichomes from the remaining leaf tissue and glass beads. Total RNA was extracted from the isolated trichomes and leaves (with trichomes removed) following the standard protocol of the TaKaRa MiniBEST Plant RNA Extraction Kit (TaKaRa, Japan). cDNA was synthesized from 1.0 μg of total RNA according to the recommendations of the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Japan). qRT-PCR (cDNA equivalent to 100 ng was used as the template in 20-μL volume) was carried out on the Applied Biosystems 7500 Real-Time PCR system. The cycling program was: 95°C for 30 sec, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec, then 95°C for 15 sec, 65°C for 1 min, 97°C for 1 sec. Actin was used as the endogenous control. Gene ID and accession number used in this study are listed in Table 3-1. The specific primers used to detect different *ALS* transcripts are listed in Table 3-2.

Table 3-1 Gene ID and Accession Number used in this study

Species	Gene	Gene ID in SGN	Accession Number in NCBI
N. tabacum	*NtALS1*	Nitab4.5_0001530g0160.1	XM_016609038.1
	NtALS2	Nitab4.5_0010961g0010.1	X07645.1
	NtALS3	Nitab4.5_0011199g0020.1	X07644.1
	NtActin	Nitab4.5_0001800g0170.1	XM_016618073.1
N. attenuata	*NaALS1*	NIATv7_g04850.t1	XM_019392063.1
	NaALS2	NIATv7_g23189.t1	XM_019368947.1
N. benthamiana	*NbALS1*	Niben101Scf01491g00004.1	-
	NbALS2	Niben101Scf05032g00009.1	-
	NbActin	Niben101Scf06087g02002.1	-
N. tomentosiformis	*NtoALS1*	mRNA_31276	XM_009613307.3
	NtoALS2	mRNA_36947	XM_009617241.3
	NtoActin	mRNA_41835	XM_009620607.3
N. sylvestris	*NsALS1*	mRNA_72505	XM_009771393.1
	NsActin	mRNA_78733	XM_009775623.1
S. lycopersicum	*SlALS1*	Solyc06g059880.3.1	XM_004242005.4
	SlALS2	Solyc03g044330.1.1	XM_004234616.3
	SlALS3	Solyc07g061940.4.1	XM_010326113.2
S. pennellii	*SpALS1*	Sopen06g019960	-
	SpALS2	Sopen03g008440	XM_015214237.2
	SpALS3	Sopen07g030210	XM_015226148.1
S. tuberosum	*StALS1*	Sotub06g018760.1.1	XM_006356659.2
	StALS2	Sotub07g025380.1.1	XM_006348295.2
	StALS3	Sotub10g015690.1.1	XM_006361678.2
P. axillaris	*PaxALS1*	Peaxi162Scf00591g00413.1	-
	PaxALS2	Peaxi162Scf00377g00074.1	-
A. thaliana	*AtALS*	AT3G48560*	NM_114714.3
Z. mays	*ZmALS1*	Zm00001d050567**	NM_001148702.2
	ZmALS2	Zm00001d016572**	NM_001158289.2

*: Gene ID comes from TAIR (www.arabidopsis.org);
**: Gene ID comes from Ensembl Plants (plants.ensembl.org/index.html/);
-: The corresponding gene accession number cannot be found in NCBI (www.ncbi.nlm.nih.gov/).

Table 3-2 All primers used in this study.

Primer name	Sequence (5' to 3')	Notes
For qRT-PCR analysis		
qRT-NtActin_F	CCACACAGGTGTGATGGTTG	The primer pairs of the control gene in *N.tabacum.*
qRT-NtActin_R	GTGGCTAACACCATCACCAG	
qRT-NtALS1_F	TCCTTCCTCCTCCGCTTTCTAT	The specific qRT-PCR primers for NtALS1.
qRT-NtALS1_R	CGTCGGTAATGGTAAAACGGC	
qRT-NtALS2_F	CACTTTGGAAAGGTTGAGTGTG	The specific qRT-PCR primers for NtALS2.
qRT-NtALS2_R	GCGCTCTATTTAGTCGCCAT	
qRT-NtALS3_F	TCAATCAAGTAGGACCCTTTCAG	The specific qRT-PCR primers for NtALS3.
qRT-NtALS3_R	TGAGAGCAGTGAGAGACGAA	
qRT-NtoActin_F	CCACACAGGTGTGATGGTTG	The primer pairs of the control gene in *N. tomentosiformis.*
qRT-NtoActin_R	GTGGCTAACACCATCACCAG	
qRT-NtoALS1_F	TTGGGACAGACGCATTTCAA	The specific qRT-PCR primers for NtoALS1.
qRT-NtoALS1_R	ATCGCGCTAGATTAAACGCT	
qRT-NtoALS2_F	TCAATCAAGTAGGACCCTTTCAG	The specific qRT-PCR primers for NtoALS2.
qRT-NtoALS2_R	TGAGAGCAGTGAGAGACGAA	
qRT-NsActin_F	CCACACAGGTGTGATGGTTG	The primer pairs of the control gene in *N. sylvestris.*
qRT-NsActin_R	GTGGCTAACACCATCACCAG	
qRT-NsALS1_F	CACTTTGGAAAGGTTGAGTGTG	The specific qRT-PCR primers for NsALS1.
qRT-NsALS1_R	GCGCTCTATTTAGTCGCCAT	
qRT-NbActin_F	CAAGGAAATCACCGCTTTGG	The primer pairs of the control gene in *N.benthamiana.*
qRT-NbActin_R	AAGGGATGCGAGGATGGA	
qRT-NbALS1_F	AGCATCTCGTATTCTTGTATTTCG	The specific qRT-PCR primers for NbALS1.
qRT-NbALS1_R	ATAAGCAGAGCCACAGCATC	
qRT-NbALS2_F	TAGTTTTTGCCGTGGCTTTG	The specific qRT-PCR primers for NbALS2.
qRT-NbALS2_R	CCAAGTGGATGTTGCAAATAGAG	
For *NtALS1* gene coning		
NtALS1_F	ATGGCGGCAGCTTCTCCAACT	The primer pairs used in NtALS1 cloning.
NtALS1_R	TCAGTAGGAAGATCTCCCATCGCC	

For gene tissue localization (pCambia35tlegfps2#4 vector, Pst1 and BamHI cut site)		
P-NtALS1_F	AGTAAGAGGGTTGCTATAGAAAGCG	The primer pairs used in NtALS1 promoter cloning.
P-NtALS1_R	GATATGGCTGATAAGGGAGCTAAAG	
TL-NtALS1_F	ATTACGAATTTCGACCTGCAGAAGAGAAGTGACG GGTGCTTGT	The primer pairs used for vector construction.
TL-NtALS1_R	CTGCGGCCGCGCCGGATCCTGTTGAGGGACAAAT GCATGAAAT	
NtALS1_394_F	TTTACACTTTATGCTTCCGGCT	The primer pairs used in correct constructs selection.
NtALS1_480_R	TTACGTCGCCGTCCAGCTCGA	
For gene subcellular localization (pCambia35tlegfps2#4 vector, Kpn1 and BamHI cut site)		
SCL-NtALS1_F	CGAACGATAGCCATGGTACCATGGCGGCAGCTTC TCCAACT	The primer pairs used in NtALS1 cloning.
SCL-NtALS1_R	CTGCGGCCGCGCCGGATCCGTAGGAAGATCTCCC ATCGCC	
NtALS1_64_F	CAATCAAGCATTCTACTTCTATTGCAGCA	The primer pairs used in correct constructs selection.
NtALS1_480_R	TTACGTCGCCGTCCAGCTCGA	
For CRISPR-Cas9 constructs and positive plants selection		
NtALS1-Cas9-Target1	CCTCTTATCCGGCCGGCCTTACC	Three CRISPR/Cas9 target sites of *NtALS1*.
NtALS1-Cas9-Target2	CCCTCAGCAACCGCCGTTTTACC	
NtALS1-Cas9-Target3	CCGTTTTACCATTACCGACGCTC	
NtALS1- Target1-F	CAGTGGTCTCAAGTGGTAAGGCCGGCCGGATAAG	The primer pairs used for Intermediate carrier construction of NtALS1-Cas9-Target1.
NtALS1-Target1-R	CAGTGGTCTCAAAACCTTATCCGGCCGGCCTTAC	
NtALS1-Target2-F	CAGTGGTCTCAATTGGTAAAACGGCGGTTGCTGA	The primer pairs used for Intermediate carrier construction of NtALS1-Cas9-Target2.
NtALS1-Target2-R	CAGTGGTCTCAAAACTCAGCAACCGCCGTTTTAC	
NtALS1-Target3-F	CAGTGGTCTCAATTGAGCGTCGGTAATGGTAAAA	The primer pairs used for Intermediate carrier

NtALS1-Target3-R	CAGTGGTCTCAAAACTTTTACCATTACCGACGCT	construction of NtALS1-Cas9-Target3.
NtALS1-zmpl-cas9-F	GCACCCGGTGGAGAACACGC	The primer pairs used for the selection of positive plants of transformation.
NtALS1-zmpl-cas9-R	GTTCAGGTACGCGTCATGGGC	
CX-NtALS1-F	GGCCATACAAACACCAGGGA	The primer pairs used for target sites sequencing and mutant screening.
CX-NtALS1-R	AATGTTGGCTGCAGAAAGCG	

1.3 Tissue and Subcellular Localization

The tissue localization reporting vectors of *NtALS1* were respectively constructed based on pCambia35tlegfps2#4 by the homologous recombination method. The promoter region of *NtALS1* was amplified from the DNA of tobacco Hongda by PCR, using primer pairs listed in Table 3-2, and the PCR product was inserted into the pCambia35tlegfps2#4 plasmid via PstI and BamHI double digestion to produce the entry vector pCambia35tlegfps2#4::pNtALS1-GFP using the ClonExpress MultiS One Step Cloning Kit (Vazyme, China). The subcellular localization reporting vectors of *NtALS1* were constructed similarly, only with the CDS of *NtALS1* amplified from the cDNA of Hongda's trichomes and inserted into the pCambia35tl:egfps2#4 plasmid via KpnI and BamHI double digestion. Primers used for these sections are listed in Table 3-2.

The above constructs were transformed into *Agrobacterium tumefaciens* strain LBA4404, followed by inverted culturing on LB media containing kanamycin (50 mg/L) and rifampicin (50 mg/L) at 28°C for 36–72 h. A positive single *Agrobacterium* colony of each vector was selected for culture growth at 28°C and 220 rpm overnight. The *Agrobacterium* cells were collected by centrifuging at 5000 rpm for 5 min, suspended and washed with sterile ddH_2O, re-collected by centrifuging, and suspended in a prepared suspension solution containing 98 mL sterile ddH_2O, 1 mL 1 M MES, 1 mL 1 M $MgCl_2$, and 100 μL 100 mM Acetylsyringone. A_{600} (the absorbance at 600 nm) of the *Agrobacterium* liquid was measured and adjusted to 0.600–0.800 by a UV-visible spectrophotometer.

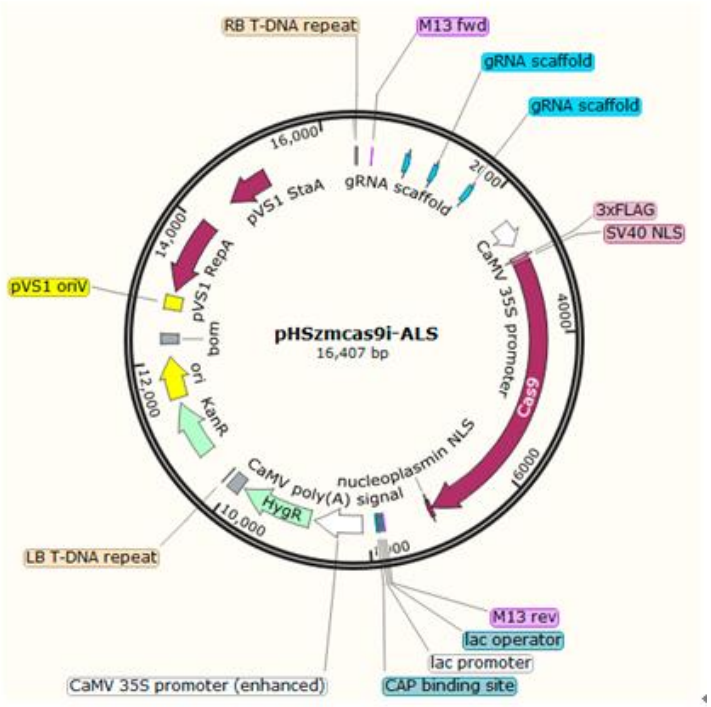
The above *Agrobacterium* preparations were incubated at 28°C in the dark for 3 h and then used to inoculate *N. benthamiana* leaves through their abaxial side (plants at the 4–5 true leaf stage) using a 1 mL injection syringe without the needle. For tissue localization analysis, the lower epidermis of the inoculated leaves was observed at 3 d post infiltration with a confocal microscope (Leica TCP S8) using an excitation wavelength of 488 nm and a receiving range of 500–540 nm. For subcellular localization analysis, the inoculated leaves were observed for chloroplast autofluorescence.

1.4 CRISPR-Cas9 Vector Construction and Plant Transformation

The Cas9 expression backbone vector pBWA(v)HS-zmcas9pl was used in this paper. For our three-target vector, the individual annealed sgRNA oligonucleotide pairs (Table 3-2 and Figure 3-1) were cloned into pBWA(V)HS-zmplcas9 and two intermediate vectors pBWD(LB)DNAi and pBWD(LA)DNAi, respectively. Following this, sgRNA2 in the intermediate vector pBWD(LB)DNAi and sgRNA3 in intermediate vector pBWD(LA)DNAi were successively inserted through Golden Gate cloning into the expression vector pBWA(v)HS-zmcas9pl carrying sgRNA1 to form the final recombinant plasmid. The constructs were transferred into *A. tumefaciens* strain LBA4404. Tobacco transformation was carried out by the leaf disc method. Transgenic tobacco lines were genetically identified by PCR analysis using specific primers. Homozygous transgenic plants were used for further

investigation. Primers used for these sections are listed in Table 3-2.

a



b

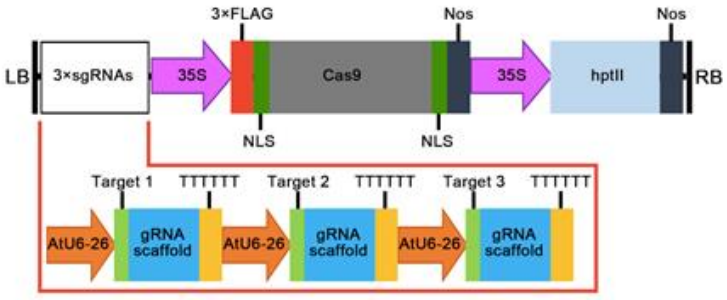


Figure 3-1 a Construct maps, b schematic diagram.

1.5 GC/MS Analysis

Sample preparation and pretreatment were carried out according to a previously reported method (Vijay et al., 2010). Using a sharpened 16-mm stainless steel punch,

twelve leaf disks were removed from fresh leaves of each sample and transferred to a 20-mL glass tube. Leaf disks were washed twice with 7.5 mL of HPLC grade dichloromethane by vibrating for 30 s and collected and pooled in another collection tube. Then, 0.5 g of anhydrous Na_2SO_4 was added into each collection tube, and the tubes were allowed to stand overnight. The extracts were then filtered through a 0.45-μm nylon membrane into a new tube, and 50 μL of an internal standard solution containing 200 μg/mL heptadecane (C-17 alkane) and 200 μg/mL sucrose octa-acetate in dichloromethane was added. Then, the solvent was evaporated by heating at 40°C under a stream of N_2. One hundred microliters of a 1:1 mixture of BSTFA:DMF was added to the tube, capped tightly under a N_2 headspace, and heated at 75°C for 30 min. After cooling, 100 μL of 1:1 BSA:pyridine solution was added, and the mixture was filtered through a 0.22 μm nylon membrane into a liner-filled GC-MS vial and stored at -20°C until subsequent analysis.

The GC-MS analysis was conducted using GC/MS-QP2010 plus (SHIMADZU) and a 30-m Rtx-5MS column (30 m × 0.25 μm × 0.25μm). The carrier gas was helium at a linear gas velocity of 38.6 cm/sec. The injector was set at 265°C. The temperature of both the ion source and transmission line was set at 280°C. The analysis consisted of a temperature increase from 165°C to 280°C at a rate of 4°C per minute, followed by a hold at 280°C for 25 min. A 1-μL sample was injected in a split mode with a split ratio 15:1. α- and β-cembratrien-diol were identified referencing the Chinese Tobacco Industry Standards YC/T 470-2013. Acylsugars were identified according to their specific ions described in the document (Severson et al., 1985).

1.6 Multiple Sequence Alignments and Phylogenetic Analyses

Multiple sequence alignments and phylogenetic analyses was performed using DNAMAN7. The construction of phylogenetic trees was based on the default parameter settings, and a bootstrap test of 1,000 replicates was used to assess the reliability of the phylogeny. Gene ID and accession number used in this study are listed in Table 3-1.

2 Results

2.1 Screening and Expression Analysis of ALS Genes in Cultivated Tobacco

To identify *ALS* genes in cultivated tobacco, we used the coding sequence (CDS) of the Arabidopsis *ALS* gene (AT3G48560, https://www.arabidopsis.org/) as a query sequence and performed homology-based gene prediction using the genomic data of the cultivated tobacco variety K326 deposited in the Solanaceae Genomics Network (SGN, https://solgenomics.net/). Three genes homologous to Arabidopsis *ALS* were identified, namely *NtALS1*, *NtALS2* (*SuRB*), and *NtALS3* (*SuRA*) (Figure 3-2). We subsequently evaluated the expression levels of *NtALS1*, *NtALS2*, and *NtALS3* in different tissues of the flue-cured tobacco cultivar Hongda, specifically the roots, glandular trichome-removed stems, glandular trichomes on the stems, glandular

trichome-removed leaves, glandular trichomes on the leaves, glandular trichome-removed flowers, and glandular trichomes on the flowers. The results showed that the expression levels of *NtALS1* in glandular trichomes on stems, leaves, and flowers were significantly higher than both *NtALS1* expression in other tissues and the expression levels of *NtALS2* (Figure 3-3). *NtALS2* was expressed weakly in each of the seven tissues tested, with no significant difference in expression level among different tissues, whereas almost no *NtALS3* expression was detected (Figure 3-3). Since acylsugars are specifically synthesized in the tips of glandular trichomes (Kandra and Wagner 1988), we speculate that *NtALS1* is involved in acylsugar biosynthesis in tobacco.

Figure 3-2 Sequence alignment of homologous *ALS* proteins from tobacco and Arabidopsis. (Highly conserved sequence is indicated by the red line above the alignment.)

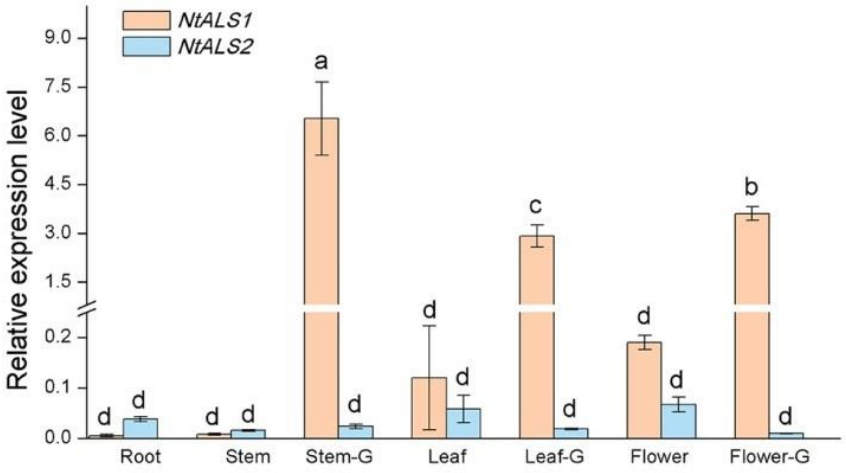


Figure 3-3 Expression pattern analysis of ALS genes in tobacco. Expression patterns of the three *NtALS* genes in seven tobacco tissues, including root, stem (with trichomes removed), stem-G (glandular trichomes from stem), leaf (with trichomes removed), leaf-G (glandular trichomes from leaf), fower (with trichomes removed), and fower-G (glandular trichomes from fower) in Hongda tobacco. The expression of *NtALS3* is too low to be detected. Three technical replicates were included for each sample, and the data are depicted

as means±standard deviation (SD). Multiple comparison was used to analyze signifcant diferences, which are indicated by diferent letters ($P<0.05$).

2.2 Tissue-Specific and Subcellular Localization of NtALS1

To further clarify the tissue and subcellular localization of NtALS1, we cloned the genomic *NtALS1* promoter (Figure 3-4) and the *NtALS1* CDS, the latter from the glandular trichome cDNA of Hongda. Using these isolated sequences, we constructed a tissue-localization reporter plasmid, with expression of the green fluorescent protein (GFP) driven by the *NtALS1* promoter. We also constructed a subcellular-localization reporter plasmid, with expression of the GFP-tagged NtALS1 protein driven by the 35S promoter. We then transiently infected *N. benthamiana* with these two reporter plasmids and analyzed the tissue and subcellular localization of NtALS1. For tissue localization analysis, the GFP signal could only be detected in the tip cells of glandular trichomes, indicating that *NtALS1* was specifically expressed in these cells (Figure 3-5a). For subcellular localization analysis, the signal from GFP-tagged NtALS1 overlapped well with chloroplast autofluorescence, indicating that NtALS1 is localized to the chloroplast (Figure 3-5b).

GATATGGCTGATAAGGGAGCTAAAGGAAAAGGAAGAGAAGTGACGGGTGCTTGTGGAGGAGAGA
AAACACACGTTGGAATTAATGGAGTATTTCCACACAAAGCCAAGAGTGGGAGTGTGTTCCCGAA
AGAGCAAAAATCAGTGAAGGCCATGGCTGTTGAAAAGATTGGGAAAGTTGTGACTTCTACTTTC
AAGAATAACAAGAAAAAGAACAATCCTCACGACAACTAGGAGTATACCACGTCTAGGACATGGT
TTGTTTCTTATTCTAGAGATGTTTGAAATTTTATTTACAAATGCAAATAAATAACTCTCTACTA
CGTGGTATTAGGATTCTACGTACTAATATACGTGCTGTTGCTACAACTTCTGTAATCTCAAATG
TGGTATTGTAATTAAAATGTTTTTCTTGGAGTCTATTTGAAAAGACACTTTGTAATTGGATTTG
GTGTAATCTTAAAAGTTACACAATATAGTAAAAATTGCCTGGATATATTTGTTTGCCATACGTA
ATTAAACTATAATTTAATGTGCCATCTTTGGTTGCACAGGTGTGTAATTACATAATCATATATT
TTTATTTTAAACTAAAGTTAATAGTAAAATTAAATAATTTTTTTTAGATGGAAAAGGTAAAACT
TTAATGTGTGACGAATATATGTGCATATAAAAAATATTAGTAATTTTTTTATAACTTATTAAGT
ATATCTAAAATATACATATTTTTGAAAAAAATAATGTTAATAATTATAAATTGGTAAGTAATAC
TATCGTAAAGAGTAATCAGTATCATCTTCCTACTCGGGTGAATATAATGAAATTTAGAATTGTC
CCATTTTTTAAAAAACAAAAAAATATGTATGTGTTTAGATATATTTTCACTATATTGCACTAAA
AGAAAGTAGAAAAAGAAGCTAGTAAGACACGAAACACCAAAGTGTTTTTAACAAGTATTTCTCC
GCATAGTTGAAATGGAAGAGATAGAGTGTGAAGAGAATATTATATTTGAAAAATTAAGAATGAA
AGAGAATTTAAAAAATTACCTACACATCATGTAACTAGTAATTACTACTAGTAATTTTCACTTT
CAACTTGAGATTTGAAAAGGTGTAATTGCTCTCCGTCAATATTACATCCGATTTCATACTTGGC
AAATAGATAGTCTGATCTTATTAAGGTTTTGTTTTACTTAATTATCCTTATTAATGGTTTTTAA
AGTTGTAACTCCAATAAATTTGAGAAGTGTGGAAATTTAATACAAGCTAAAAGTAATATTAGAA
GAAAATAATAATTTTTTATTTATTTACTAAATCGGACTAGTCAAAGGAAATGAGGGGTATTAAA
TTACTTTTTTACATCAATTAACACAACAACAATATATTCAATGAAATTTTATAACTGGAGTCTG
ACAGATCATGCAGTTACATTGGACAGATAGAGAGGTTATTTCCACACTCTCGACTCAAAGACAA
GCATATTCACGTTGTGGTGCAAACATGATGCGTACAAAAATATTTTTTTTCCTGATTGACGCAC
TAATGAAACTTAATTATACGGCGTGCATCAAACGGAGATCGAGATGATCTGTAGAAGTTTGGTT
TCTAGATGTTGGAAGAAGTTAGCTAAAAGTTGGCAGAACAGTGTAAATGTTGGCTGCAGAAAGC
GGTACCTCTACATTTGAACTTTGTTAAAAAGCTGCTACTACTTTTGACGGCTACCCATTATAGT
ACAACCTGAATCCTTGATGTGGCTTGATCGAGTTGATATTATGCCGCTATTTTTATCACTTTAA
TTTCCTGCCTAAATTCATTGGACCCGTATCAAAGTCAATTTTTTCTTTTATTACGATTAGTACT
TTTTAGATCAGTTATACTGTAATAATAAAAATCGTTTGCTCTCTAATGCAATTTCCATTCTCTT
TTGAGTCTCGTGATGCACCCTATGCTAGCGTCTTAATTATATATCAACATTCCATTTAGTATAA
AGTGGACTCTCAAATCTCAGCTGTGAAACTGACCTCTCTTAATTTCATGCATTTGTCCCTCAAC
AATG

Figure 3-4 Promoter sequence of *NtALS1*.

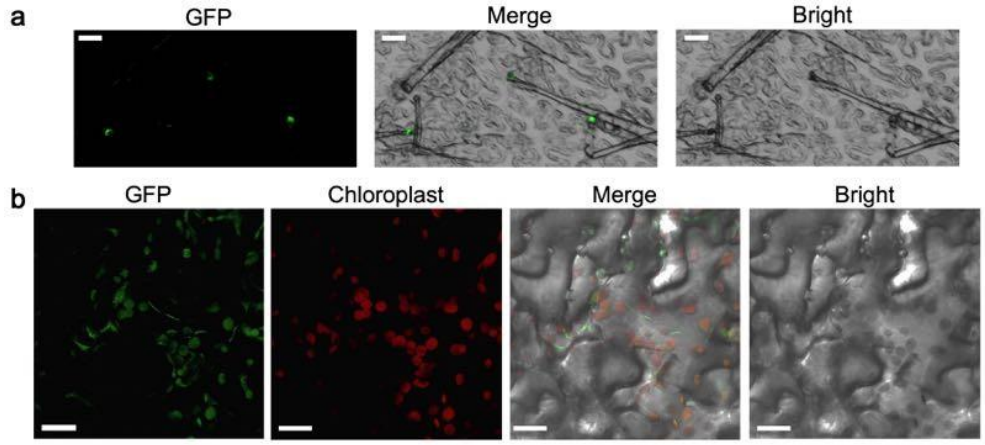


Figure 3-5 Tissue-specific localization and subcellular localization of NtALS1. (The red font represents the start codon.) **a** GFP forescence in the tip cells of trichomes following transient expression of GFP driven by the NtALS1 promoter in N. benthamiana. The images represent the abaxial epidermis of inoculated leaves. Bar=100 μm. **b** The experiment was conducted by using transient expression in N. benthamiana, and the photos were obtained using inoculated leaves. NtALS1 was localized at the chloroplast. Bar=20 μm

2.3 Functional Analysis of NtALS1

To verify whether *NtALS1* is involved in acylsugar biosynthesis in tobacco, we used CRISPR-Cas9 technology to knock out *NtALS1* in the flue-cured tobacco cultivar K326. Since NtALS1 has three exons, two introns and 586 conserved C-terminal amino acids (Figure 3-6a), we designed and constructed plasmid carrying three guide RNAs (gRNAs) that specifically targeted the first exon of *NtALS1* (Figures 3-1b, 3-6b) and introduced it into K326 through Agrobacterium-mediated transformation. From the T_0 transgenic lines, two heterozygous mutant strains were obtained and named *ntals1-1* and *ntals1-2*. The gRNA target sites in ten plants from each T_1 mutant population were sequenced. Finally, three plants homozygous for a 2-bp insertion mutation were identified from the T_1 plants of *ntals1-1*, and two plants homozygous for a 40-bp deletion were identified from the T_1 plants of *ntals1-2* (Figure 3-6b-c).Subsequently, we selected plants of the wild type and two mutant K326 lines for trichome secretion profiling. Care was taken to select control plants that displayed comparable growth and development and were at the same developmental stage as the mutants at the time of sampling. Surface secretions of these plants were extracted with dichloromethane and analyzed by gas chromatography-mass spectrometry (GC-MS) (Figure 3-6d-e). As cembranoid diterpenes are the most abundant components in trichome's secretions and they are also trichome-sepecific metabolites (Yan et al., 2016), in addition to analyzing acylsugars, we also payed attention to the cembranoid diterpenes. The results showed that, compared with the wild-type K326 plants, the mutant plants had similar contents of α-cembratrien-diol and β-cembratrien-diol but significantly lower

contents of acylsugars (Figure 3-6f, Figure 3-7). These results indicate that *NtALS1* plays an essential role in acylsugar biosynthesis in tobacco.

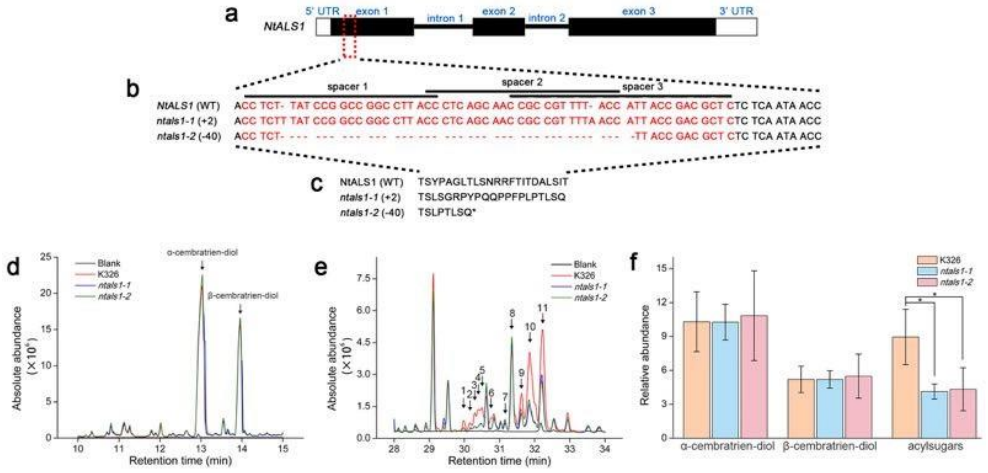


Figure 3-6 Genotypic and phenotypic characterization of the wild type and *ntals1* loss-of-function mutants. a Targeted mutagenesis of NtALS1 in K326 using the CRISPR-Cas9 system. A schematic of gene structure of NtALS1 is shown. The spacer location (target sites) is marked by red dotted box. **b** sgRNA:Cas9-induced tobacco NtALS1 gene mutations at the target site in the mutants. The sequence in red font under the black line indicates the spacer sequence. **c** Amino acid sequence alignment of the wild-type NtALS1 protein and those in the ntals1 mutants. GC–MS chromatogram comparison of the diterpenes **d** and acylsugars **e** profle of the wild type K326 and ntals1 mutants. Black arrows indicate the peaks of two diterpenes **e** and different acylsugars **e** (indicated by the arrows, peak 1 to peak 6 belong to group I acylsugar and peak 7 to peak 11 belong to group II acylsugar). **f** Comparison of relative abundances of acylsugars and diterpenes in the wild type and ntals1 mutants. Three plants were tested for each line and data are mean values±SD. Values labeled with asterisks are signifcantly different (*P<0.05).

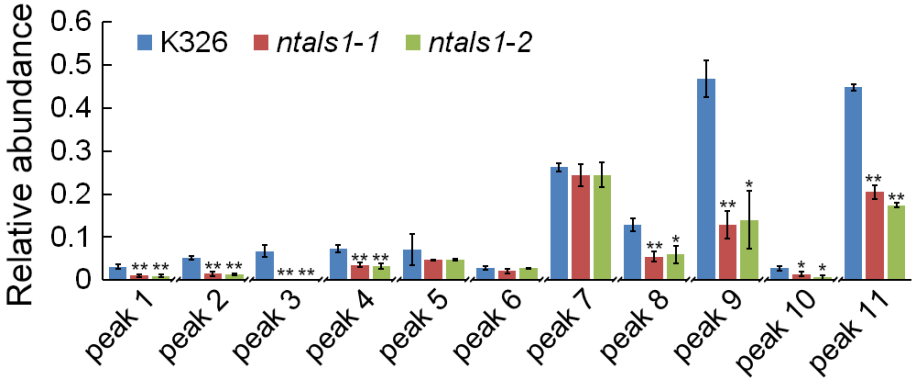


Figure 3-7 Relative abundance of each peak of group I and group II acylsugars in the wild type and ntals1 loss-of-function mutants. (Peak 1 to peak 6 belong to group I

acylsugars and peak 7 to peak 11 belong to group II acylsugars. Data were statistically analyzed with *t*-test. $*P < 0.05$, $**P < 0.01$.)

2.4 Phylogenetic Analysis of ALS and Expression Analysis of ALS Genes in Diploid Tobacco Varieties

N. tabacum has two diploid ancestors, namely *N. tomentosiformis*, which contains acylsugars, and *N. sylvestris*, which does not contain acylsugars (Moghe et al., 2017). To determine the source of *NtALS1* and further explore whether other acylsugar-containing Solanaceae crop species have *NtALS1* homologs, we used the NtALS1 protein sequence as the query sequence to consult the *N. tomentosiformis*, *N. benthamiana*, *N. attenuata*, *S. lycopersicum*, *S. pennellii*, *P. axillaris*, *S.tuberosum*, and *N. sylvestris* protein databases in SGN using the BLASTP algorithm. We identified three ALS homologs in both *S. lycopersicum* and *S. pennellii*, two ALS homologs in each of *P. axillaris*, *N. tomentosiformis*, *N. benthamiana*, and *N. attenuata*, and one ALS homolog in *N. sylvestris* (Figure 3-8). These identified protein sequences were combined with those ALS sequences in *N. tabacum*, Arabidopsis, and maize (*Zea mays*) in a phylogenetic analysis of ALS homologs (Figure 3-9). The results showed that the ALS homologs of the Solanaceae family are clearly separated from those of the Brassicaceae and Poaceae families. ALSs in Solanaceae species have also differentiated during evolution and exhibited obvious clustering into two major categories. Moreover, in the above-mentioned Solanaceae species, each acylsugar-containing species possessed an ALS homolog that clustered together with NtALS1, whereas the ALS homolog of N. *sylvestris*, a species that does not contain acylsugars (Moghe et al., 2017), did not cluster with NtALS1. As *NtALS1* is a trichome-specific expression gene, we speculate that the ALS homologs that cluster together with *NtALS1*, including *NaALS1*, *NtoALS1*, *NbALS1*, *SlALS1*, *SpALS1*, *PaxALS1*, and *StALS1*, are highly expressed in the glandular trichome.

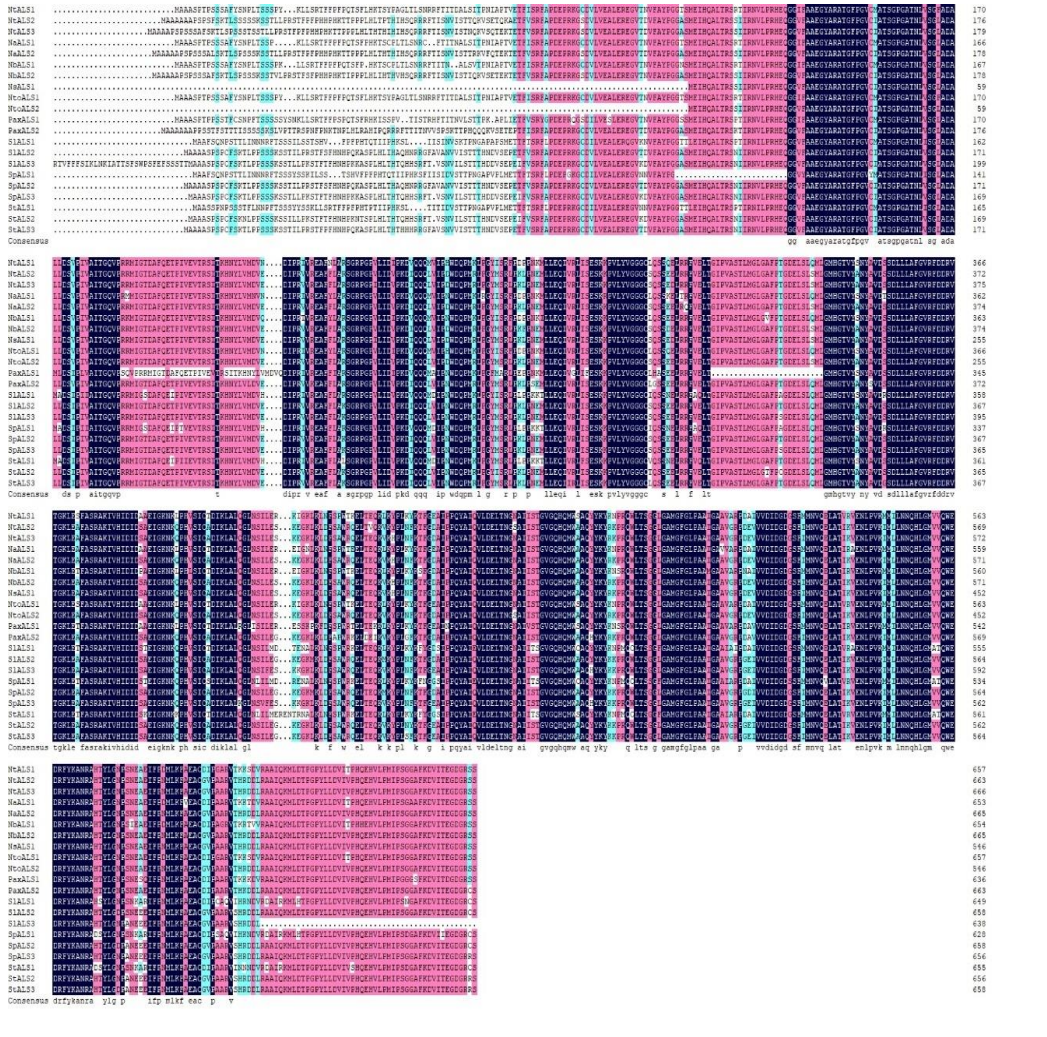


Figure 3-8 Sequence alignment of ALS in Solanaceae. Nt, N. *tabacum*; Na, N. *attenuata*; Nb, N. *benthamiana*; Ns, N. *sylvestris*; Nto, N. *tomentosiformis*; Sl, S. *lycopersicum*; Sp, S. *pennellii*; Pax, *Petunia axillaris*; St, *Solanum tuberosum*.

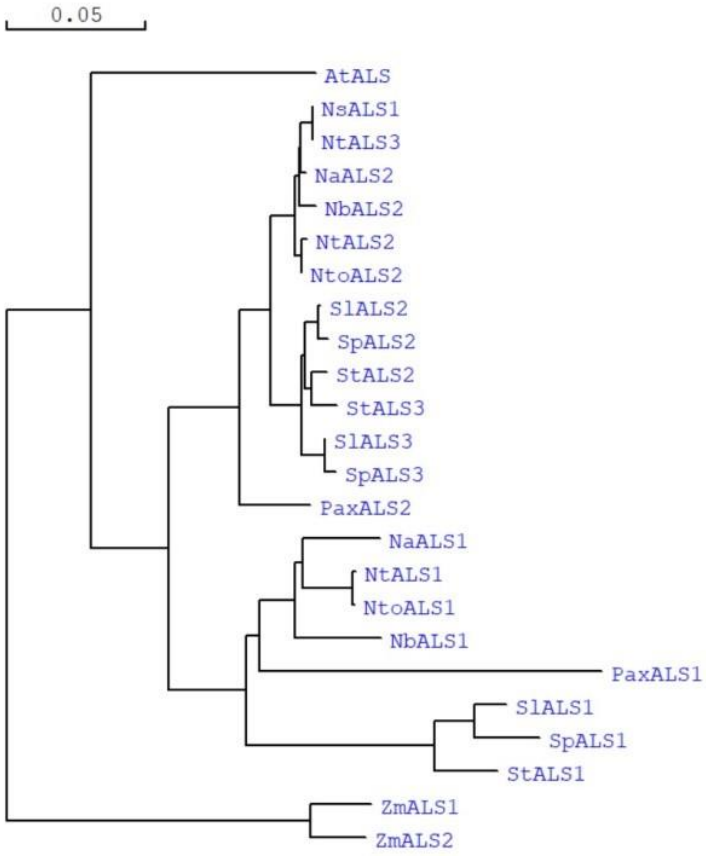


Figure 3-9 A phylogenetic analysis of ALS homologs indicates that there are potential trichome-specifc ALS genes in other acylsugar-containing Solanaceae species. A phylogenetic tree was created using predicted ALS proteins from Arabidopsis, maize, and several other Solanaceae species. *At Arabidopsis thaliana, Zm Zea mays, Nb N. benthamiana, Na N. attenuata, Ns N. sylvestris, Nto N. tomentosiformis, Nt N. tabacum, Sl S. lycopersicum, Sp S. pennellii, Pax Petunia axillaris, St Solanum tuberosum*.

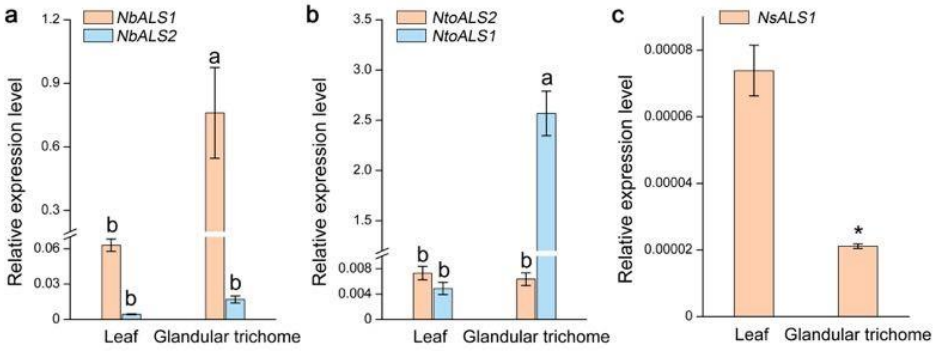


Figure 3-10 Expression analysis of ALS homologs. The expression levels of two ALS genes of **a** *N. benthamiana* and **b** *N. tomentosiformis* were respectively compared in corresponding trichomes and trichomeremoved leaves using multiple comparison. Different letters indicate significant differences (*P<0.05*). **c** Comparison of *ALS* expressionin *N. sylvestris* trichome-removed leaves and isolated trichomes. Values labeled with asterisks are significantly different (**P<0.05*). There were three technical replicates for each sample, and data are means±SD.

To test this hypothesis, we examined *ALS* gene expression in *N. benthamiana*, *N. tomentosiformis*, and *N. sylvestris*. We extracted RNA from the glandular trichomes and trichome-removed leaves of the three species and performed qRT-PCR analysis. Consistent with our hypothesis, genes encoding *NbALS1* and *NtoALS1*, which were clustered together with NtALS1 in our phylogenetic analysis, were highly expressed in glandular trichomes compared to the expression of ALS genes encoding proteins that did not cluster with NtALS1 (Figure 3-10a, b). By contrast, the gene encoding *NsALS1* was lowly expressed in glandular trichomes, which was notably lower than that in leaves (Figure 3-10c). These results indicate that all other genes encoding ALS homologs within the NtALS1 cluster may also express in glandular trichomes and may function in acylsugar biosynthesis.

3 Discussion

Two ALS genes, *NtALS2* and *NtALS3*, have been identified in tobacco previously (Lee et al., 1988; Keeler et al., 1993). In this study, the expression patterns of *NtALS2* and *NtALS3* are investigated by qRT-PCR. The results showed that *NtALS2* is consistently higher than that of *NtALS3* in each tissue, which were consistent with previous studies (Lee et al., 1988; Keeler et al., 1993). However, there is no significant difference in the expression level of *NtALS2* among different tissues, and almost no expression level of *NtALS3* was detected in each tissue (Figure 3-3). These results are slightly different from previous results (Keeler et al., 1993), which may be due to different tobacco varieties, different sampling methods, detection methods, especially whether there are tender tissues in the samples.

Acylsugars are a class of secondary metabolites that were first identified in the glandular trichome secretions of Solanaceous species. Acylsugars play important

roles in plant defense and have high-potential prospects for plant breeding programs and commercial development. Thus, regarding secondary metabolism in Solanaceae species, the characterization of acylsugar metabolism and the genes involved in their biosynthesis are highly active areas of research (Slocombe et al., 2008; Schilmiller et al., 2016, 2012, 2015; Ning et al., 2015; Fan et al., 2016, 2017, 2019; Moghe et al., 2017; Nadakuduti et al., 2017; Leong et al., 2019; Mandal et al., 2020). Long-term research has shown that the BCAA metabolic pathway is an important source of fatty acid chains required for acylsugar biosynthesis (Kandra and Wagner 1990; Kandra et al., 1990; Walters and Steffens 1990; Slocombe et al., 2008; Mandal et al., 2020). However, only a few acylsugar biosynthetic genes have been identified that are highly and specifically expressed in glandular trichomes, including those encoding the BCKD E1-β subunit, *KAS I*, *KAS II*, *KAS III*, and *SlIPMS3* (Slocombe et al., 2008; Ning et al., 2015; Mandal et al., 2020), and the complete acylsugar metabolic pathway remains unclear. As a key regulatory gene in BCAA metabolism, *ALS* has been extensively studied as a herbicide target (Mazur et al., 1987; Lee et al., 1988; Duggleby et al., 2008; Shimizu et al., 2008; Xie et al., 2019). In addition, in previous transcriptomic studies of Solanaceae genes involved in acylsugar metabolism, no specific high-level gene expression corresponding to the large subunit of ALS has been detected in glandular trichomes (Slocombe et al., 2008; Mandal et al., 2020). In this study, we identified a glandular trichome-specific *NtALS1* gene in tobacco through homology-based gene prediction and subsequent gene expression analysis. Furthermore, we used CRISPR-Cas9 to knock out *NtALS1*, thus demonstrating its involvement in acylsugar biosynthesis in tobacco glandular trichomes (Figure 3-6f, Figure 3-7). In addition, phylogenetic analysis revealed that the *ALS* genes in Solanaceae species have differentiated significantly (Figure 3-9), and NtALS1 homologs were found only in Solanaceae species that produce acylsugars. *N. sylvestris* does not produce acylsugars (Moghe et al., 2017), and its ALS protein was not clustered together with NtALS1 (Figure 3-9). Moreover, *NtALS1* is trichome-specific, and genes encoding NbALS1 and NtALS1, which phylogenetically clustered together with NtALS1, were also found to be highly and specifically expressed in glandular trichomes (Figure 3-10). Whereas the gene encoding *N. sylvestris* ALS had very low expression level in trichomes (Figure 3-10). Based on the above results and previous studies (Ning et al., 2015; Leong et al., 2019), we speculate that acylsugar biosynthesis-related genes involved in primary metabolism (such as *SlIPMS3*, *SpASFF1*, and *NtALS1*) may have experienced gene duplication and specialization via spatiotemporal expression to participate in tissue-specific biosynthesis of acylsugars that thereby help the plants to adapt to changes in external environmental conditions during long-term evolution. Therefore, homology-based gene prediction and tissue-specific gene expression pattern analysis are effective ways to discover unknown genes involved in acylsugar biosynthesis from primary metabolic pathways such as the BCAA synthetic pathway.

In previous studies, following plant treatment with ALS inhibitors, the contents of diterpenoids in tobacco leaf surface secretions were unchanged, whereas the contents of acylsugars decreased significantly (Kandra and Wagner 1990). Moreover, among the acylsugar fatty acids, the contents of branched-chain fatty acids were

significantly decreased, while the contents of linear-chain fatty acids were significantly increased (Kandra and Wagner 1990). In this study, the contents of acylsugars in the *NtALS1* knockout mutants were significantly reduced, whereas the contents of diterpenes remained unchanged (Figure 3-6f, Figure 3-7), which is largely consistent with the results of previous studies (Kandra and Wagner 1990). According to the chromatogram of the surface secretion chemicals of *NtALS1* knockout plants, the peak positions of various types of acylsugars were not significantly different from those in the control group (Figure 3-6e). This indicated that the composition of acylsugar fatty acids in the mutant lines did not change significantly. As disruption of *NtALS1* had the potential to alter the composition of acylsugar fatty acids, we predicted that maybe *NtALS1* is not the only *ALS* gene involved in acylsugar biosynthetic pathway in tobacco, because we found that *NtALS2* is also expressed in glandular trichomes and may also participate in the biosynthesis of acylsugar fatty acid chains, despite its lower expression in glandular trichomes than in leaves (Figure 3-3). In addition, linear-chain fatty acids were rarely detected in tobacco acylsugars (Kroumova et al., 2016), which lead us to predict that acyltransferases involved in acylsugar assembly may have specificity, such as a potential preference for branched-chain fatty acid substrates, and furtherly, if other *ALS* genes can replace *NtALS1* to synthesize branched-chain fatty acid precursors, the acylsugar structures in tobacco may not change much and just decrease in content. Whether the structural types of acylsugars in *NtALS1* knockout mutants have changed remains to be determined.

In this study, we found there is no trichome-specific *NtALS1* homolog in *N. sylvestris* (Figure 3-9). As acylsugars in *NtALS1* knockout mutants are only significantly reduced, and there is no acylsugars at all in *N. sylvestris* (Moghe et al., 2017), we predict that *NtALS1* homolog may affect the biosynthesis of some acyl-chains and thus affects the quantity of acylsugars, but which may not be the key gene responsible for the lack of acylsugars in *N. sylvestris*. As *N. tomentosiformis* and *N. sylvestris* are two ancestors of *N.tabacum* (Leitch et al., 2008), and the trichomes of *N.tabacum* contain abundant acylsugars, we predict that the *NtALS1* in *N.tabacum* should be from *N. tomentosiformis*. Moreover, based on the phylogenetic analysis of ALS homologous genes in Solanaceae and the expression level test of *NtALS1* homologs in several tobacco species, we obtained potential information that genes encoding other Solanaceae ALS proteins (NaALS1, NtoALS1, NbALS1, SlALS1, SpALS1, PaxALS1, and StALS1) that cluster together with *NtALS1* may also be highly and specifically expressed in glandular trichomes and participate in acylsugar biosynthesis, but this need further verification in tomato and petunia *etc*.

Reference

Amme S, Rutten T, Melzer M, et al (2005) A proteome approach defines protective functions of tobacco leaf trichomes. Proteomics 5:2508–2518. https://doi.org/10.1002/pmic.200401274

Arrendale RF, Severson RF, Sisson VA, et al (1990) Characterization of the

Sucrose Ester Fraction from Nicotiana glutinosa. J Agric Food Chem 38:75–85. https://doi.org/10.1021/jf00091a015

Ashraf-Khorassani M, Nazem N, Taylor L, Coleman W (2016) Separation and Identification of Sucrose Esters from Turkish Tobacco Using Liquid Chromatography-Mass Spectrometry. Beiträge zur Tab Int to Tob Res 21:381–389. https://doi.org/10.2478/cttr-2013-0804

Ben-Mahmoud S, Smeda JR, Chappell TM, et al (2018) Acylsugar amount and fatty acid profile differentially suppress oviposition by western flower thrips, frankliniella occidentalis, on tomato and interspecific hybrid flowers. PLoS One 13:. https://doi.org/10.1371/journal.pone.0201583

Binder S (2010) Branched-Chain Amino Acid Metabolism in Arabidopsis thaliana . Arab B 8:e0137. https://doi.org/10.1199/tab.0137

Binder S, Knill T, Schuster J (2007) Branched-chain amino acid metabolism in higher plants. Physiol Plant 129:68–78. https://doi.org/10.1111/j.1399-3054.2006.00800.x

Bonierbale MW, Plaisted RL, Pineda O, Tanksley SD (1994) QTL analysis of trichome-mediated insect resistance in potato. Theor Appl Genet Int J Plant Breed Res 87:973–987. https://doi.org/10.1007/BF00225792

Chortyk OT, Kays SJ, Teng Q (1997) Characterization of Insecticidal Sugar Esters of Petunia. J Agric Food Chem 45:270–275. https://doi.org/10.1021/jf960322f

Chortyk OT, Pomonis JG, Johnson AW (1996) Syntheses and characterizations of insecticidal sucrose esters. J Agric Food Chem 44:1551–1557. https://doi.org/10.1021/jf950615t

Chortyk OT, Severson RF, Cutler HC, Sisson VA (1993) Antibiotic Activities of Sugar Esters Isolated from Selected Nicotiana Species. Biosci Biotechnol Biochem 57:1355–1356. https://doi.org/10.1271/bbb.57.1355

Duggleby RG, McCourt JA, Guddat LW (2008) Structure and mechanism of inhibition of plant acetohydroxyacid synthase. Plant Physiol Biochem 46:309–324. https://doi.org/10.1016/j.plaphy.2007.12.004

Fan P, Leong BJ, Last RL (2019) Tip of the trichome: evolution of acylsugar metabolic diversity in Solanaceae. Curr Opin Plant Biol 49:8–16. https://doi.org/10.1016/j.pbi.2019.03.005

Fan P, Miller AM, Liu X, et al (2017) Evolution of a flipped pathway creates metabolic innovation in tomato trichomes through BAHD enzyme promiscuity. Nat Commun 8:2080. https://doi.org/10.1038/s41467-017-02045-7

Fan P, Miller AM, Schilmiller AL, et al (2016) In vitro reconstruction and analysis of evolutionary variation of the tomato acylsucrose metabolic network. Proc Natl Acad Sci U S A 113:E239–E248. https://doi.org/10.1073/pnas.1517930113

Ghosh B, Jones AD (2017) Profiling, characterization, and analysis of natural and synthetic acylsugars (sugar esters). Anal Methods 9:892–905. https://doi.org/10.1039/c6ay02944b

Ghosh B, Westbrook TC, Jones AD (2014) Comparative structural profiling of

trichome specialized metabolites in tomato (Solanum lycopersicum) and S. habrochaites: Acylsugar profiles revealed by UHPLC/MS and NMR. Metabolomics 10:496–507. https://doi.org/10.1007/s11306-013-0585-y

Jackson DM, Chortyk OT, Stephenson MG, et al (1998) Potential of Nicotiana species for production of sugar esters. Tob Sci 42:1–9

Johnson A, Severson R (1984) Leaf Surface Chemistry of Tobacco Budwormi Resistant Tobacc02. J Agric Entomol 1:

Kandra L, Severson R, Wagner GJ (1990) Modified branched-chain amino acid pathways give rise to acyl acids of sucrose esters exuded from tobacco leaf trichomes. Eur J Biochem 188:385–391. https://doi.org/10.1111/j.1432-1033.1990.tb15415.x

Kandra L, Wagner GJ (1990) Chlorsulfuron modifies biosynthesis of acyl acid substituents of sucrose esters secreted by tobacco trichomes. Plant Physiol 94:905–912. https://doi.org/10.1104/pp.94.3.906

Kandra L, Wagner GJ (1988) Studies of the site and mode of biosynthesis of tobacco trichome exudate components. Arch Biochem Biophys 265:425–432. https://doi.org/10.1016/0003-9861(88)90145-2

Keeler SJ, Sanders P, Smith JK, Mazur BJ (1993) Regulation of tobacco acetolactate synthase gene expression. Plant Physiol 102:1009–1018. https://doi.org/10.1104/pp.102.3.1009

Kroumova AB, Wagner GJ (2003) Different elongation pathways in the biosynthesis of acyl groups of trichome exudate sugar esters from various solanaceous plants. Planta 216:1013–1021. https://doi.org/10.1007/s00425-002-0954-7

Kroumova ABM, Zaitlin D, Wagner GJ (2016) Natural variability in acyl moieties of sugar esters produced by certain tobacco and other Solanaceae species. Phytochemistry 130:218–227. https://doi.org/10.1016/j.phytochem.2016.05.008

Lawson DM, Lunde CF, Mutschler MA (1997) Marker-assisted transfer of acylsugar-mediated pest resistance from the wild tomato, Lycopersicon pennellii, to the cultivated tomato, Lycopersicon esculentum. Mol Breed 3:307–317. https://doi.org/10.1023/A:1009677412902

Leckie BM, de Jong DM, Mutschler MA (2012) Quantitative trait loci increasing acylsugars in tomato breeding lines and their impacts on silverleaf whiteflies. Mol Breed 30:1621–1634. https://doi.org/10.1007/s11032-012-9746-3

Leckie BM, Halitschke R, De Jong DM, et al (2014) Quantitative trait loci regulating the fatty acid profile of acylsugars in tomato. Mol Breed 34:1201–1213. https://doi.org/10.1007/s11032-014-0110-7

Lee KY, Townsend J, Tepperman J, et al (1988) The molecular basis of sulfonylurea herbicide resistance in tobacco. EMBO J 7:1241–1248. https://doi.org/10.1002/j.1460-2075.1988.tb02937.x

Leitch IJ, Hanson L, Lim KY, et al (2008) The ups and downs of genome size evolution in polyploid species of Nicotiana (Solanaceae). Ann Bot 101:805–814.

https://doi.org/10.1093/aob/mcm326

Leong BJ, Lybrand DB, Lou YR, et al (2019) Evolution of metabolic novelty: A trichome-expressed invertase creates specialized metabolic diversity in wild tomato. Sci Adv 5:. https://doi.org/10.1126/sciadv.aaw3754

Liu Y, Jing SX, Luo SH, Li SH (2019) Non-volatile natural products in plant glandular trichomes: Chemistry, biological activities and biosynthesis. Nat Prod Rep 36:626–665. https://doi.org/10.1039/c8np00077h

Luu VT, Weinhold A, Ullah C, et al (2017) O-Acyl sugars protect a wild Tobacco from both native fungal pathogens and a specialist herbivore. Plant Physiol 174:370–386. https://doi.org/10.1104/pp.16.01904

Mandal S, Ji W, McKnight TD (2020) Candidate Gene Networks for Acylsugar Metabolism and Plant Defense in Wild Tomato Solanum pennellii. Plant Cell 32:81–99. https://doi.org/10.1105/tpc.19.00552

Mazur BJ, Chui C-F, Smith JK (1987) Isolation and Characterization of Plant Genes Coding for Acetolactate Synthase, the Target Enzyme for Two Classes of Herbicides. Plant Physiol 85:1110–1117. https://doi.org/10.1104/pp.85.4.1110

Moghe GD, Leong BJ, Hurney SM, et al (2017) Evolutionary routes to biochemical innovation revealed by integrative analysis of a plant-defense related specialized metabolic pathway. Elife 6:. https://doi.org/10.7554/eLife.28468

Nadakuduti SS, Uebler JB, Liu X, et al (2017) Characterization of trichome-expressed BAHD acyltransferases in petunia axillaris reveals distinct acylsugar assembly mechanisms within the solanaceae. Plant Physiol 175:36–50. https://doi.org/10.1104/pp.17.00538

Ning J, Moghe GD, Leong B, et al (2015) A feedback-insensitive isopropylmalate synthase affects acylsugar composition in cultivated and wild tomato. Plant Physiol 169:1821–1835. https://doi.org/10.1104/pp.15.00474

Puterka GJ, Farone W, Palmer T, Barrington A (2003) Structure-Function Relationships Affecting the Insecticidal and Miticidal Activity of Sugar Esters. J Econ Entomol 96:636–644. https://doi.org/10.1093/jee/96.3.636

Rodríguez-López MJ, Garzo E, Bonani JP, et al (2012) Acylsucrose-producing tomato plants forces Bemisia tabaci to shift its preferred settling and feeding site. PLoS One 7:. https://doi.org/10.1371/journal.pone.0033064

Rodriguez AE, Tingey WM, Mutschler MA (1993) Acylsugars of Lycopersicon pennellii Deter Settling and Feeding of the Green Peach Aphid (Homoptera: Aphididae). J Econ Entomol 86:34–39. https://doi.org/10.1093/jee/86.1.34

Schilmiller AL, Charbonneau AL, Last RL (2012) Identification of a BAHD acetyltransferase that produces protective acyl sugars in tomato trichomes. Proc Natl Acad Sci U S A 109:16377–16382. https://doi.org/10.1073/pnas.1207906109

Schilmiller AL, Gilgallon K, Ghosh B, et al (2016) Acylsugar acylhydrolases: Carboxylesterase-catalyzed hydrolysis of acylsugars in tomato trichomes. Plant Physiol 170:1331–1344. https://doi.org/10.1104/pp.15.01348

Schilmiller AL, Moghe GD, Fan P, et al (2015) Functionally divergent alleles and

duplicated loci encoding an acyltransferase contribute to acylsugar metabolite diversity in Solanum trichomes. Plant Cell 27:1002–1017. https://doi.org/10.1105/tpc.15.00087

Severson RF, Arrendale RF, Chortyk OT, et al (1985) Isolation and Characterization of the Sucrose Esters of the Cuticular Waxes of Green Tobacco Leaf. J Agric Food Chem 33:870–875. https://doi.org/10.1021/jf00065a026

Shimizu M, Goto M, Hanai M, et al (2008) Selectable tolerance to herbicides by mutated acetolactate synthase genes integrated into the chloroplast genome of tobacco. Plant Physiol 147:1976–1983. https://doi.org/10.1104/pp.108.120519

Slocombe SP, Schauvinhold I, McQuinn RP, et al (2008) Transcriptomic and reverse genetic analyses of branched-chain fatty acid and acyl sugar production in Solanum pennellii and Nicotiana benthamiana. Plant Physiol 148:1830–1846. https://doi.org/10.1104/pp.108.129510

Smeda JR, Schilmiller AL, Anderson T, et al (2018) Combination of Acylglucose QTL reveals additive and epistatic genetic interactions and impacts insect oviposition and virus infection. Mol Breed 38:3. https://doi.org/10.1007/s11032-017-0756-z

Smeda JR, Schilmiller AL, Last RL, Mutschler MA (2016) Introgression of acylsugar chemistry QTL modifies the composition and structure of acylsugars produced by high-accumulating tomato lines. Mol Breed 36:160. https://doi.org/10.1007/s11032-016-0584-6

Stubenrauch C, Sottmann T (2008) Microemulsions stabilized by sugar surfactants. Sugar-Based Surfactants Fundam Appl 463–499. https://doi.org/10.1201/9781420051674

Van der Hoeven RS, Steffens JC (2000) Biosynthesis and elongation of short- and medium-chain-length fatty acids. Plant Physiol 122:275–282. https://doi.org/10.1104/pp.122.1.275

Vijay V, Danehower DA, Tyler S, et al (2010) Analysis of a nicotiana tabacum l. genomic region controlling two leaf surface chemistry traits. J Agric Food Chem 58:294–300. https://doi.org/10.1021/jf903256h

Walters DS, Steffens JC (1990) Branched chain amino acid metabolism in the biosynthesis of Lycopersicon pennellii glucose esters. Plant Physiol 93:1544–1551. https://doi.org/10.1104/pp.93.4.1544

Weinhold A, Baldwin IT (2011) Trichome-derived O-acyl sugars are a first meal for caterpillars that tags them for predation. Proc Natl Acad Sci U S A 108:7855–7859. https://doi.org/10.1073/pnas.1101306108

Xia Y (1998) Toxicity of natural and synthetic sugar esters against tobacco insects with an emphasis on the tobacco aphid, Myzus nicotianae Blackman,(Homoptera: Aphididae).

Xie Y, Zhang C, Wang Z, et al (2019) Fluorogenic Assay for Acetohydroxyacid Synthase: Design and Applications. Anal Chem 91:13582–13590. https://doi.org/10.1021/acs.analchem.9b02739

Yan N, Du Y, Liu X, et al (2016) Chemical structures, biosynthesis, bioactivities, biocatalysis and semisynthesis of tobacco cembranoids: An overview. Ind Crops Prod 83:66–80. https://doi.org/10.1016/j.indcrop.2015.12.031

4

Characterization of Trichome-specific BAHD Acyltransferases Involved in Acylsugar Biosynthesis in *Nicotiana Tabacum*

Chapter Four Characterization of Trichome-specific BAHD Acyltransferases involved in Acylsugar Biosynthesis in *Nicotiana tabacum*

Aixia Chang1,3,*,†, Zhongyi Hu2,*, Biao Chen1, Herve Vanderschuren3,6, Ming Chen1, Yafang Qu1, Weisong Yu1, Yangyang Li4, Huiqing Sun1, Jianmin Cao1, Kumar Vasudevan 3, Chenying Li1, Yanan Cao1, Jianye Zhang5, Yeming Shen5, Aiguo Yang1,†, Yuanying Wang1,†

[1] Tobacco Research Institute, Chinese Academy of Agricultural Sciences, Qingdao, 266101, China
[2] Jiangxi Food Inspection and Testing Research Institute, Nanchang, 330001, China
[3] Plant Genetics Laboratory, Gembloux Agro-Bio Tech, University of Liège, 5030 Gembloux, Belgium
[4] Hunan Tobacco Research Institute, Changsha, 410004, China;
[5] Green Catalysis Center, College of Chemistry, Zhengzhou University, Zhengzhou, 450001, China
[6] Tropical Crop Improvement Lab, Department of Biosystems, KU Leuven, Heverlee, Belgium
[*] These authors contributed equally to this study.

Adapted from the reference: Chang AX, Hu ZY, Chen B, Vanderschuren H, Chen M, Qu YF, Yu WS, Li YY, Sun HQ, Cao JM, Vasudevan K, Li CY, Cao YN, Zhang JY, Shen YM, Yang AG, Wang YY. (2022). Characterization of trichome-specific BAHD acyltransferases involved in acylsugar biosynthesis in *Nicotiana tabacum*. *Journal of experimental botany* (https://doi.org/10.1093/jxb/erac095).

Abstract

Glandular trichomes of tobacco (*Nicotiana tabacum*) produce blends of acylsucroses that contribute to defence against pathogens and herbivorous insects, but the mechanism of assembly of these acylsugars has not yet been determined. In this study, we isolated and characterized two trichome-specific acylsugar acyltransferases that are localized in the endoplasmic reticulum, NtASAT1 and NtASAT2. They sequentially catalyse two additive steps of acyl donors to sucrose to produce di-acylsucrose. Knocking out of *NtASAT1* or *NtASAT2* resulted in deficiency of acylsucrose; however, there was no effect on acylsugar accumulation in plants overexpressing *NtASAT1* or *NtASAT2*. Genomic analysis and profiling revealed that *NtASAT*s originated from the T subgenome, which is derived from the acylsugar-producing diploid ancestor *N. tomentosiformis*. Our identification of NtASAT1 and NtASAT2 as enzymes involved in acylsugar assembly in tobacco potentially provides a new approach and target genes for improving crop resistance against pathogens and insects.

Key words

Acylsugar biosynthesis, glandular trichome, *Nicotiana tabacum* (tobacco), *NtASAT1*, *NtASAT2*, plant defence

Introduction

Pathogens and pests are estimated to be responsible for 20–30% of crop yield losses annually, putting great pressure on global food security (Savary et al., 2019). Plants synthesize an array of defensive secondary metabolites (phytoalexins) to improve their adaptation to the environment (Bednarek and Osbourn, 2009; Pichersky and Lewinsohn, 2011; Weng, 2014; Moghe and Last, 2015). Acylsugars are specialized metabolites that were first identified in secretions of glandular trichomes in Solanaceae species. They consist of a sucrose, glucose, or inositol core with 2–6 straight- or branched-chain acyl groups (of C2 to ~C20) attached to different positions (Arrendale et al., 1990; Haliński and Stepnowski, 2013; Ghosh et al., 2014; Kroumova et al., 2016; Liu et al., 2017; Moghe et al., 2017; Fan et al., 2019; Leong et al., 2020; Lou et al., 2021). Because of their anti-oviposition, anti-feedant, and other toxic effects on insects (Johnson and Severson, 1984; Kennedy et al., 1992; Rodriguez et al., 1993; Chortyk et al., 1997; Puterka et al., 2003; Simmons et al., 2004; Weinhold and Baldwin, 2011; Rodríguez-López et al., 2012; Leckie et al., 2016; Luu et al., 2017; Ben-Mahmoud et al., 2018; Feng, et al., 2022), acylsugars have been considered as potential target phytochemicals for improving insect resistance of *Solanaceae* plants by increasing their contents (Chortyk et al., 1997; Lawson et al., 1997; McKenzie et al., 2005; Maluf et al., 2010; Leckie et al., 2012, 2014; Smeda et al., 2016, 2018). In addition to being pesticides (Puterka et al., 2003), acylsugars also show potential for use as anti-inflammatory compounds (Perez-Castorena et al., 2010), antibiotics (Chortyk et al., 1993), and as additives in cosmetic products and food (Hill and Rhode, 1999).

In tobacco (*Nicotiana tabacum*), acylsugars represent the second major class of surface chemicals (Severson et al., 1984). They are different from those of other Solanaceae species in terms of the number, position, and length of acyl chains, as well as the sugar core (Ghosh et al., 2014; Liu et al., 2017; Moghe et al., 2017; Leong et al., 2020; Lou et al., 2021; Schenck et al., 2022, Preprint). For example, in contrast to the tri- and tetra-acylsucroses of *Solanum lycopersicum* (tomato; Ghosh et al., 2014), the tetra- and penta-acylsucroses of *Petunia axillaris* (Liu et al., 2017), and the tetra-, penta-, and hexa-acylsucroses of *Salpiglossis sinuata* (Moghe et al., 2017), tobacco accumulates only tetra-acylsucroses (Wagner, 1999; Jia et al., 2013). Moreover, both tobacco and petunia produce tetra-acylsucroses with acyl chains on the pyranose ring (Wagner, 1999; Jia et al., 2013; Liu et al., 2017), whereas the tetra-acylsucroses of tomato and *Salpiglossis* have three acyl chains on the pyranose ring and one on the furanose ring (Moghe et al., 2017) (Figure 4-1). Such diversity of acylsugar structures within the Solanaceae family might be due to variation of acyl pools and divergent substrate specificities of acylsugar assembly enzymes.

Studies in tobacco and tomato have indicated that the branched-chain amino acid (BCAA) metabolic pathway is an important source of keto acid precursors for acylsugar acyl chains (Kandra et al., 1990; Kandra and Wagner, 1990; Walters and Steffens, 1990). Short-chain acyl groups act as primers to produce extended-chain fatty acids. There are two different mechanisms for fatty acid chain elongation. In

several tobacco and petunia species, branched-chain fatty acids (BCFAs) extend via a one-carbon mechanism termed α-keto acid elongation (α-KAE) (Kroumova et al., 1994; Kroumova and Wagner, 2003; Slocombe et al., 2008), while plastid fatty acid synthase (FAS) is the route for BCFA elongation in tomato (van der Hoeven and Steffens, 2000; Kroumova and Wagner, 2003; Slocombe et al., 2008). In addition, studies have revealed that the trichome-enriched acylsugar acyl-CoA synthetase and acylsugar enoyl- CoA hydratase are also involved in generating medium-chain acyl-CoAs (Fan et al., 2020). The variations in sequences and activities of key enzymes in the BCAA pathway (Ning et al., 2015) and differences in acyl chain extension mechanisms (van der Hoeven and Steffens, 2000; Kroumova and Wagner, 2003; Slocombe et al., 2008; Fan et al., 2020) are key factors that affect the diversity of acylsugar structures in Solanaceae plants. A group of BAHD acyltransferases, known as acylsucrose acyltransferases (ASATs), have been identified and characterized in *Solanum* (Figure 4-1A), *Petunia* (Figure 4-1B), and *Salpiglossis* (Figure 4-1C) (Schilmiller et al., 2012, 2015; Fan et al., 2016, 2017; Moghe et al., 2017; Nadakuduti et al., 2017). They catalyse the attachment of single or multiple acyl-CoA donors to various hydroxyl groups of sucrose to produce acylsucroses (Kandra et al., 1990; Walters and Steffens, 1990; Slocombe et al., 2008; Ning et al., 2015). Although several acyltransferases (NbASAT1/2 and NacASAT1/2/3/4) have been found to contribute to acylsucrose biosynthesis in *N. benthamiana* and *N. acuminata*, their catalytic activities and *in vivo* functions remain to be elucidated (Feng, et al., 2022; Schenck et al., 2022, Preprint). In *N. tabacum*, the enzymes that catalyse the assembly of side-chains remain unknown. Here, we report the isolation and characterization of two trichome-specific BAHD acyltransferases, NtASAT1 and NtASAT2, in *N. tabacum*. This study not only extends our understanding of acylsucrose biosynthesis in plants, but also identifies potential new genes for engineering crop resistance against pathogens and insects.

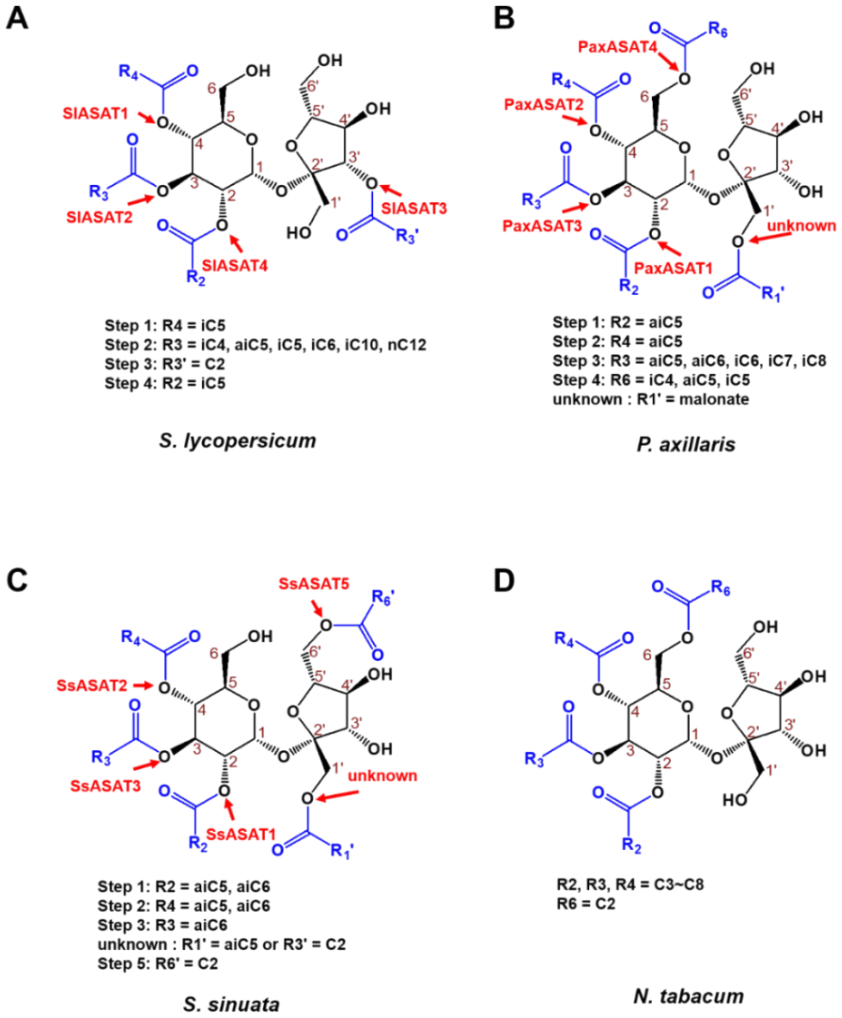


Figure 4-1 Structures of acylsugars produced by members of the Solanaceae. (A) *Solanum lycopersicum*, (B) *Petunia axillaris*, and (C) *Salpiglossis sinuate*. Details of the length of the acyl chains typically found at each position are summarized from previous reports, and together with the identities of the ASAT enzymes responsible for acylsugar assembly and the order of the acylation reactions that they catalyse (Ghosh et al., 2014; Schilmiller et al., 2015; Fan et al., 2016; Liu et al., 2017; Moghe et al., 2017; Nadakuduti et al., 2017). (D) Structures of acylsugars from *Nicotiana tabacum*. Details of the length of the acyl chains typically found at each position are derived from previous structural characterizations (Wagner, 1999; Jia et al., 2013). The order of assembly of *N. tabacum* acylsucroses and the corresponding ASAT enzymes are currently unknown.

1 Materials and Methods

1.1 Plant materials and Growth Conditions

Two cultivars of *Nicotiana tabacum*, Hongda (HD) and Beinhart1000-1 (BH), and the ancestry diploid wild species *N. tomentosiformis* and *N. sylvestris* were planted in the field in Jimo, Shandong Province, China, and grown according to standard agricultural practices. *Nicotiana benthamiana* was planted in a greenhouse with a photoperiod of 16/8 h light/dark at 24 °C and 65% relative humidity.

1.2 Trichome Isolation, RNA Extraction, and Real-time Quantitative Reverse Transcription-PCR

Trichomes were collected from upper leaves (10-15 cm in length) of 50-day-old transplanted plants using a mechanical abrading method and glass beads as described by Amme et al. (2005). The leaves from which the trichomes were removed were also used for expression analysis. Total RNAs were isolated using a MiniBEST Plant RNA Extraction Kit and cDNAs were synthesized using PrimeScript™ RT reagent (both TaKaRa), according to manufacturer's instructions. Real-time quantitative reverse-transcription PCR (RT-qPCR) was performed using an Applied Biosystems 7500 Real-Time PCR System. Melting curves were used to ensure that only a single PCR product was amplified and to verify the absence of primer–dimer artefacts. Each sample was run in triplicate. Relative gene quantification was performed using the $\Delta\Delta CT$ method (Livak and Schmittgen, 2001), and the results were normalized to *Actin*.

1.3 Identification of NtASATs and Phylogenetic Analysis

To identify *N. tabacum ASAT* genes, we used the following known acyltransferase genes in other species as query sequences to search for homologs in the NCBI database (http://www.ncbi.nlm.nih.gov/) and the Solanaceae Genomics Network (SGN, https://solgenomics.net/): *SlASAT1* (Solyc12g006330), *SlASAT2* (Solyc04g012020), *SlASAT3* (Solyc11g067270), and *SlASAT4* (Solyc01g105580) of *Solanum lycopersicum*; *PaxASAT1* (KT716258), *PaxASAT2* (KT716259), *PaxASAT3* (KT716260), and *PaxASAT4* (KT716261) of *Petunia axillaris*; and *SsASAT1*(KY978746), *SsASAT2* (KY978747), *SsASAT3* (KY978748), and *SsASAT5* (KY978749) of *Salpiglossis sinuata*. The putative amino acid sequences of NtASATs and other Solanaceae ASATs were subjected to phylogenetic analysis using the maximum-likelihood method, with bootstrap=2000 (Kumar et al., 2016). Conserved amino acid residences were analysed using the MEME server (http://meme.nbcr.net) (Bailey et al., 2009).

1.4 Expression in E. coli and Enzyme Activity Assays

The ORFs of *NtASAT*s were amplified and inserted into the BamHI and XhoI sites of the pATX-sumo vector, using the primers listed in Table 4-1, and transformed into *E. coli* strain Rosetta. The cells were induced by the addition of 0.05 mM

isopropyl *β*-D-1- thiogalactopyranoside (IPTG) when OD_{600}=0.6 and this was followed by cultivating overnight at 16 °C with shaking (120 rpm). Then, the cells were lysated by sonication and the recombinant proteins were purified using Ni-affinity chromatography, as described previously (Schilmiller et al., 2012).

Enzyme activity assays were conducted by incubating 2 μg of purified recombinant protein in a 60-μL reaction volume containing 50 mM ammonium acetate buffer (pH 6.0), 100 μM acyl-CoA, and 1 mM sucrose or glucose. The reaction mixtures were incubated at 30 °C for 30 min, and the reaction was stopped by adding 120 μL stop solution (acetonitrile: isopropanol: formic acid at 1: 1: 0.001). The mixture was centrifuged at 17000 *g* for 10 min, filtered through a 0.22-μm mesh, and then used for analysis.

For the sequential enzyme activity assay, NtASAT1 was incubated with acyl-CoA and sucrose for 30 min at 30 °C, and the reaction was stopped by heating at 65 °C for 5 min followed by cooling on ice. Then, NtASAT2 and 50 μM acyl-CoA were added to the mixture and it was again incubated at 30°C for 30 min before the reaction was stopped by the addition of 120 μL of stop solution, as described above.

1.5 Purification of Acylsugars from Enzyme Reactions for Nuclear Magnetic Resonance (NMR) Analysis

We examined the catalytic activities of NtASAT1 and NtASAT2 *in vitro* using sucrose as the acyl acceptor together with various acyl donors. When NtASAT1 was incubated with 100 μM isovaleryl-CoA and 1 mM sucrose in a 60-mL reaction mixture buffered by 50 mM ammonium acetate (pH 6.0) at 30 °C for 4 h, it resulted in the production of the monoacylsucrose S1:5 (iC5). (Acylsucrose nomenclature is as follows: the first letter indicates the sucrose core; the number before the colon indicates the number of acyl chains; the number after the colon indicates the sum of carbons in all acyl chains; and individual acyl chains are shown in parentheses.) The NtASAT1 reaction was stopped at 65 °C for 10 min followed by cooling on ice, and then 100 μM isovaleryl-CoA and NtASAT2 were added and incubated at 30 °C for 7 h, resulting in the production of the diacylsucrose S2:10 (iC5, iC5). All the reactions were then stopped by adding 120 mL of stop solution, as described above.

For preparation for NMR, the solutions were dried using a rotary vacuum evaporator, then re-dissolved in 2 mL of distilled water under sonication for 10 min. After centrifugation at 4000 *g* for 2 min, the supernatants were loaded into a Waters Prep 150 LC system equipped with a Waters fraction collector III. For S1:5 (iC5) and S2:10 (iC5, iC5) separations, a Waters Symmetry Prep™ C18 HPLC column (4.6 × 150 mm, 5 μm) was used, with column temperature set at 40 °C. The mobile phase consisted of solvent A (0.1% formic acid in acetonitrile) and solvent B (0.1% formic acid in water), at a flow rate of 1.0 mL/min. For S1:5 (iC5), the ratio A:B was set at 6:94 for 15 min, and for S2:10 (iC5, iC5) it was set at 21:79 for 20 min. Eluted fractions were collected every 30 s using a volume of 100 μL for each injection. Fractions were first analysed using LC-MS/MS, and then pooled for NMR analysis.

Determination of NMR spectra was carried out as described previously by Ghosh et al. (2014). The purified acylsugars were dissolved in 50 mL of deuterium oxide

and transferred into 1.7-mm NMR capillary tubes. The spectra were recorded using a Bruker Avance III 600 NMR spectrometer equipped with a TCI triple-resonance inverse detection cryoprobe. The NMR experiments included ^{1}H measurement at 600.13 MHz, correlation spectroscopy, and HSQC spectroscopy (F1, 150.91 MHz; F2, 600.13 MHz). The acylsugar substitution patterns were determined via chemical shifts of H, based on the ^{1}H and HSQC spectroscopy.

1.6 Liquid Chromatography Quadrupole Time-of-flight Mass Spectrometry (LC Q-TOF MS)

The products of *in vitro* enzyme activity assays were analyzed using the Waters Acquity UPLC system connected to Waters VION IMS LC Q-TOF MS (Waters, Milford, MA, USA). The method was optimized on the basis of previous report (Liu et al., 2017). To analyze NtASAT1-produced monoacylsucrose, 10 μL of enzyme assay solution was injected into a fused core ACQUITY UPLC ethylene bridged hybrid (BEH) C18 column (2.1 mm × 50 mm, 1.7 μm, Waters), and the column oven was set to 40°C. The flow rate was set to 0.3 mL/min, with starting conditions of 95% A and 5% B (A: 0.1% formic acid H_2O, B: acetonitrile). The elution gradient was as follows: start at 95% A, ramp to 95% B at 13 min, return to 95% A at 13.01 min, and hold until 14 min. Collision-induced dissociation MS in the positive-ion mode was used.

1.7 Gas Chromatography-Mass Spectrometry (GC-MS)

Sample preparation and GC-MS analysis was carried out as described previously by Chang et al. (2020). Using a sharpened 16-mm diameter stainless steel punch, 12 disks were collected from three upper leaves (10-15 cm in length) from three plants at 50-day-old after transplaning and placed in a 20-mL glass tube. The disks were washed twice with 7.5 mL of dichloromethane by vibrating for 30 s and dried by adding 0.5 g of anhydrous Na_2SO_4. The extracts were then filtered through a 0.45-μm nylon membrane, and 50 μL internal standard solution was added (200 μg mL^{-1} sucrose octaacetate in dichloromethane). The solvent was then evaporated by heating at 40 °C under a stream of N_2, after which 100 μL of a 1:1 mixture of BSTFA: DMF was added to the tube, it was capped tightly under a N_2 headspace, and heated at 75 °C for 30 min. After cooling, 100 μL of 1:1 BSA:pyridine solution was added, and the mixture was filtered through a 0.22-μm nylon membrane and stored at -20 °C until use.

Samples were analysed on GC/MS-QP2010 Plus system (Shimadzu) with a Rtx-5MS column (30 m ×0.25 × 0.25 μm). The carrier gas was helium at a linear velocity of 38.6 cm s^{-1}. The injector was set at 265 °C. The temperature of both the ion source and transmission line was set at 280 °C. The analysis consisted of a temperature increase from 165 °C to 280 °C at a rate of 4 °C min^{-1}, followed by a hold at 280 °C for 25 min. A 1-μL sample was injected in a split mode with a split ratio of 15:1. Acylsugars were identified according to their specific ions (*m*/*z* 361 for a furanose ring, and *m*/*z* 443, 457, 471, 485, 499, and 513 for pyranose rings) (Severson et al., 1985; Cai et al., 2009). Sucrose octaacetate was used as the reference internal standard for semi-quantitative determination. The ratio of the total

ion peak area of the target sucrose ester component and the internal standard sucrose octaacetate was multiplied by the mass of sucrose octaacetate to calculate the relative content of the target sucrose ester component. The correction factor was assumed to be 1.

1.8 Expression Pattern and Subcellular Localization

To analyse the expression patterns of *NtASAT*s, their promoter regions were amplified from genomic DNA and ligated into the PstI/KpnI sites of the pCambia35tl:egfps2#4 vector to make promoter::*GFP* constructs. To analyse the subcellular localization of NtASATs, the ORFs without the stop codon were amplified from trichome cDNA and inserted into the BamHI/KpnI sites of the pCambia35tl:egfps2#4 vector to make GFP fusion proteins. The endoplasmic reticulum (ER) marker vector fused with mCherry was obtained from the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences. Details of the pCambia35tl-egfps2#4 vector are given in Figure 4-2. The primers used are listed in Table 4-1.

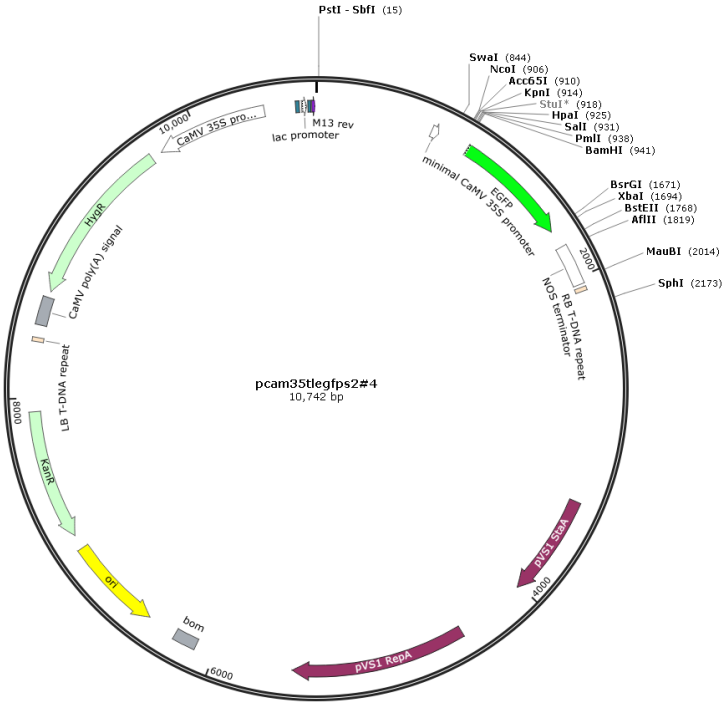


Figure 4-2 Details of the pCambia35tl:egfps2#4 vector. Which was used for the construction of tissue localization, subcellular localization and overexpression vectors.

All constructs were transformed into *Agrobacterium tumefaciens* strain LBA4404. The liquid cultures of *Agrobacterium* were incubated overnight at 28°C with 220

rpm agitation, and were then harvested and re-suspended in buffer containing 10 mM MES, 10 mM $MgCl_2$, and 100 µM acetosyringone at OD_{600}=0.6-0.8. The suspensions were infiltrated into leaves of 4-week-old *N. benthamiana* plants. Fluorescence of GFP and mCherry were observed 72-96 h post-infiltration using confocal microscopy (Leica TCP S8).

Table 4-1 Primers used in the study. *NtASAT1* and *NtASAT2* contained in the primer names represent *Nitab4.5_0004970g0010* and *XM_01659769* respectively.

Primer name	Sequence (5' to 3')
For qRT-PCR analysis	
Actin_F	CCACACAGGTGTGATGGTTG
Actin_R	GTGGCTAACACCATCACCAG
qRT-NtASAT1_F	CTTTCCACCTACTAATGAACC
qRT-NtASAT1_R	TGCTGTGAGTAATTCTACCC
qRT-NtASAT2_F	GCCAAATTCCTCCTACAACTG
qRT-NtASAT2_R	AATTGCATTTCCTACCAAGCC
For protein expression constructs（pATX-sumo vector）	
P-NtASAT1_F	gaacagattggtggccaaggatccATGGCTGCCTCAGCTCTAGTTTCTTTA
P-NtASAT1_R	tcagtggtggtggtggtggtgctcgagtcaACATCGAGCTTCCATTTTAATTTC
P-NtASAT2_F	gaacagattggtggccaaggatccATGGCTATTTCAAGGCTTGTTTTAC
P-NtASAT2_R	tcagtggtggtggtggtggtgctcgagtcaGAGTCCTGAGCTTGGAGAAGCAAAC
For gene tissue localization(pCambia35tlegfps2#4 vector, Pst1 and Kpn1 cut site)	
TL-NtASAT1_F	ATTACGAATTTCGACCTGCAGATTCATCATTGTTAGGAGG
TL-NtASAT1_R	ATGTTAACAAGGCCTGGTACCTTCTTACTGAAATTAATTGAGGA
TL-NtASAT2_F	ATTACGAATTTCGACCTGCAGACACATAGGGTAGCTTGAA
TL-NtASAT2_R	ATGTTAACAAGGCCTGGTACCCTTAGTTGAATTATAAC
For gene subcelllular localization(pCambia35tlegfps2#4 vector, BamHI and Kpn1 cut site)	
SCL-NtASAT1_F	TTACGAACGATAGCCATGGTACCATGGCTGCCTCAGCTCTAG
SCL-NtASAT1_R	GCCTGCGGCCGCGCCGGATCCACATCGAGCTTCCATTTTAATTTC
SCL-NtASAT2_F	TTACGAACGATAGCCATGGTACCATGGCTATTTCAAGGCTTG
SCL-NtASAT2_R	GCCTGCGGCCGCGCCGGATCCGAGTCCTGAGCTTGGAGAAG
For CRISPR-Cas9 constructs and positive plants selection (pORE-Cas9/gRNA vector, BsaI cut site)	

NtASAT1-SP1-F	GATTGCTGTATGATAACAAGACAG
NtASAT1-SP1-R	AAACCTGTCTTGTTATCATACAGC
NtASAT1-SP2-F	GATTGCCAGCAGTATTATCTGGCAG
NtASAT1-SP2-R	AAACCTGCCAGATAATACTGCTGGC
NtASAT1-SP3-F	GATTGTCACTTGTGATGGTTCAGAG
NtASAT1-SP3-R	AAACCTCTGAACCATCACAAGTGAC
NtASAT2-SP1-F	GATTGAGAAGAGTTACTATGTTCAA
NtASAT2-SP1-R	AAACTTGAACATAGTAACTCTTCTC
NtASAT2-SP2-F	GATTGTTTGGGTAGAAGAAAGAAAT
NtASAT2-SP2-R	AAACATTTCTTTCTTCTACCCAAAC
NtASAT2-SP3-F	GATTGATTCTATGTGAAAATGGGGT
NtASAT2-SP3-R	AAACACCCCATTTTCACATAGAATC
Construct-selection-F (Used for correct construct selection)	TTAGGTTTACCCGCCAATA
Construct-selection-R	The reverse primer of the target sequences listed above.
NtASAT1-P-plants-selection_F	TAGCTCTAAGTACATGTGTGT
NtASAT1-P-plants-selection_R	GTACTTTCCTCATCCAAACAT
NtASAT2-P-plants-selection_F	ATGGCTATTTCAAGGCTTGTT
NtASAT2-P-plants-selection_R	GGAGGCCGAGAAATGGTACCT
For gene overexpression constructs and positive plants selection (pCambia35tlegfps2#4 vector, Pst1 and Xba1 cut site)	
O-P_F1（CYP71D16_F）	ATTACGAATTTCGACCTGCAGTAAGTTGATAAAGCTAATTTCTC
O-P- NtASAT1_R1	ACTAGAGCTGAGGCAGCCATCCATGGACCTGGAGGCAATCT
O-NtASAT1-F	ATGGCTGCCTCAGCTCTAGTTTCTT
O-NtASAT1-X_R	ACCGGCGCTCAGTTGGAATTCTAGATCAACATCGAGCTTCCATTT
O-P- NtASAT2_R1	ACAAGCCTTGAAATAGCCATCCATGGACCTGGAGGCAATCT
O-NtASAT2-F	ATGGCTATTTCAAGGCTTGT
O-NtASAT2-X_R	ACCGGCGCTCAGTTGGAATTCTAGACTAGAGTCCTGAGCTTGGA
S-PJ_F1	AAATTTCATATTTGGCTCCACA
S- NtASAT1_R	AGCCTTATAAAAATCAGCACC
S- NtASAT2_R	TGAGCTACGATTAAACTGTCC

1.9 CRISPR/Cas9 Mutagenesis

For CRISPR mutagenesis of *NtASAT1* and *NtASAT2*, three sgRNAs for each gene were selected using the (http://www.multicrispr.net/ index.html) (Table 4-1), and inserted into the pORE-Cas9/gRNA vector (Figure 4-3). *Agrobacterium tumefaciens* strain LBA4404 harboring each of these constructs were used for the transformation of *N. tabacum*. The transformants were analysed by PCR amplification of the target spanning regions using PrimeStar Max DNA Polymerase (TaKaRa) followed by

sequencing. Heterozygous and homozygous mutants were identified by comparing the sequences with those of the wild-type plants.

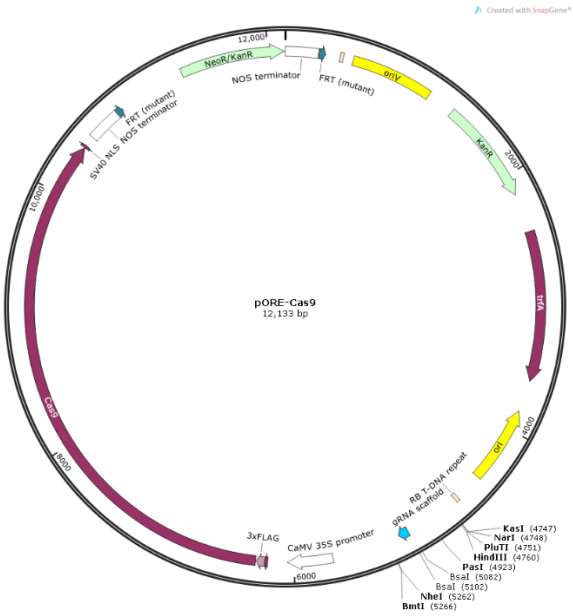


Figure 4-3 Details of the pORE-Cas9/gRNA vector. Which was used for the construction of CRISPR vector.

1.10 Construction and Transformation of The Overexpression Vectors

The trichome-specific *CYP71D16* promoter (Wang et al., 2002) and ORFs of *NtASAT*s were ligated into the pCambia35tl-egfps2#4 vector at the PstI and XbaI sites (see Table 4-1 for primers), introduced into *A. tumefaciens* strain LBA4404, and transformed into *N. tabacum* (cv. HD) and *N. sylvestris*. The transgenic plants were selected through germination on MS medium containing kanamycin, and confirmed by PCR.

1.11 Statistical Analyses

All data analysis was performed using the SPSS software v.25.0. Significant differences between means of dependent variables were determined by one-way ANOVA followed by Duncan's multiple range test.

2 Results

2.1 Identification of NtASATs and Phylogenetic Analyses

To identify *ASAT*s from the *N. tabacum* genome, 12 known *ASAT* genes from *S. lycopersicum*, *P. axill3aris*, and *S. sinuate* were used as queries to search the NCBI database and the *N. tabacum* genomes in the Solanaceae Genomics Network (SGN) using the blastn algorithm. Three hits were obtained in the NCBI database, namely XM_016625428, XM_016595769, and XM_016649597 (Table 4-2). Comparison of these sequences showed consistency of the coding sequences of *XM_01659769* and *XM_016649597* in both the NCBI and SGN websites, whereas the *XM_016625428* coding sequence was found to be different from that of *Nitab4.5_0004970g0010* (Table 4-2, Figures 4-4, 4-5). PCR amplification of these genes using *N. tabacum* trichome cDNA and leaf DNA as templates resulted in the identification *Nitab4.5_0004970g0010* and *XM_016595769*, which were designated as *NtASAT1* and *NtASAT2*, respectively, and they were used for further functional characterization.

Table 4-2 *ASAT* homologous genes searched in NCBI and SGN databases using *SlASATs*, *PaxASATs*, and *SsASATs* as query sequences

Query sequences	Database	Gene NO. of the hits	Length of the coding sequence (bp)
PaxASAT1, *SsASAT1*	NCBI	XM_016625428	1584
	SGN(K326)	Nitab4.5_0004970g0010	1305
	SGN(BX)	mRNA_88047	1305（2 base differences from Nitab4.5_0004970g0010）
	SGN(TN90)	mRNA_56961	1305（1 base difference from Nitab4.5_0004970g0010）
SlASAT1, *PaxASAT2*, *SsASAT2*	NCBI	XM_016595769	1263
	SGN(K326)	Nitab4.5_0004862g0030	1176
	SGN(BX)	mRNA_16888	1263 (same as XM_016595769)
	SGN(TN90)	mRNA_31613	1263 (same as XM_016595769)
SlASAT2, *PaxASAT3*,*Ss ASAT3*	NCBI	XM_016649597	1440 (containing the 1299 bp CDS in K326)
	SGN(K326)	mRNA_58770	1299
	SGN(BX)	mRNA_27780	1299（1 base difference from the CDS of mRNA_58770）
	SGN(TN90)	mRNA_86166	1440 (same as XM_016649597)
SlASAT3, *SlASAT4*, *PaxASAT4*, *SsASAT5*	NCBI	No hits	/
	SGN(K326)	No hits	/
	SGN(BX)	No hits	/
	SGN(TN90)	No hits	/

K326, BX, and TN90 are different tobacco varieties belonging to *Nicotiana tabacum*.

Figure 4-4 Sequence alignments of *PaxASAT1*, *SsASAT1*, and search hits for *N.tabacum* in the NCBI and SGN databases (Table 4-2). *PaxASAT1* and *SsASAT1* are the query sequences; *XM_016625428* is the hit from NCBI; *Nitab4.5_0004970g0010*, *BX_mRNA_88047* and *TN90_mRNA_56961* are three hits respectively from the genome data of three tobacco varieties (K326, BX and TN90) in the SGN databas.

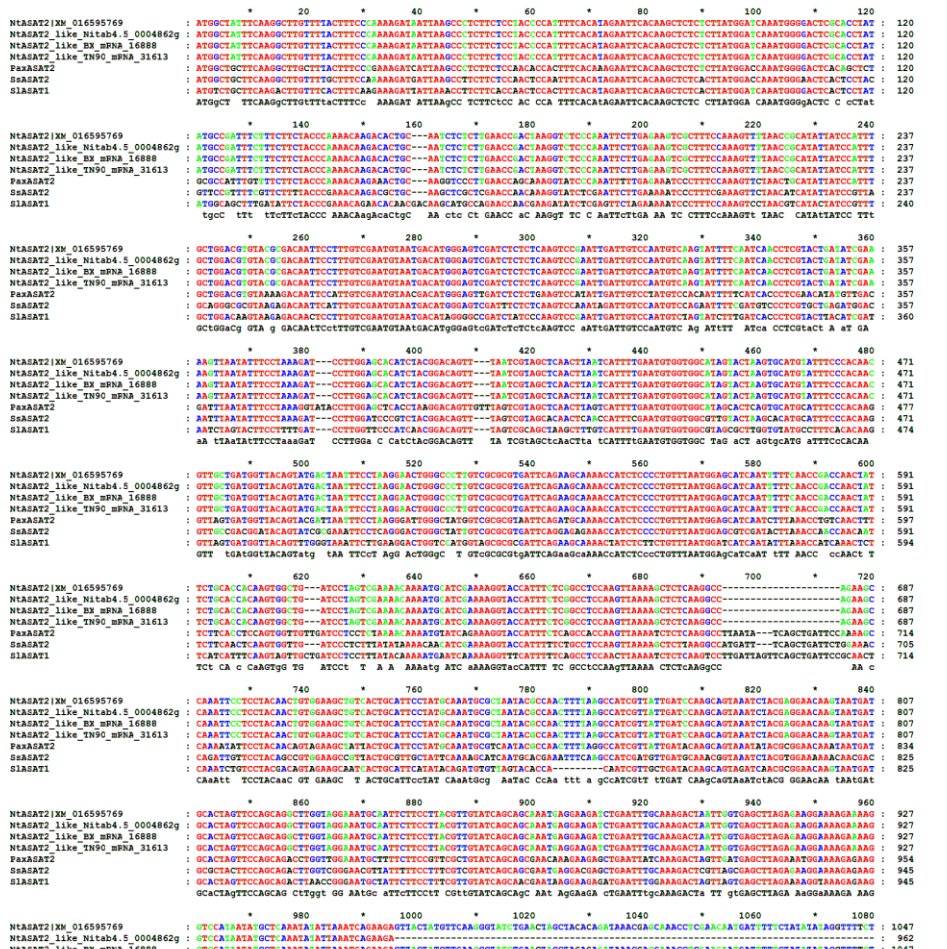


Figure 4-5 Sequence alignments of *SlASAT1*, *PaxASAT2*, *SsASAT2*, and search hits for *Nicotiana tabacum* in the NCBI and SGN database (Table 4-2). *SlASAT1*, *PaxASAT2* and *SsASAT2* are the query sequences; *XM_016595769* is the hit from NCBI; *Nitab4.5_0004862g0030*, *BX_mRNA_16888* and *TN90_mRNA_31613* are three hits respectively from the genome data of three tobacco varieties (K326, BX and TN90) in the SGN database. The coding sequences of *XM_016595769*, *BX_mRNA_16888* and *TN90_mRNA_31613* are completely same.

The ORFs of *NtASAT1* and *NtASAT2* are 1305 bp and 1263 bp long, and encode peptides of 434 and 420 amino acid residues, respectively. They share amino acid sequence identity of 60-75% with the other Solanaceae ASATs that we examined. Comparative analysis of these ASATs led to the identification of 12 putative motifs with 6-50 conserved amino acid residues (Figure 4-6A). Phylogenetic analysis using all *N. tabacum* BAHD family proteins annotated from the genome together with previously reported Solanaceae functional ASATs revealed that NtASAT1 and NtASAT2 were closely clustered with PaxASAT1 and PaxASAT2, respectively, indicating that they have similar functions (Figures 4-6B, 4-7).

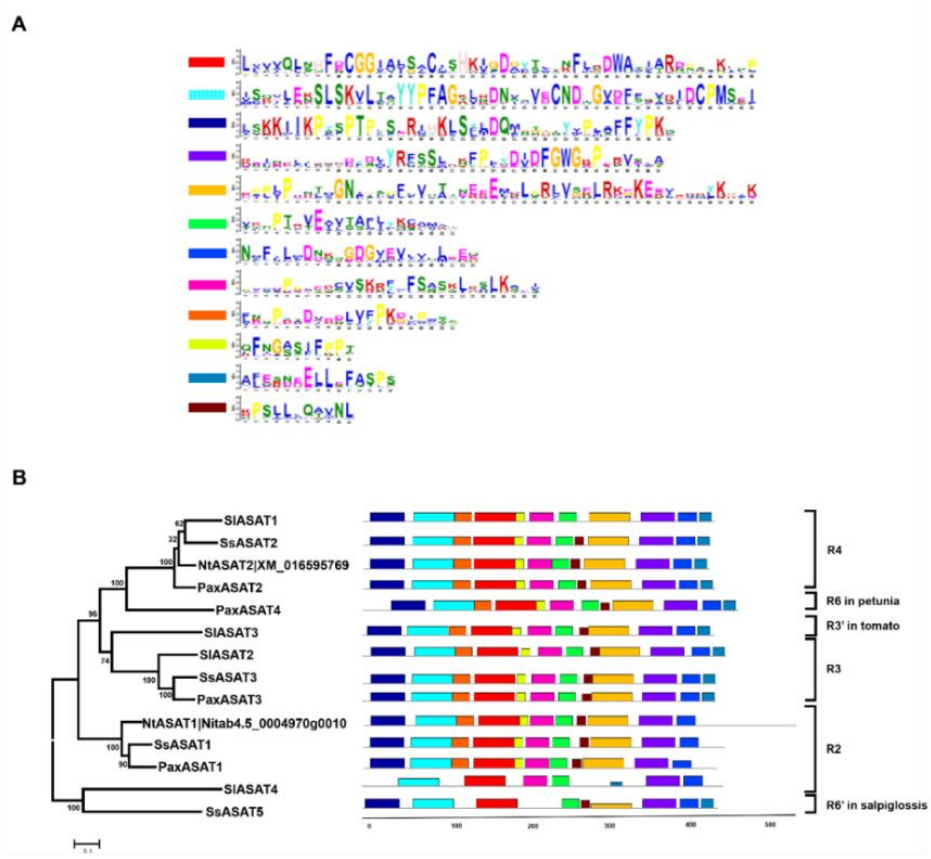


Figure 4-6 Motifs and phylogenetic analysis of putative ASATs of *Nicotiana tabacum*. (A) Identification of 12 conserved motifs in the amino acid sequences of ASATs from Solanaceae species. (B) Phylogenetic analysis of NtASAT1 and NtASAT2 from *N. tabacum* (Nt), together with acyltransferases from *Solanum lycopersicum* (Sl), *P. axillaris* (Pax), and *Salpiglossis sinuata* (Ss) that have been previously validated (Schilmiller et al., 2015; Moghe et al., 2017; Nadakuduti et al., 2017). The corresponding catalytic positions on sucrose are indicated. Each group of the phylogenetic tree shared a similar motif organization, and the motif pattern diversity was in agreement with the phylogenetic analysis.

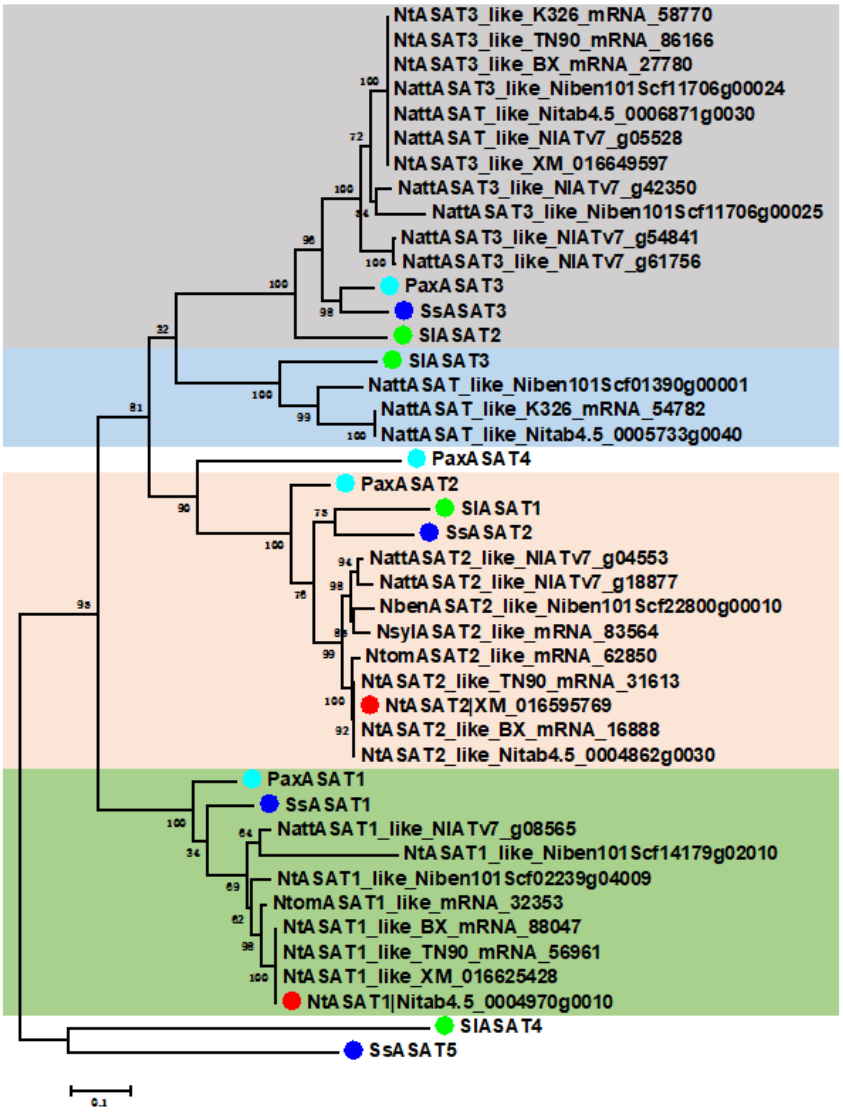


Figure 4-7 Phylogenetic analyses of annotated *ASAT*s from *Solanaceae* species. The *ASAT* Nucleic acid sequences were used to construct the tree from Solanaceae species, including *Nicotiana tobacum* (Nt, two NtASATs were showed in red solid circle), *Nicotiana tomentosiformis* (Ntom), *Nicotiana sylvesis* (Nsyl), *Nicotiana benthamiana* (Nben), *Nicotiana attenuate* (Natt), *Solanum lycopersicum* (Sl), *Petunia axillaris* (Pax), *Salpiglossis sinuata* (Ss). The *ASAT* homologous genes searched in the NCBI and SGN databases using *SlASATs*, *PaxASATs*, and *SsASATs* as query sequences (identities > 70%), the evolutionary history of *ASATs* from Solanaceae species was inferred by using the Maximum likelihood method in MEGA 7 (bootstrap of 1000).

2.2 NtASATs are Localized in The ER of Trichome Head Cells

RT-qPCR showed that the transcript levels of *NtASAT1* and *NtASAT2* were 180-1100-fold greater in trichomes than in leaves with the trichomes removed (Figure 4-8A). To further examine their expression patterns, their promoters were isolated and fused to *GFP* (green fluorescent protein), and the resulting transgenic *N. benthamiana* plants showed strong fluorescence signals in the glandular trichome head cells (Figure 4-8B). To investigate the subcellular localizations of the two NtASATs, they were each fused to *GFP* under the control of the CaMV *35S* promoter, and transiently expressed in *N. benthamiana*. The GFP fluorescence overlapped with that of the ER marker mCherry (Figure 4-8C), suggesting that both of the NtASATs were localized in the ER.

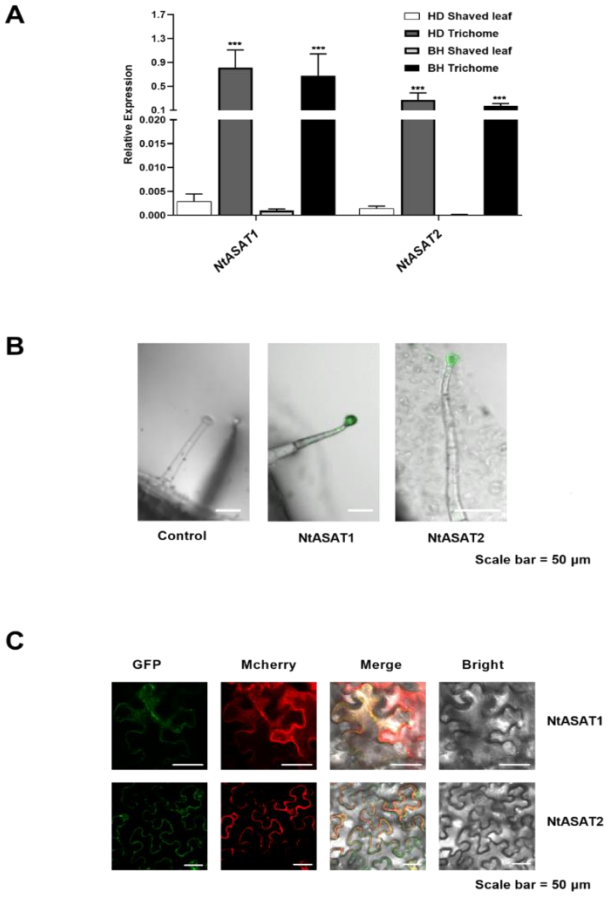


Figure 4-8 Expression patterns and subcellular localization of NtASATs. (A) qRT-PCR analysis of the Relative expression levels of NtASATs in glandular trichomes and trichome-shaved leaves of the *Nicotiana tabucum* cultivars Hongda (HD) and Beinhart1000-1 (BH). Upper leaves (10-15 cm in length) were sampled from 50-day-old plants after transplanting. Data are means (±SD), with three biological and three technical

replicates being performed for each sample. Values labeled with asterisks are significantly different; Significant differences between the leaves and trichomes were determined using one-way ANOVA followed by Duncan's multiple range test (***P<0.01). (B) Localization of NtASAT in trichomes, as determined by transient expression of green fluorescent protein (GFP) driven by the *NtASAT* promoters in *N. benthamiana*. Representative images of the dorsal epidermis are shown. (C) Subcellular localization of the NtASATs, as determined using transient expression in leaves of *N. benthamiana*. GFP and the endoplasmic reticulum marker mCherry were expressed under the control of the CaMV *35S* promoter.

2.3 Biochemical Characterization of N. tabacum ASATs in vitro

To analyse the catalytic activities of NtASAT1 and NtASAT2 *in vitro*, their recombinant proteins were produced in *E.coli*, purified, and their activities were tested using sucrose as the acyl acceptor together with various acyl donors, including acetyl-CoA (C2-CoA), isobutyryl-CoA (iC4-CoA), and isovaleryl-CoA (iC5-CoA) (Table 4-3), which are common acyl groups in *N. tabacum* acylsucroses (Wang et al, 2002). We found that NtASAT1 could accept all the tested acyl-CoA substrates towards the sucrose core, producing the monoacylsucroses S1:2 with acetyl-CoA, S1:4 with iC4- CoA, and S1:5 with iC5-CoA (Figures 4-9, 4-10; Table 4-3). Moreover, while a single product was detected with the acetyl-CoA donor, for iC4-CoA and iC5-CoA we also detected a few minor products with different retention times in addition to the major product, and this phenomenon might have been caused by the enzymes having multiple catalytic sites or by non-enzyme rearrangement (Fan et al., 2016; Lou et al., 2021). To determine the position of acylation, we scaled-up the reaction using sucrose and iC5-CoA as substrates and purified the S1:5 product. NMR analysis then revealed that the acyl group was attached to the R2 position of sucrose (Table 4-4). In contrast, NtASAT2 did not show activity towards sucrose or any acyl donor, indicating that it is not involved in the biosynthesis of monoacylsucroses.

To elucidate the secondary step of acylsucrose biosynthesis, a sequential reaction was performed by adding the NtASAT1/2 enzymes and C2/iC4/iC5-CoA donors into the NtASAT1 reaction mixtures containing monoacylsucroses acceptors S1:2, S1:4, or S1:5 (Table 4-3). Addition of NtASAT2 in the presence of S1:5 and iC5-CoA lead to a major product of diacylsucrose (S2:10, *m/z* 533.2209, $[M^+Na]^+$) as well as several minor products (Figure 4-9B), whereas no product was detected when C2-CoA and iC4-CoA were used as donors. NMR analysis revealed that at least three acylsucroses products were present in the major product peak, as purified by LC (Figure 4-11). No catalytic activity was detected for NtASAT1 with any combinations of monoacylsucrose and acyl-group donor.

When glucose was used as the sugar substrate, no catalytic activity was detected for either of the two NtASATs with the C2/iC4/iC5-CoA donors (Table 4-3), suggesting that they possess acyl-transfer activities towards sucrose but not glucose, which is consistent with the presence of large amounts of acylsucroses on the surfaces of *N. tabacum* plants, rather than acylglucoses.

Table 4-3 Summary of ASAT activities using different acyl acceptors and acyl-CoA donors as substrates.

Enzymes	Acyl acceptors	Acyl-CoA donors	Products
Nitab4.5_0004970g0010 / **NtASAT1**	Sucrose	C2	+
	Sucrose	iC4	+
	Sucrose	iC5	+
XM_016595769 / **NtASAT2**	Sucrose	C2	-
	Sucrose	iC4	-
	Sucrose	iC5	-
Nitab4.5_0004970g0010 / **NtASAT1**	S1:2(C2)	C2	-
	S1:2(C2)	iC4	-
	S1:2(C2)	iC5	-
	S1:4(iC4)	C2	-
	S1:4(iC4)	iC4	-
	S1:4(iC4)	iC5	-
	S1:5(iC5)	C2	-
	S1:5(iC5)	iC4	-
	S1:5(iC5)	iC5	-
XM_016595769 / **NtASAT2**	S1:2(C2)	C2	-
	S1:2(C2)	iC4	-
	S1:2(C2)	iC5	-
	S1:4(iC4)	C2	-
	S1:4(iC4)	iC4	-
	S1:4(iC4)	iC5	-
	S1:5(iC5)	C2	-
	S1:5(iC5)	iC4	-
	S1:5(iC5)	iC5	+
	S1:5(iC5)	iC4	-
	S1:5(iC5)	iC5	-
Nitab4.5_0004970g0010/ **NtASAT1**	S2:10(iC5,iC5)	C2	-
	S2:10(iC5,iC5)	iC4	-
	S2:10(iC5,iC5)	iC5	-
XM_016595769 /	S2:10(iC5,iC5)	C2	-

NtASAT2	S2:10(iC5,iC5)	iC4	-
	S2:10(iC5,iC5)	iC5	-
	S2:10(iC5,iC5)	iC5	-
Nitab4.5_0004970g0010 / **NtASAT1**	glucose	C2	-
	glucose	iC4	-
	glucose	iC5	-
XM_016595769 / **NtASAT2**	glucose	C2	-
	glucose	iC4	-
	glucose	iC5	-

"+" means active with corresponding product peaks. "-" refers to no acylsugar product was detected.

Table 4-4 Nuclear magnetic resonance chemical shifts of S1:5 produced *in vitro*

Carbon	^{1}H(ppm)	^{13}C(ppm)[a]
1(CH)	5.45(br)	89.7
2(CH)	4.66(dd,J=3.5Hz,10.3Hz)	72.38
2-O-	-	-
-1(CO)	-	-
-2(CH2)	2.23,2.32(m)	42.86
-3(CH)	1.99(m)	25.11
-4(CH3)	0.87(br,m)	21.53
3(CH)	3.85(t,J=9.6Hz)	70.35
4(CH)	3.47(m)	69.25
5(CH)	3.80(br)[b]	72.41
6(CH)	3.72(br,m)[a]	62.43
1'(CH2)	3.42,3.50(m)	60.87
2'(C)	-	-
3'(CH)	4.13(d,J=8.8Hz)	75.44
4'(CH)	3.95(t,J=8.5Hz)	73.74
5'(CH)	3.79(br)[a]	81.56
6'(CH2)	3.74(br,m)[a]	60.07

[a]Determined by HSQC. [b]Determined by COSY

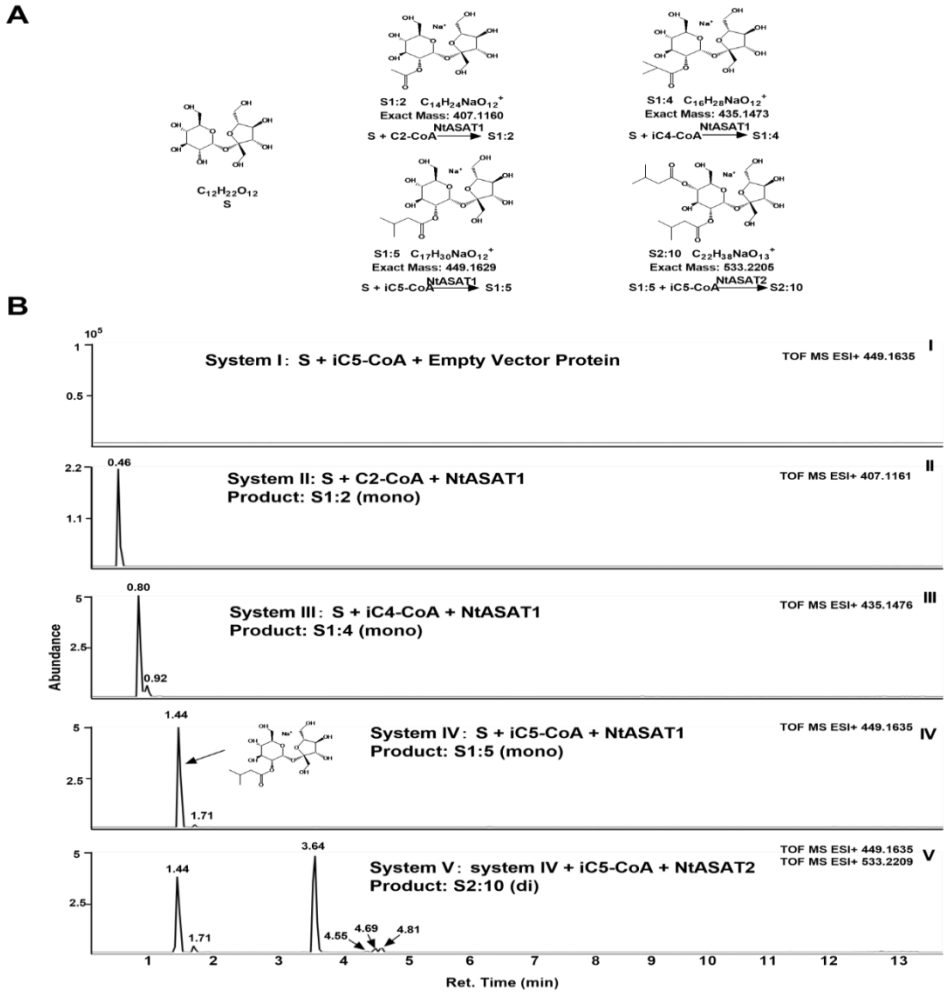


Figure 4-9 Functional characterization of NtASAT1 and NtASAT2 *in vitro*. (A) Structures and exact masses of mono- and di-acylsucroses as characterized by protein assays. The structures shown were only used for exact mass calculations, and all isomers had the same mass. The exact mass was calculated based on the predicted molecular formula of singly charged molecular ions with Na^+ as adduct in positive ion mode. The reactions that produced the S1:2, S1:4, S1:5, and S2:10 acylsucroses are shown below each structure. (B) Extracted ion chromatograms from liquid-chromatography quadrupole time-of-flight mass spectrometry in positive ion mode for reactions *in vitro*. (I) Control assay of the pATX-sumo empty vector protein using sucrose (S) as the acyl acceptor and iC5-CoA as the acyl donor. The same result was also found when using C2-CoA or iC4-CoA. (II) Formation of S1:2 using sucrose as the acyl acceptor and C2-CoA as the acyl donor, (III) formation of S1:4 using sucrose and iC4-CoA, and (IV) formation of S1:5 using sucrose and iC5-CoA. The mass-to-charge ratios (*m/z*) of the reaction products are shown. (V) The consecutive reactions with NtASAT1 and NtASAT2 that produce S2:10 diacylsucrose (the finished reaction

catalysed by NtASAT1 is also shown in IV). The *m/z* values of the sequential reaction products are shown. The numbers above the peaks in (II–V) represent the retention times of different isomers. The structure of S1:5 (retention time 1.44 min) was verified using NMR spectroscopy (Table 4-4).

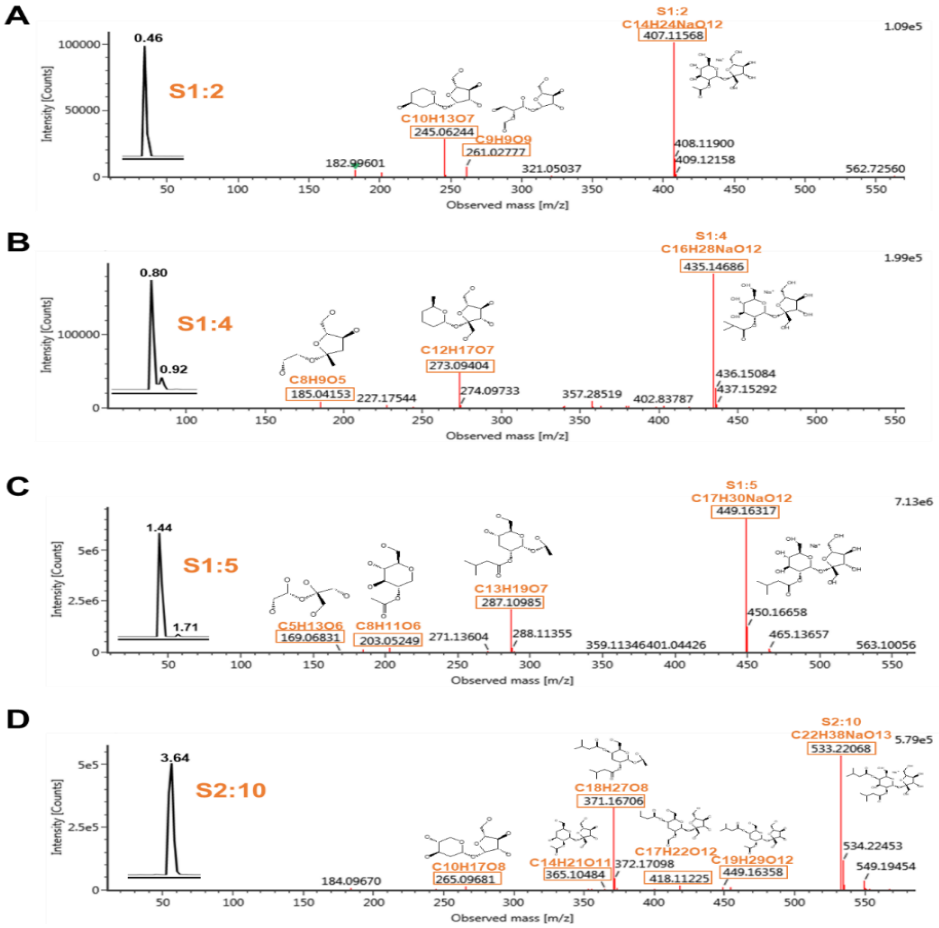


Figure 4-10 LC-MS/MS analysis of mono- and di-acylsugars in protein assays. (**A**), (**B**), (**C**), (**D**) showed the LC-MS/MS analysis of the acylsugars in protein assays shown in figure 4, respectively. The names of the acylsugars are highlighted in orange and the chromatography is aligned on the x axis based on m/z. (**A**), (**B**), (**C**) showed S1:2, S1:4, and S1:5 via NtASAT1, using sucrose as the acyl acceptor and C2-CoA, iC4-CoA, and iC5-CoA, respectively, as the acyl donors. (**D**) showed S2:10 via NtASAT2, using S1:5 (produced from the first step) as the acyl acceptor and iC5-CoA as the acyl donor. Highlighted in orange boxes are some of the abundant characteristic mass features, with their compositions above.

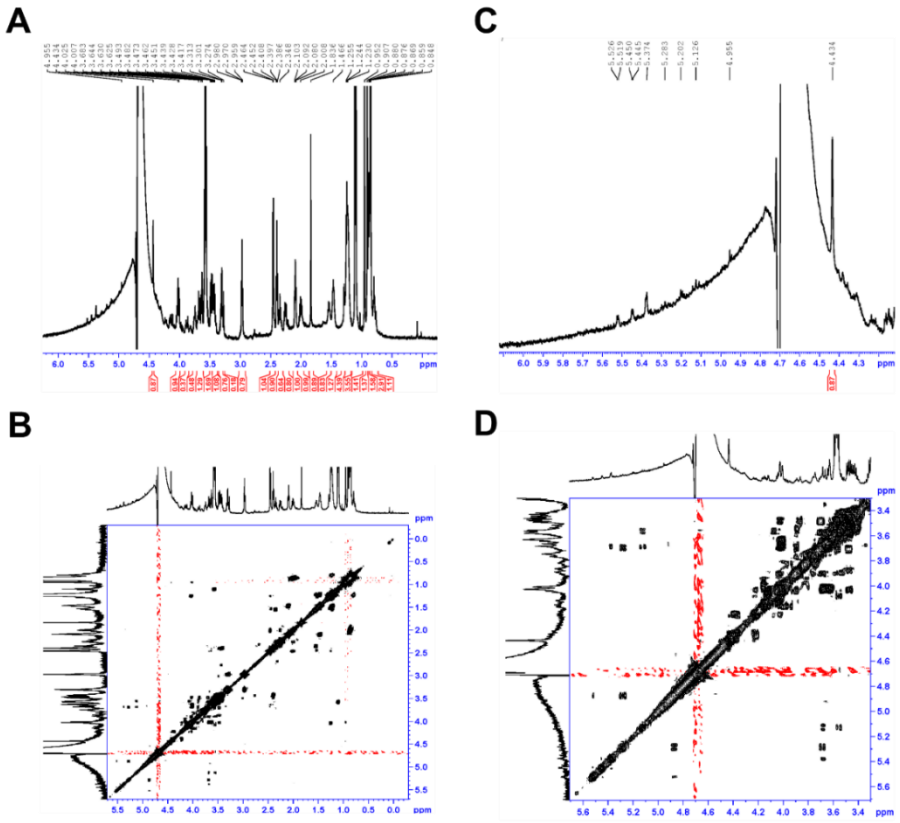


Figure 4-11 ^{1}H NMR and ^{1}H-^{1}H COSY spectra of isolated diacylsucrose products (S2:10). (**A**) ^{1}H NMR spectra (600 MHz) of S2:10 in D_2O. (**B**) ^{1}H-^{1}H COSY spectra (600 MHz) of S2:10 in D_2O. (**C**) Partial ^{1}H NMR spectra (600 MHz) of S2:10 in D_2O. (**D**) Partial ^{1}H-^{1}H COSY spectra (600 MHz) of S2:10 in D_2O.

2.4 Silencing of NtASATs Impairs Acylsugar Biosynthesis in Tobacco

To examine their functions *in vivo*, *NtASAT1* and *NtASAT2* were mutated by the CRISPR/Cas9 method in the *N. tabacum* cultivars Hongda (HD) and Beinhart1000-1 (BH). For each of the genes, three specific target sites in the exon region (named as *NtASAT1*-SP1, *NtASAT1*-SP2, and *NtASAT1*-SP3 for *NtASAT1*, and *NtASAT2*-SP1, *NtASAT2*-SP2, and *NtASAT2*-SP3 for *NtASAT2*) were selected to produce binary vectors and introduced into HD and BH, which possess different acylsucrose profiles (Qu et al., 2017; Figures 4-12, 4-13). In terms of nomenclature, the mutants of *NtASAT1* and *NtASAT2* from the different target sites in HD are referred to in the form H-*asat1*-SP1-3 and H-*asat2*-SP1-3, respectively, and in BH as B-*asat1*-SP1-3 and B-*asat2*-SP1-3, respectively. In the T0 generation, one heterozygous and four homozygous mutant plants of BH were obtained, and two heterozygous mutant lines of HD, namely H-*asat1*-SP2-T0#6 and

H-*asat2*-SP2-T0#24 (Figure 4-14, Tables 4-5, 4-6). Self-pollinated seeds collected from H-*asat1*-SP2-T0#6 and H-*asat2*-SP2-T0#24 were subsequently planted (named as H-*asat1*-SP2-T1#6 and H-*asat2*-SP2-T1#24, respectively) and the mutations of their target sites were confirmed by sequencing (Table 4-5, Figure 4-14B, C). We then selected 20 plants with different mutation types for acylsugar profiling (Table 4-6).

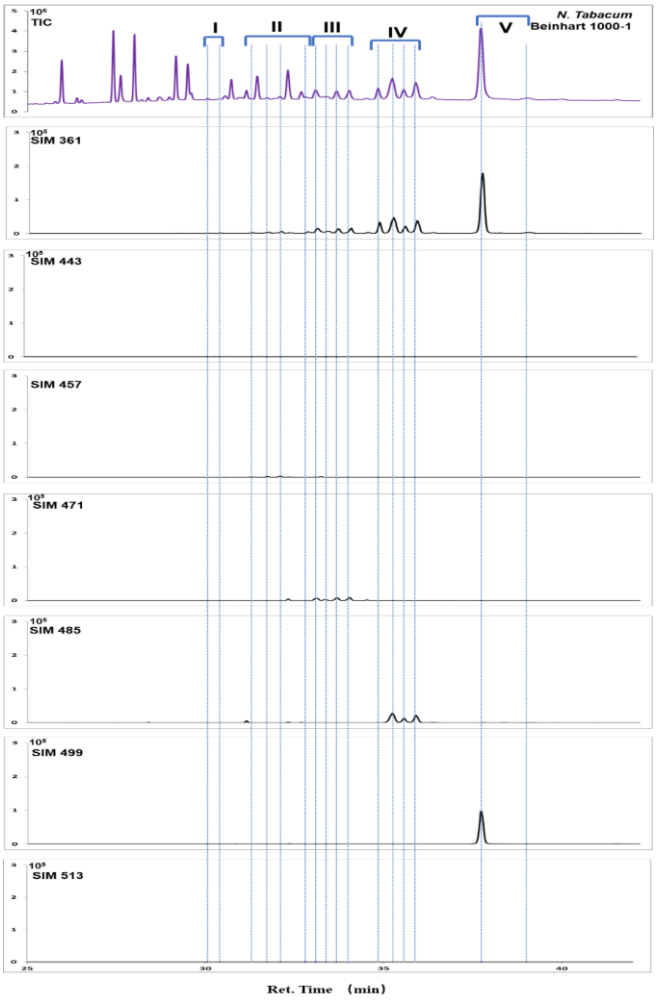


Figure 4-12 Type I-V acylsugars in *N. tabacum* cultivar Beinhart1000-1 (BH).

Acylsugars types were identified according to extraction specific ions (Severson et al., 1985), 361 was the specific ion from furanose ring, 443, 457, 471, 485, 499 and 513 were the specific ion from pyranose rings.

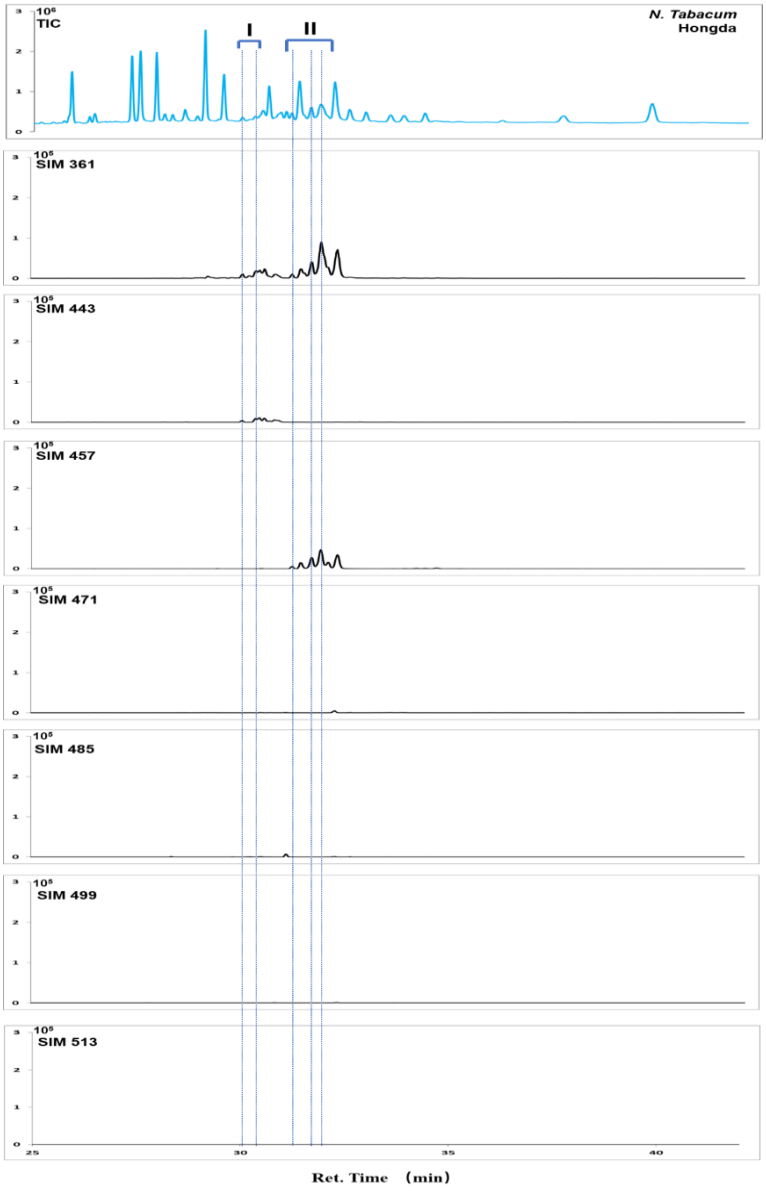


Figure 4-13 Type I and II acylsugars in *N. tabacum* cultivar Hongda (HD). Acylsugars types were identified according to extraction specific ions (Severson et al., 1985), 361 was the specific ion from furanose ring, 443, 457, 471, 485, 499 and 513 were the specific ion from pyranose rings.

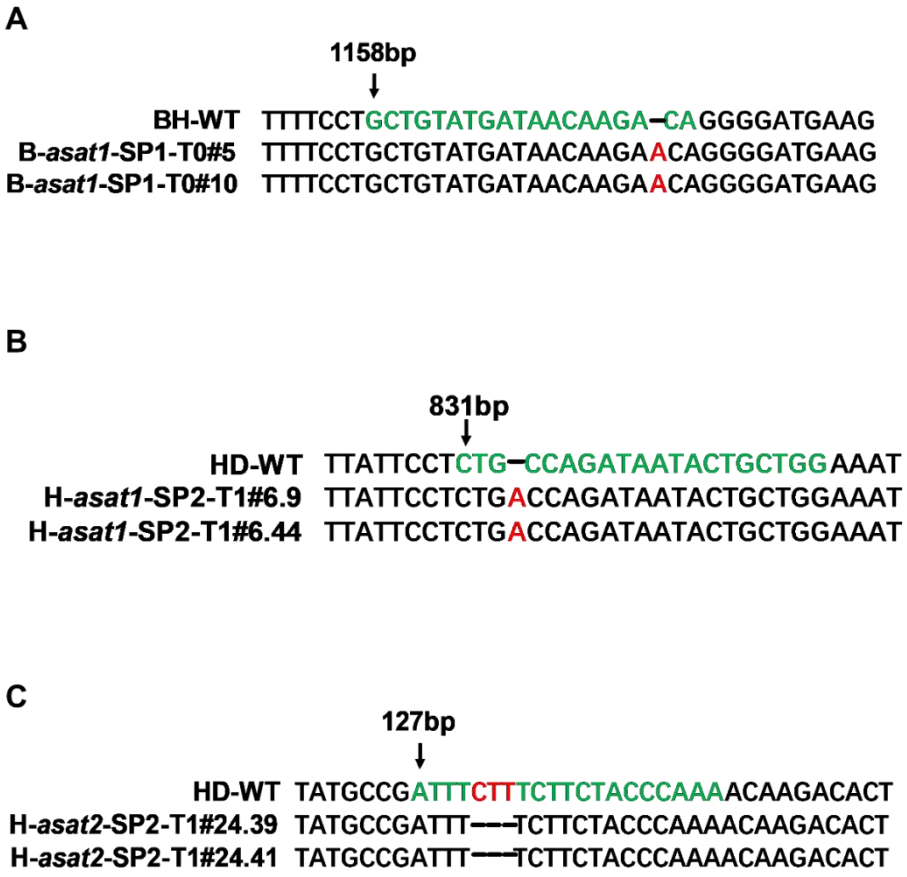


Figure 4-14 Targeted inversions and deletions in *NtASAT1* and *NtASAT2* loci by CRISPR-Cas9 technology. (A) Sequence comparison of *NtASAT1*-SP1 from *N. tabacum* cultivar Beinhart1000-1 wildtype (BH-WT) and two T0 homozygous mutants (B-*asat1*-SP1-T0#5, B-*asat1*-SP1-T0#10). **(B)** Sequence comparison of *NtASAT1*-SP2 from *N. tabacum* cultivar Hongda wildtype (HD-WT) and two T1 homozygous mutants (H-*asat1*-SP2-T1#6.9, H-*asat1*-SP2-T1#6.44). **(C)** Sequence comparison of *NtASAT2*-SP2 from HD-WT and two T1 homozygous mutants (H-*asat2*-SP2-T1#24.39, H-*asat2*-SP2-T1#24.41). In **a**, **b** and **c**, the arrows marked with numbers pointed to the beginning position of the gRNA target sequences in the target gene. The gRNA target sequences were shown in green letters, DNA insertions are shown in red letters and DNA deletions are shown in dashes. All the homozygous mutants for the same target site have the same edition type, but only two mutants for each target were shown here.

Table 4-5 CRISPR/Cas9-mediated mutagenesis of *NtASAT1* and *NtASAT2* in T0 and T1 generations.

Name of lines	No. of plants sequenced	No. of homozygous mutants (with vectors)	No. of homozygous mutants (without vectors)	No. of heterozygous mutants (with vectors)	No. of plants with no mutation (with vectors)
B-*asat1*-SP1-T0	12	4 (+ 1 bp insertion)	0	1	7
H-*asat1*-SP2-T0	4	0	0	1	3
H-*asat2*-SP2-T0	5	0	0	1	4
H-*asat1*-SP2-T1#6	50	8 (+ 1 bp insertion)	2 (+ 1 bp insertion)	32	8
H-*asat2*-SP2-T1#24	50	7 (- 3 bp deletion)	1 (- 3 bp deletion)	34	8

Table 4-6 Information of all *NtASAT1* and *NtASAT2* knockout plants.

Number of acylsugar detected plants	Generation	Genetic background	Mutation types at the target site	With vector or not
B-asat1-SP1-T0#2	T0	Beinhart1000-1	heterozygous mutants	with vector
B-asat1-SP1-T0#1	T0	Beinhart1000-1	homozygous mutants (+1 bp insertion)	with vector
B-asat1-SP1-T0#4	T0	Beinhart1000-1	homozygous mutants (+1 bp insertion)	with vector
B-asat1-SP1-T0#5	T0	Beinhart1000-1	homozygous mutants (+1 bp insertion)	with vector
B-asat1-SP1-T0#10	T0	Beinhart1000-1	homozygous mutants (+1 bp insertion)	with vector
H-asat1-SP2-T1#6.9	T1	Hongda	homozygous mutants (+1 bp insertion)	without vector
H-asat1-SP2-T1#6.16	T1	Hongda	homozygous mutants (+1 bp insertion)	without vector
H-asat1-SP2-T1#6.29	T1	Hongda	homozygous mutants (+1 bp insertion)	with vector
H-asat1-SP2-T1#6.44	T1	Hongda	homozygous mutants (+1 bp insertion)	with vector
H-asat1-SP2-T1#6.45	T1	Hongda	homozygous mutants (+1 bp insertion)	with vector
H-asat1-SP2-T1#6.6	T1	Hongda	No mutation	with vector
H-asat1-SP2-T1#6.17	T1	Hongda	No mutation	with vector
H-asat1-SP2-T1#6.18	T1	Hongda	No mutation	with vector
H-asat2-SP2-T1#24.36	T1	Hongda	homozygous mutants (-3 bp deletion)	with vector
H-asat2-SP2-T1#24.38	T1	Hongda	homozygous mutants (-3 bp deletion)	with vector

H-asat2-SP2-T1#24.39	T1	Hongda	homozygous mutants (-3 bp deletion)	with vector
H-asat2-SP2-T1#24.41	T1	Hongda	homozygous mutants (-3 bp deletion)	without vector
H-asat2-SP2-T1#24.12	T1	Hongda	No mutation	with vector
H-asat2-SP2-T1#24.20	T1	Hongda	No mutation	with vector
H-asat2-SP2-T1#24.23	T1	Hongda	No mutation	with vector
Beinhart1000-1	Wild type	/	/	/
Hongda	Wild type	/	/	/

For the B-*asat1*-SP1-T0 plants, acylsugar profiling showed that the phenotypes of the heterozygous mutants were similar to that of the BH wild-type, and no acylsugars were detected in any of a total of four homozygous mutants (Figures 4-15A, 4-16). For the H-*asat1*-SP2-T1#6 population, no acylsugars were detected in homozygous mutants, while two types of acylsucroses, similar to those of the HD wild-type, were detected in plants with vectors but without mutations (Figures 4-15B, 4-17). This indicated that *NtASAT1* mutations, but not vector insertions, affected the acylsugar phenotypes. The results for the H-*asat2*-SP2-T1#24 acylsugar profile were the same as those for H-*asat1*-SP2-T1#6 T1 plants (Figure 4-15B), and no acylsugars were detected in the homozygous mutants and two acylsucrose types were detected in plants without mutations (Figures 4-15B, 4-18). Taken together, these results suggest that *NtASAT1* and *NtASAT2* are involved in acylsugar assembly *in planta*.

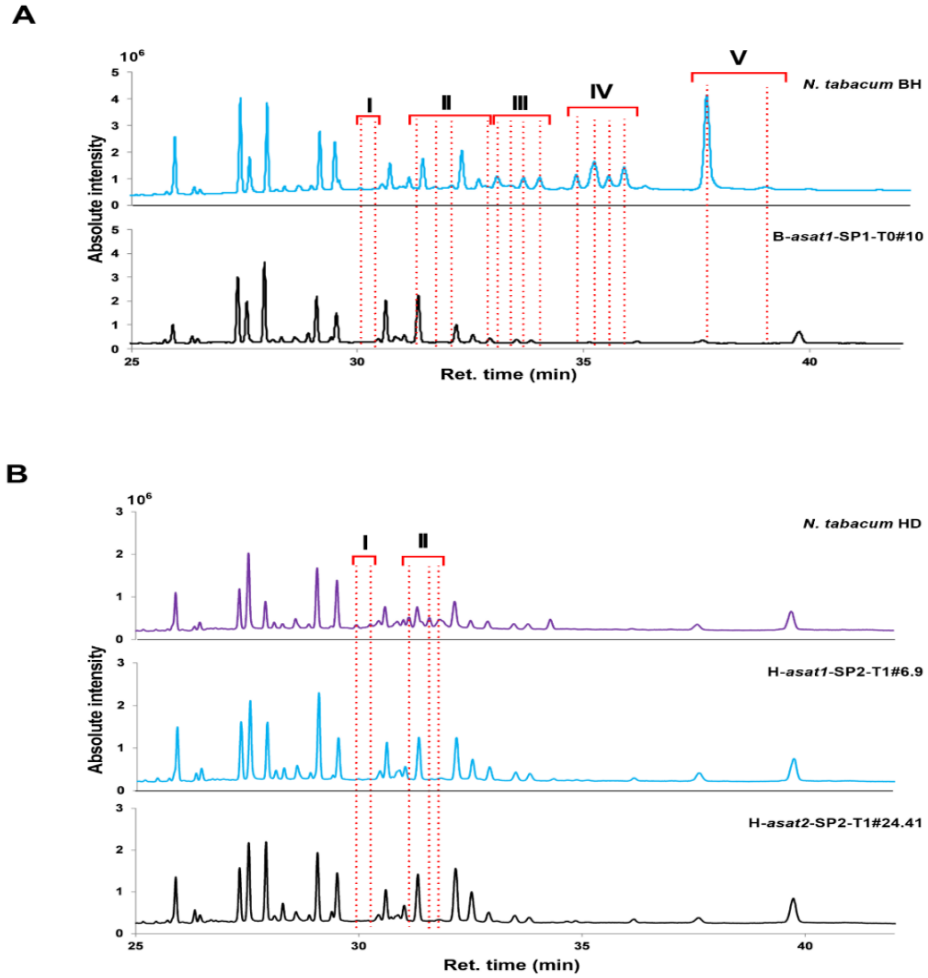


Figure 4-15 Acylsugar profiles of CRISPR/Cas9-edited *NtASAT* mutants of the *Nicotiana tabacum* cultivars Hongda (HD) and Beinhart1000-1 (BH). Samples were taken from upper leaves (10-15 cm in length) of 50-day-old plants grown in the field. (A) GC-MS analysis of acylsucrose profiles of the BH wild-type and the T0 homozygous *asat1* mutant (B-*asat1*-SP1-T0#10). The dashed lines indicate the five different acylsugar types, as classified according to Severson et al. (1985). (B) GC-MS analysis of acylsucrose profiles of the HD wild-type and T1 homozygous *asat1* mutant (H-*asat1*-SP2-T1#6.9) and *asat2* mutant (H-*asat2*-SP2-T1#24.41). The acylsugar types are indicated as in (A). The profiles in (A) and (B) show that no acylsugars could be detected in the homozygous *asat1* or *asat2* mutants.

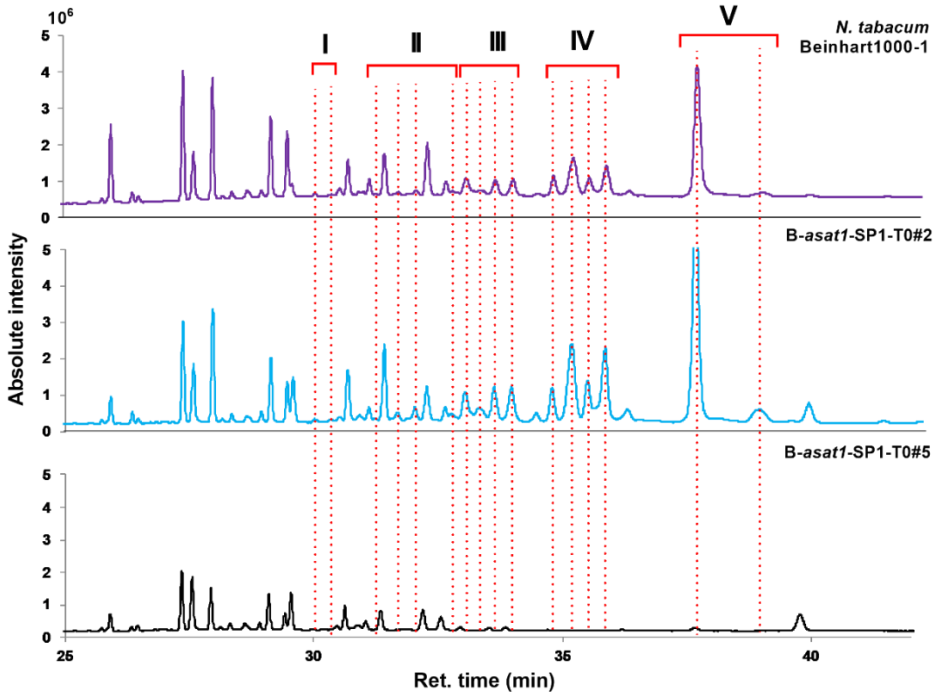


Figure 4-16 Acylsugar profiles of the *N. tabacum* cultivar Beinhart1000-1 (BH) wild-type and T0 heterozygous and homozygous mutants of the *NtASAT1* locus. The GC-MS chromatograms are shown. The chromatographic peaks on the dashed line position are acylsugars, and all the marks on the figure are same as the above on Figure 4-12. The acylsugar profile of heterozygous mutant (B-*asat1*-SP1-T0#2) is similar to that of wildtype, and there are no acylsugars detected in all homozygous mutants (B-*asat1*-SP1-T0#5 is shown here. Another one homozygous mutant is shown in Figure 4-15, others are similar and not shown).

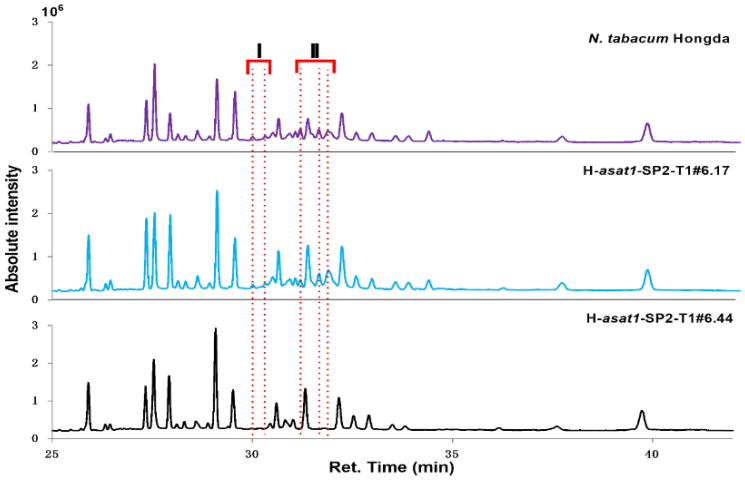


Figure 4-17 Acylsugar profiles of the *N. tabacum* cultivar Hongda (HD) wild-type, and *NtASAT1* mutated and un-mutated T1 homozygous plants. The GC-MS chromatograms are shown. All the marks on the figure are same as the above on Figure 4-13. The acylsugar profile of the unmutated homozygous plants (here one plant with vector, H-*asat1*-SP2-T1#6.17, is shown) are all similar to that of wildtype, and there are no acylsugars detected in all homozygous mutants with or without vector (here one plant with vector, H-*asat1*-SP2-T1#6.44, is shown. One homozygous mutant without vector, H-*asat1*-SP2-T1#6.9, is also shown in Figure 4-15, others are similar and not shown).

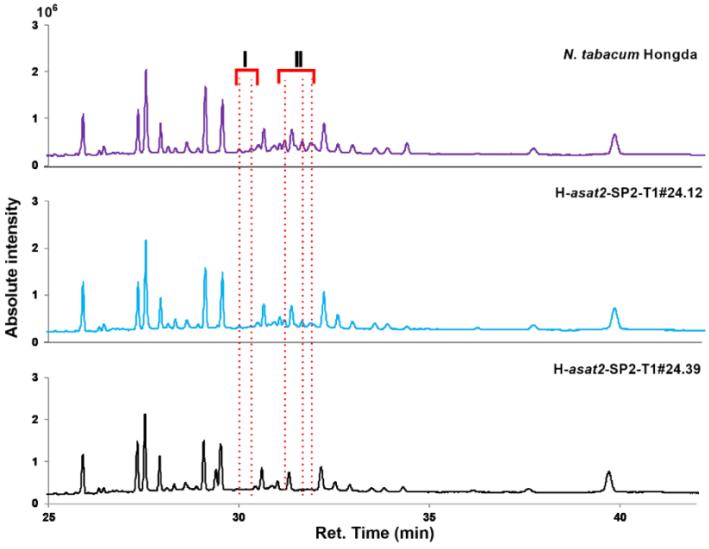


Figure 4-18 Acylsugar profiles of the *N. tabacum* cultivar Hongda (HD) wild-type, and *NtASAT2* mutated and un-mutated T1 homozygous plants. The GC-MS chromatograms

are shown. All the marks on the figure are same as the above on Figure 4-13 and 4-17. The acylsugar profile of the unmutated homozygous plants (here one plant with vector, H-*asat2*-SP2-T1#24.12, is shown) are all similar to that of wildtype, and there are no acylsugars detected in all homozygous mutants with or without vector (here one plant with vector, H-*asat2*-SP2-T1#24.39, is shown. One homozygous mutant without vector H-*asat2*-SP2-T1#24.41 is shown in Figure 4-15, others are similar and not shown).

2.5 Overexpression of NtASATs Does Not Increase The Acylsugar Content of N. tabacum

To further examine the role of *NtASAT*s in acylsugar biosynthesis, we generated transgenic *N. tabacum* lines overexpressing *NtASAT1* or *NtASAT2* in the HD background (Figure 4-19). Three *NtASAT1* and four *NtASAT2* independent lines in the T1 generation with significantly increased expression levels were selected and planted in the field for analysis. qRT-PCR indicated that the expression levels of *NtASAT1* in the T1-ASAT1 lines and of *NtASAT2* in the T1- ASAT2 lines were up-regulated significantly (Figure 4-20A, B), whilst the expression levels of *NtASAT2* in the T1-ASAT1 lines and of *NtASAT1* in the T1-ASAT2 lines were not improved compared to those of wild-type plants (Figure 4-21). No differences in acylsugar content were observed between the overexpression lines and the wild-type (Figure 4-20C). We subsequently repeated the experiment in a greenhouse using three *NtASAT2* overexpression lines, and obtained similar results (Figure 4-22).

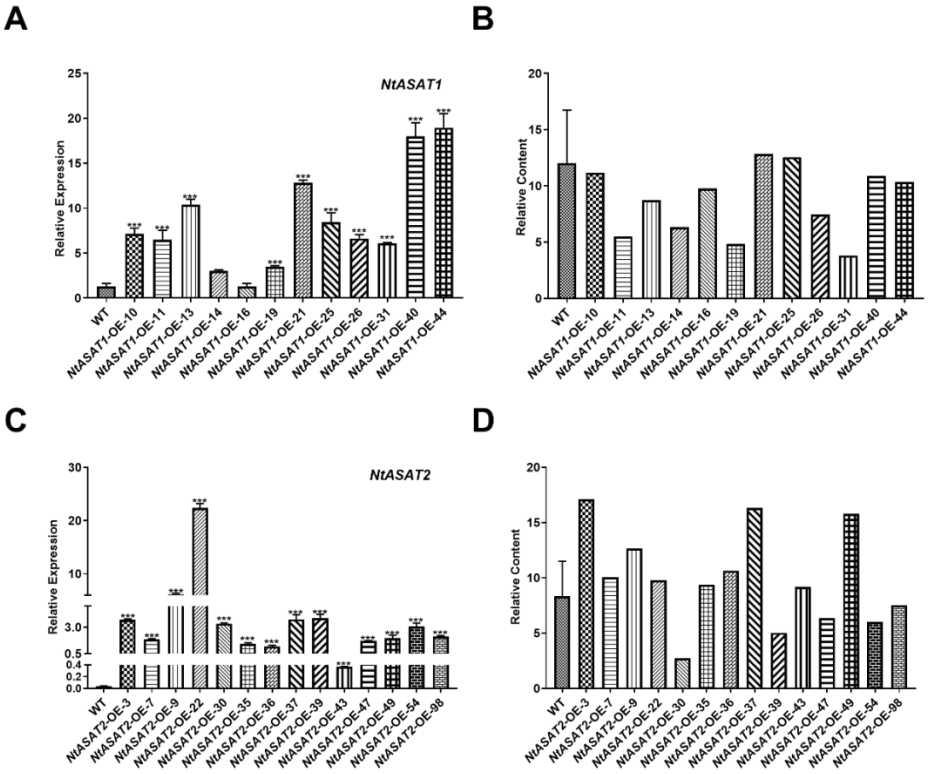


Figure 4-19 Analysis of *NtASAT1* and *NtASAT2* over-expression plants of *N. tabacum* cultivar Hongda (HD). (**A**) *NtASAT1* expression level comparison of the thichomes of Hongda wild type (WT) and 12 independent T0 *NtASAT1* transgenic positive plants. (**B**) The acylsugar contents of the WT and the corresponding independent *NtASAT1* overexpression plants. (**C**) *NtASAT2* expression level comparison of the thichomes of Hongda wild type (WT) and 14 independent T0 transgenic positive plants. (**D**) The Acylsugar content data for the WT and the corresponding independent T0 *NtASAT2* overexpression plants. **A, C,** the expression level data of which are the average of three technical replicates, asterisks denote significant differences; ***, $P < 0.01$. **B, D** are single measurements of each T0 primary transformant. Data for Hongda WT are the average of three biological replicates.

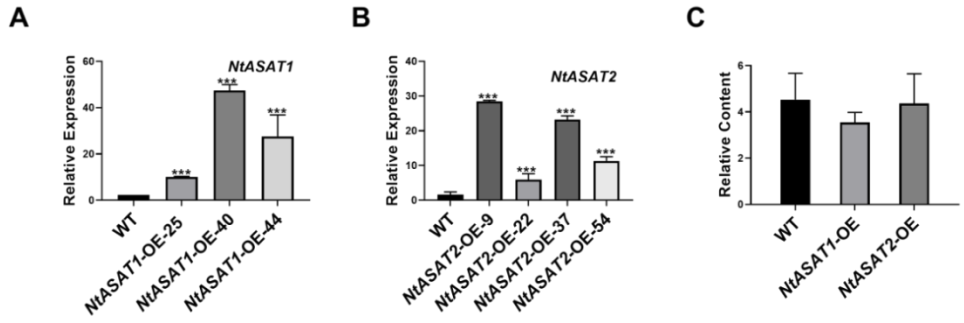


Figure 4-20 Relative expression and acylsugar contents in the *Nicotiana tabacum* wild-type cultivar Hongda, and *ASAT1* and *ASAT2*-overexpression lines. Samples were taken from upper leaves (10-15 cm in length) of 50-day-old plants grown in the field. (A, B) Relative expression of *ASAT1* in the trichomes of the wild-type (WT) and three *NtASAT1*-overexpression (OE) lines, and (B) relative expression of *ASAT2* in the trichomes of the WT and four *NtASAT2*- overexpression (OE) lines. Expression was determined by qRT-PCR and is relative to that in the WT, the value of which was set as 1. For the OE lines, the expression data are means of three technical replicates of a single pooled sample consisting of three individual plants. (C) Acylsugar contents in the WT and *ASAT*s-overexpression lines. The data are for T1 generation lines and are mean values for the OE lines shown in (A, B). For the OE lines, a pooled three-plant sample was taken for each of the individual lines, and the data is the mean overall value (i.e. n=3 for *ASAT1* and n=4 for *ASAT2*). The error bars are all ± SD, and significant differences compared with the WT were determined using one-way ANOVA followed by Duncan's multiple range test (***P<0.01). All data for the WT are means of three replicates, each of which consisted of pooled samples from three individual plants.

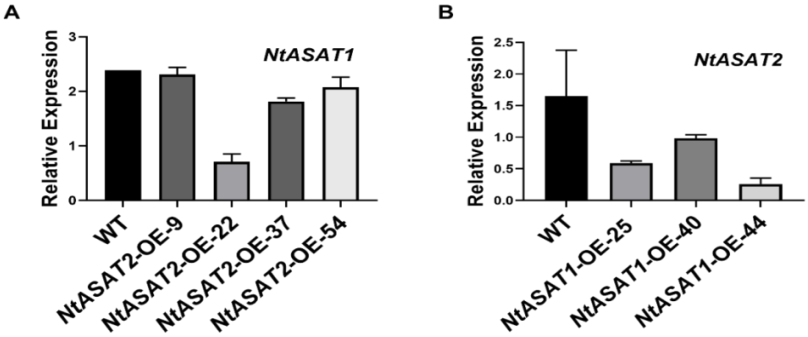


Figure 4-21 *NtASAT1* expression in *NtASAT2*-overexpression lines and *NtASAT2* expression in *NtASAT1*-overexpression lines of *N. tabacum* cultivar Hongda (HD).

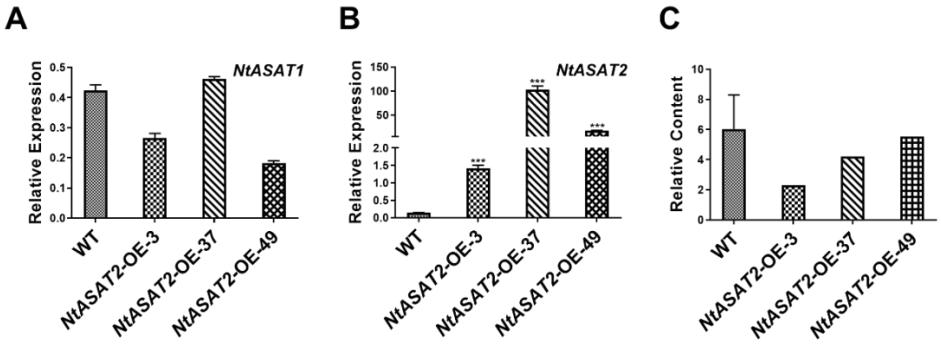


Figure 4-22 Analysis of *NtASAT2* over-expression plants of *N. tabacum* cultivar Hongda (HD) grown in a greenhouse. (A), (B), *NtASAT1* and *NtASAT2* expression level comparison of the thichomes of Hongda wild type (WT) and three independent T1 *NtASAT2* transgenic lines. Data for each transgenic line are the average of three technical replicates of the mixed sample of three plants. Data for Hongda WT are the average of three biological replicates and each replicate has mixed sampling of three plants, asterisks denote significant differences; ***, $P < 0.01$. **(C)** The acylsugar content comparison of WT and three independent T1 *NtASAT2* transgenic lines. Acylsugar content data for 3 independent *NtASAT2* overexpression T1 lines are single measurements of the mixed sample of three plants of each line, Data for Hongda WT are the average of three biological replicates and each replicate has mixed sampling of three plants.

2.6 NtASATs in N. tabacum Show a Connection to The Ancestor N. tomentosiformis

Nicotiana tabacum is a tetraploid consisting of T and S subgenomes, which originate from the diploid ancestors *N. tomentosiformis* and *N. sylvestris* (Leitch et al., 2008). Our GC-MS analysis revealed the presence of large amounts of acylsugars on the leaf surface of *N. tomentosiformis*, whereas they were barely detected on the leaf surface of *N. sylvestris* (Figure 4-23). Searching the diploid genomes led to the identification of orthologs of *NtASAT1* and *NtASAT2* in *N. tomentosiformis* (98% and 97.9% nucleotide identity, respectively), whereas no ortholog of *NtASAT1* and only an incomplete orthologous gene of *NtASAT2* were found in *N. sylvestris* (Figures 4-24, 4-25). These findings were further confirmed by successful amplification of NtASAT orthologs in the *N. tomentosiformis* leaves and glandular trichomes, but failure of amplification in *N. sylvestris* (Figure 4-26). To examine whether the deficiency of a *NtASAT1* homolog resulted in the absence of acylsugars, we expressed it ectopically in *N. sylvestris*, and found that no acylsucrose could be detected in the transgenic plants (Figure 4-23). Given that the *NtASAT2* homolog in *N. sylvestris* was not able to express the full-length intact protein, we conclude that the absence of detectable acylsugars was probably due to the lack of *NtASAT1* combined with the presence of non-functional *NtASAT2*. Our results indicated that *NtASAT1* and *NtASAT2* of *N. tabacum* are likely to have been derived from the ancestor *N. tomentosiformis*.

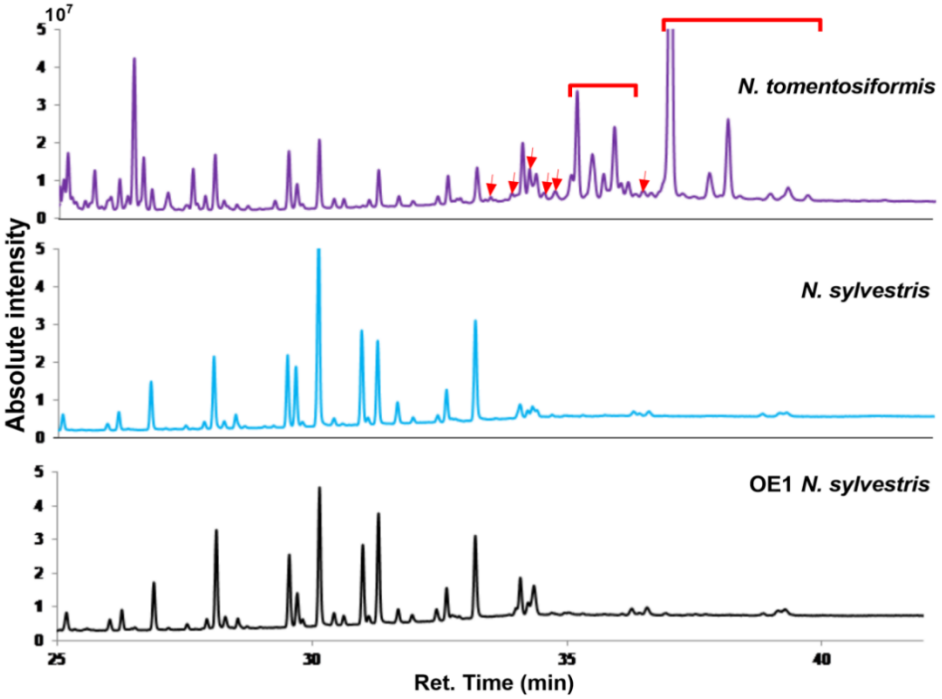


Figure 4-23 Acylsugar profiles in *Nicotiana tomentosiformis*, *N. sylvestris*, and a *N. sylvestris* transgenic line overexpressing *NtASAT1* (OE-1). Samples were taken from upper leaves (10-15 cm in length) of 50-day-old plants grown in the field. The profiles were determined by GC-MS analysis. The arrows indicate chromatographic peaks, and the retention times for different types of acylsugars are indicated by the brackets. No acylsugars were detected in *N. sylvestris* or OE1 *N. sylvestris*.

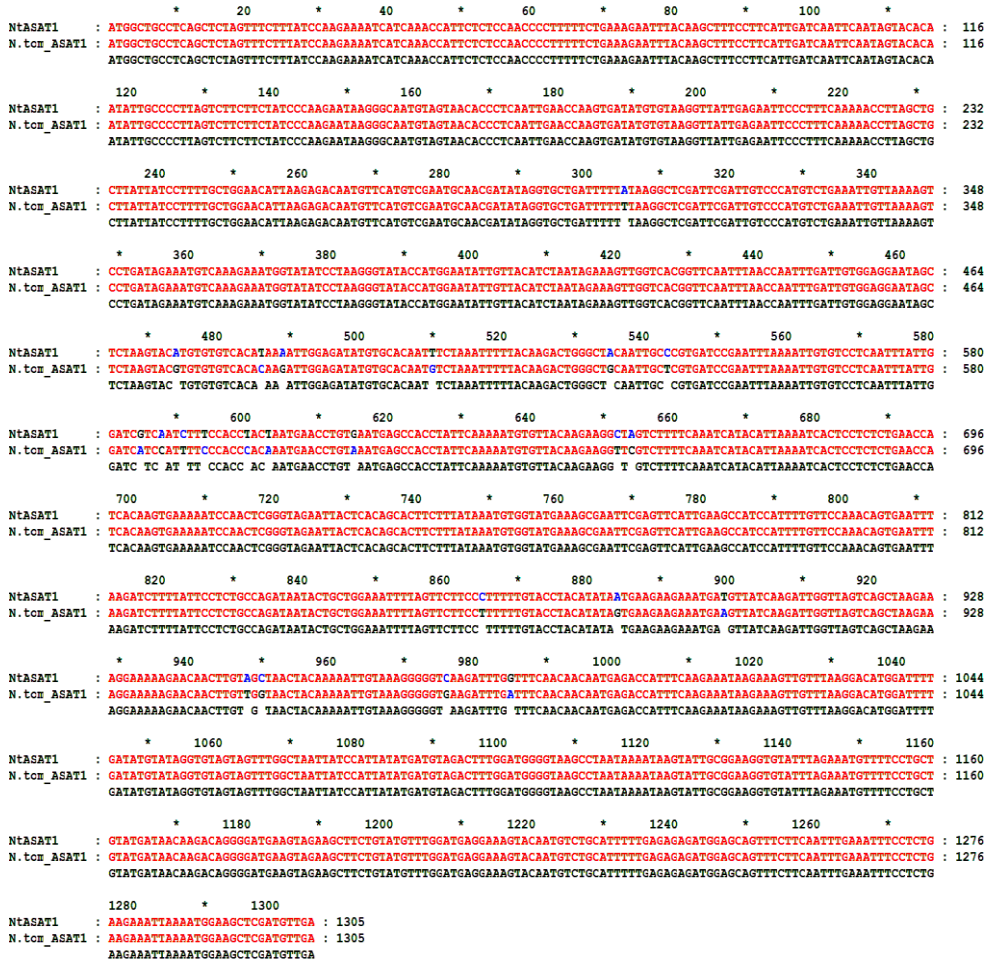


Figure 4-24 Sequence alignment of *ASAT1* cloned from *N. tabacum* Hongda and *N. tomentosiformis*.

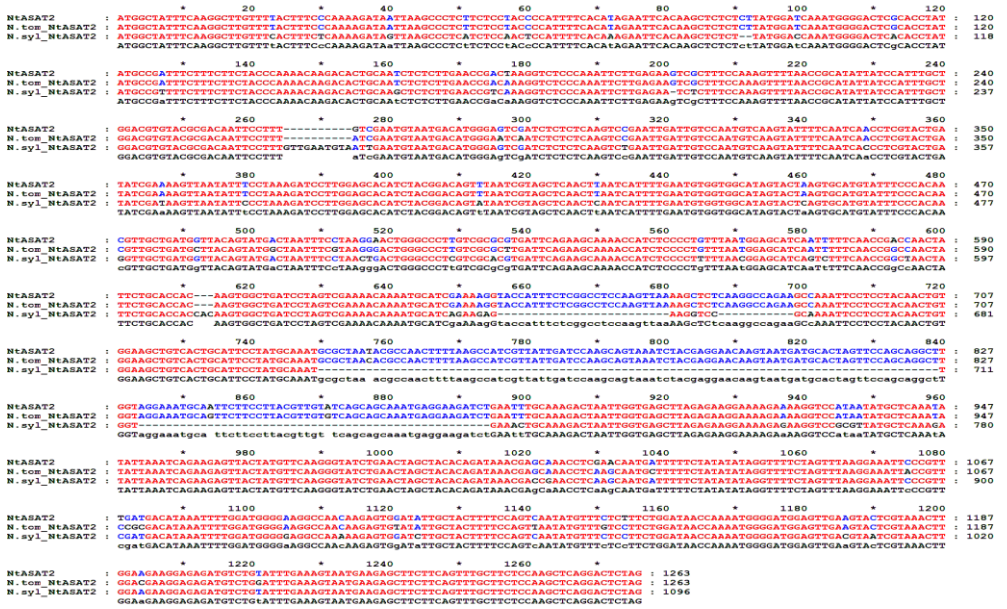


Figure 4-25 Sequence alignment of *ASAT2* cloned from *N. tabacum* Hongda, *N. tomentosiformis*, and *N. sylvestris*.

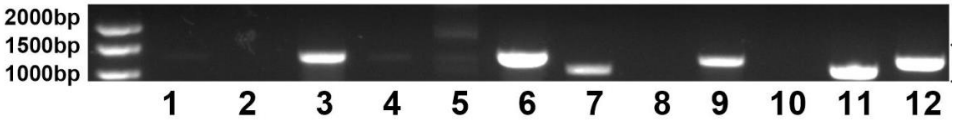


Figure 4-26 PCR analysis of *NtASAT1* and *NtASAT2* in the leaves and glandular trichomes of *N. sylvestris* and *N. tomentosiformis*. The primers used in 1-6 lanes are the clone primers of *NtASAT1* and in 7-12 lanes are the clone primers of *NtASAT2*. The PCR template of 1-6 and also 7-12 lanes in order, are the cDNA of the trichomes of *N. sylvestris,* the cDNA of *N. sylvestris* leaves with thichomes removed, the cDNA of the trichomes of *N. tomentosiformis,* the cDNA of the *N. tomentosiformis* leaves with thichomes removed, the DNA of *N. sylvestris* leaves and the DNA of *N. tomentosiformis* leaves，respectively.

3 Discussion

Acylsugars are phytoalexins that are widely distributed in Solanaceae species, and they hold great potential as natural, biorational insecticides in crop breeding (Jackson et al., 1998). According to Severson et al. (1985), C2, iC4, and iC5 are common acyl chains of type I and II acylsugars in tobacco, and C2 and aiC6 are major acyl chains of types III-V. Here, we isolated two acyltransferases, NtASAT1 and NtASAT2, which were expressed specifically in glandular trichomes in *N. tabacum* (Figure 4-8B). We found that NtASAT1 utilized C2, iC4, and iC5 to produce monoacylsucrose *in vitro*, with the highest activity when iC5 was used as the acyl-donor (Figures 4-9, 4-10). NMR analysis of the purified monoacylsucrose (S1:5) revealed that the acyl group was attached to position R2 of sucrose (Table 4-4), suggesting that NtASAT1 catalyses the attachment of acyl chains to the pyranose ring. Although acylation activity at this position has been reported previously in *Solanaceae* family enzymes such as PaxASAT1, SsASAT1 and NacASAT1, different acyl donors were identified, for example iC4 acyl-donors for NtASAT1 but not for PaxASAT1 (Fan et al., 2016; Moghe et al., 2017; Nadakuduti et al., 2017; Schenck et al., 2022, Preprint). NtASAT2 catalysed the attachment of the iC5 acyl-donor to iC5 monoacylsucrose, producing diacylsucrose (S2:10; Figure 4-9). These results are consistent with the fact that iC5 is frequently found as an acyl side-chain in cultivated tobacco (Severson et al., 1985; Arrendale et al., 1990). However, we failed to identify the structure of di-acylsucrose, because the isolated S2:10 product contained isomers that led to preliminary NMR results showing at least three acylsucrose structures in the mixture (Figure 4-11). This was consistent with the results of LC-Q-TOF-MS screening (Figure 4-9). Since NtASAT2 was closest to PaxASAT2, SsASAT2, and SlASAT1, which catalyse acylation at position R4, we deduce that it might also catalyse the attachment of acyl donors to the R4 position of the pyranose ring. It is currently unknown whether NtASAT1 and NtASAT2 have catalytic activity for the aiC6 acyl chain. Jia et al. (2013) identified the structures of types III-V acylsugars in oriental tobacco (*N. tabacum*), and all three types contain an aiC6 acyl chain on the R2 position of sucrose, and the R3 and R4 positions of type V are also aiC6 acyl chains. Consequently, we predict that NtASAT1 might be active in attaching aiC6 acyl-CoA to the R2 position of sucrose whilst NtASAT2 might be active in attaching aiC6 acyl-CoA to R4.

Through sequence alignment in the tobacco genome data, we obtained the sequence of *XM_016649597*, which is homologous with *PaxASAT3*, *SsASAT3*, and *SlASAT2* (Figure 4-27). Whether this gene is involved in acylsugar assembly in *N. tabacum* remains to be verified. Although tobacco acylsucroses have a similar pyranose ring structure to those of *Petunia*, no gene has been identified that is orthologous to *PaxASAT4* that catalyses the acylation at the R6 position of the pyranose ring to form tetra-acylsucrose. This is possibly due to the imperfect assembly of the *N. tabacum* reference genome, or it could indicate that an alternative gene with low sequence identity with *PaxASAT4* is responsible for the acylation of R6 in tobacco. The acylsucroses that have been identified in *N. tabacum* are all

esterified with an acetyl group at position R6, whereas in *P. axillaris* iC4 and iC5 acyl chains are present (Nadakuduti et al., 2017). We found that NtASAT1 could use acetyl-CoA and sucrose to produce monoacylsucrose, and showed the possibility for it to catalyse the addition of iC5 acyl groups to both the R2 position of sucrose (as the major product) and another unknown position (minor product) (Figure 4-9). However, to date, there have been no acetyl chains found at positions R2, R3, or R4 of any identified acylsucroses in *N. tabacum*. It is still unclear whether NtASAT1 can add acetyl chains to position R6. In addition, although our LC-Q-TOF-MS results showed different retention times for the S2:10 products, their structure was not determined. Hence, further in-depth studies are required to isolate acylsucroses, to determine their structures, and to identify the genes responsible for their biosynthesis and assembly.

As expected, knockout of *NtASAT1* led to blocking of the biosynthesis of acylsucroses in *N. tabacum* (Figures 4-15, 4-16, 4-17), which was consistent with the role of NtASAT1 in the first step of acylsugar assembly, and was similar to the significant reductions of acylsugar content that have been reported previously in virus-induced gene-silencing lines of *SlASAT1*, *PaxASAT1*, *SsASAT1*, and *NacASAT1* (Fan et al., 2016; Moghe et al., 2017; Nadakuduti et al., 2017; Schenck et al., 2022, Preprint). We also observed deficiencies of both di- and monoacylsucroses in *NtASAT2* mutants, in which accumulation of the monoacylsucrose precursor was expected. In addition, overexpression of *NtASAT1* in *N. sylvestris* did not result in the accumulation of acylsugars, especially monoacylsucroses (Figure 4-23). These results could indicate that a feedback regulation among biosynthetic enzymes in tobacco, or that monoacylsugars are hydrolysed by acylsugar acylhydrolases that can remove acyl groups from specific acylsugar positions, like those reported in tomato (Schilmiller et al., 2016). Another possibility is that the precursors might be further metabolized into other compounds that were not detected by our identification method. A different potential mechanism has recently been reported by Feng, et al. (2022), who found that *NbASAT1*-knockout mutations in *N. benthamiana* had reduced abundance of two predominant acylsucroses, whilst *NbASAT2* mutations caused almost complete depletion of acylsucroses. The different synthesis mechanisms might be an important reason for the great structural differences observed between the acylsucroses of *N. benthamiana* and *N. tabacum*.

The results of our overexpression experiments indicated that although the expression levels of *NtASAT1* in the T1-ASAT1 lines and of *NtASAT2* in the T1-ASAT2 lines increased significantly, the expression levels of *NtASAT2* in the T1-ASAT1 lines and of *NtASAT1* in T1-ASAT2 lines were not improved synergistically (Figures 4-20, 4-21, 4-22). Hence, there might be no accumulation of intermediate products in these lines, and consequently no significant increase in tetra-acylsucroses. Although the two NtASATs showed subcellular localization in the ER (Figure 4-8C), a previously proposed model of acylsugar biosynthesis has suggested that the enzymes involved in acyl-chain biosynthesis are localized in plastids or (Slocombe et al., 2008), so perhaps *NtASAT* overexpression could not effectively induce an increase in the acyl pool. According to our results, the acylsugar content could not be increased by the expression of a single assembly gene

in tobacco. Co-expression the key genes involved in acylsugar biosynthesis in the same organelle might be a way to increase acylsugar content, as has been demonstrated for taxane biosynthesis in *N. benthamiana* (Li et al., 2019).

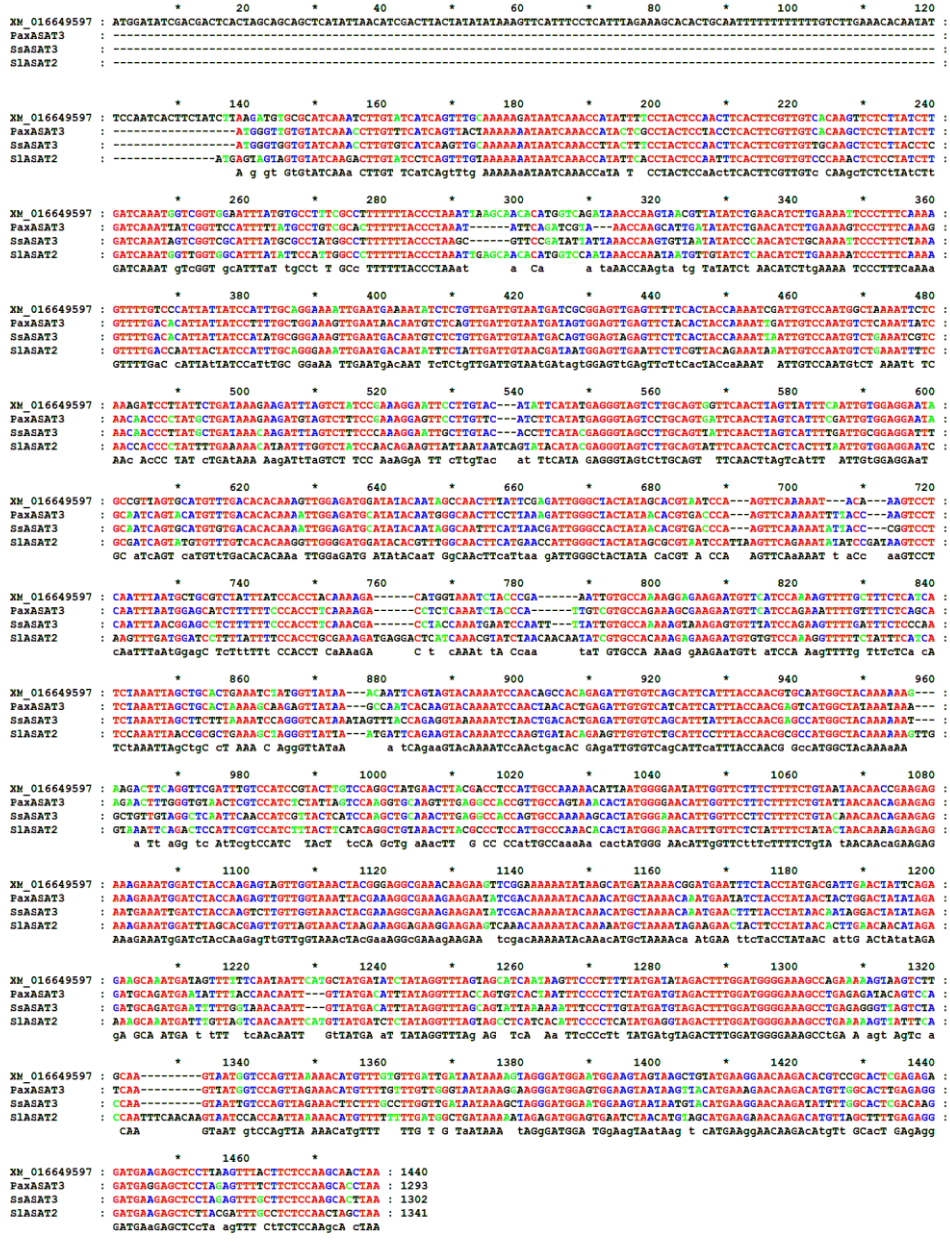


Figure 4-27 Sequence alignment of *SlASAT2*, *PaxASAT3*, *SsASAT3*, and search hits for *N. tabacum* in the NCBI database.

We have previously found that acetolactate synthase (NtALS1), a key enzyme involved in the BCAA metabolic pathway, is trichome-specific and participates in acylsugar synthesis (Chang et al., 2020). We also investigated its distribution in *N. sylvestris* and *N. tomentosiformis*, and found that it is only present in *N. tomentosiformis*. As acylsugars are significantly reduced, but still present, in *NtALS1* mutants, we speculated that loss of *NtALS1* might affect acylsugar content, but that it is not the key factor responsible for the absence of acylsugars in *N. sylvestris* (Chang et al., 2020). In the present study, the *NtASAT1*- and *NtASAT2*-knockout mutants completely lost acylsugars (Figures 4-15, 4-16, 4-17). As *N. sylvestris* had no gene homologous to *NtASAT1* together with non-functional *NtASAT2*, this could be the key reason for its lack of acylsugars. In addition, although *NtALS1*, *NtASAT1*, and *NtASAT2*, derived from *N. tomentosiformis*, were present in both the *N. tabacum* cultivars Beinhart1000-1 (BH, cigar tobacco) and Hongda (HD, flue-cured tobacco), the acylsugar phenotype of BH was basically similar to that of *N. tomentosiformis* (Figures 4-15 4-23), whereas in HD the types of sucrose ester containing 3-methylvaleryl (III-V in Figures 4-12, 4-15A) were not present (Vontimitta et al., 2010). We speculate that during the evolution of *N. tabacum*, genes that regulate 3-methylvaleryl synthesis might have been lost or mutated in the HD cultivar. Great diversity of acylsugars is found across different species in the genus *Nicotiana* (Severson et al., 1985; Arrendale et al., 1990; Chortyk et al., 1993; Haliński and Stepnowski, 2013; Kroumova et al., 2016; Schenck et al., 2022, Preprint), as well as diversity in BAHD acyltransferase genes (Figure 4-5), highlighting the possibility of different mechanisms of acylsugar biosynthesis. Hence, additional research is needed across different *Nicotiana* species to determine the degree of acylsugar diversity, to clarify the synthesis mechanisms, and to reveal the biochemical evolution of the genes involved. Once this information is obtained, it should open up an array of possibilities for future metabolic engineering, and help to both increase pest resistance in crops and to develop effective bio-insecticides.

References

Amme S, Rutten T, Melzer M, Sonsmann G, Vissers JP, Schlesier B, Mock HP. 2005. A proteome approach defines protective functions of tobacco leaf trichomes. Proteomics 5, 2508–2518.

Arrendale RF, Severson RF, Sisson VA, Costello CE, Leary JA, Himmelsbach DS, Van Halbeek H. 1990. Characterization of the sucrose ester fraction from *Nicotiana glutinosa*. Journal of Agricultural and Food Chemistry 38, 75–85.

Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS. 2009. MEME SUITE: tools for motif discovery and searching. Nucleic Acids Research 37, W202–W208.

Bednarek P, Osbourn A. 2009. Plant–microbe interactions: chemical diversity in plant defense. Science 324, 746–748.

Ben-Mahmoud S, Smeda JR, Chappell TM, Stafford-Banks C, Kaplinsky CH,

Anderson T, Mutschler MA, Kennedy GG, Ullman DE. 2018. Acylsugar amount and fatty acid profile differentially suppress oviposition by western flower thrips, *Frankliniella occidentalis*, on tomato and interspecific hybrid flowers. PLoS ONE 13, e0201583.

Cai LL, Xie WF, Liu KJ, Zhang Y, Xie JP. 2009. Analysis of sucrose esters in oriental tobacco by GC/MS. Tobacco Science and Technology 260, 40– 44. [In Chinese].

Chang AX, Chen B, Yang AG, Hu RS, Feng QF, Chen M, Yang XN, Luo CG, Li YY, Wang YY. 2020. The trichome-specific acetolactate synthase *NtALS1* gene, is involved in acylsugar biosynthesis in tobacco (*Nicotiana tabacum* L.). Planta 252, 13.

Chortyk OT, Kays SJ, Teng Q. 1997. Characterization of insecticidal sugar esters of *Petunia*. Journal of Agricultural and Food Chemistry 45, 270–275.

Chortyk OT, Severson RF, Cutler HC, Sisson VA. 1993. Antibiotic activities of sugar esters isolated from selected *Nicotiana* species. Bioscience, Biotechnology, and Biochemistry 57, 1355–1356.

Fan PX, Leong BJ, Last RL. 2019. Tip of the trichome: evolution of acylsugar metabolic diversity in Solanaceae. Current Opinion in Plant Biology 49, 8–16.

Fan PX, Miller AM, Liu X, Jones AD, Last RL. 2017. Evolution of a flipped pathway creates metabolic innovation in tomato trichomes through BAHD enzyme promiscuity. Nature Communications 8, 2080.

Fan PX, Miller AM, Schilmiller AL, Liu X, Ofner I, Jones AD, Zamir D, Last RL. 2016. *In vitro* reconstruction and analysis of evolutionary variation of the tomato acylsucrose metabolic network. Proceedings of the National Academy of Sciences, USA 113, E239–E248.

Fan PX, Wang PP, Lou YR, *et al.* 2020. Evolution of a plant gene cluster in Solanaceae and emergence of metabolic diversity. eLife 9, e56717.

Feng HL, Acosta-Gamboa L, Kruse LH, *et al.* 2022. Acylsugars protect *Nicotiana benthamiana* against insect herbivory and desiccation. Plant Molecular Biology. https://doi.org/10.1007/s11103-021-01191-3

Ghosh B, Westbrook TC, Jones AD. 2014. Comparative structural profiling of trichome specialized metabolites in tomato (*Solanum lycopersicum*) and *S. habrochaites*: acylsugar profiles revealed by UHPLC/MS and NMR. Metabolomics 10, 496–507.

Haliński ŁP, Stepnowski P. 2013. GC-MS and MALDI-TOF MS profiling of sucrose esters from *Nicotiana tabacum* and *N. rustica*. Zeitschrift fur Naturforschung C 68, 210–222.

Hill K, Rhode O. 1999. Sugar-based surfactants for consumer products and technical applications. Fett/Lipid 101, 25–33.

Jackson DM, Chortyk OT, Stephenson MG, Johnson AW, Harlow CD, Simmons AM, Sisson VA. 1998. Potential of *Nicotiana* species for production of sugar esters. Tobacco Science 42, 1–9.

Jia C, Wang Y, Zhu Y, Xu C, Mao D. 2013. Preparative isolation and structural characterization of sucrose ester isomers from oriental tobacco. Carbohydrate Research 372, 73–77.

Johnson AW, Severson RF. 1984. Leaf surface chemistry of tobacco budworm resistant tobacco. Journal of Agricultural Entomology 1, 23–84.

Kandra G, Severson R, Wagner GJ. 1990. Modified branched-chain amino acid pathways give rise to acyl acids of sucrose esters exuded from tobacco leaf trichomes. European Journal of Biochemistry 188, 385–391.

Kandra L, Wagner GJ. 1990. Chlorsulfuron modifies biosynthesis of acyl acid substituents of sucrose esters secreted by tobacco trichomes. Plant Physiology 94, 906–912.

Kennedy BS, Nielsen MT, Severson RF, Sisson VA, Stephenson MK, Jackson DM. 1992. Leaf surface chemicals from *Nicotiana* affecting germination of *Peronospora tabacina* (adam) sporangia. Journal of Chemical Ecology 18, 1467–1479.

Kroumova AB, Wagner GJ. 2003. Different elongation pathways in the biosynthesis of acyl groups of trichome exudate sugar esters from various solanaceous plants. Planta 216, 1013–1021.

Kroumova AB, Xie Z, Wagner GJ. 1994. A pathway for the biosynthesis of straight and branched, odd-and even-length, medium-chain fatty acids in plants. Proceedings of the National Academy of Sciences, USA 91, 11437–11441.

Kroumova AB, Zaitlin D, Wagner GJ. 2016. Natural variability in acyl moieties of sugar esters produced by certain tobacco and other Solanaceae species. Phytochemistry 130, 218–227.

Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33, 1870–1874.

Lawson DM, Lunde CF, Mutschler MA. 1997. Marker-assisted transfer of acylsugar-mediated pest resistance from the wild tomato, *Lycopersicon pennellii*, to the cultivated tomato, *Lycopersicon esculentum*. Molecular Breeding 3, 307–317.

Leckie BM, D'Ambrosio DA, Chappell TM, Halitschke R, De Jong DM, Kessler A, Kennedy GG, Mutschler MA. 2016. Differential and synergistic functionality of acylsugars in suppressing oviposition by insect herbivores. PLoS ONE 11, e0153345.

Leckie BM, De Jong DM, Mutschler MA. 2012. Quantitative trait loci increasing acylsugars in tomato breeding lines and their impacts on silverleaf whiteflies. Molecular Breeding 30, 1621–1634.

Leckie BM, Halitschke R, De Jong DM, Smeda JR, Kessler A, Mutschler MA. 2014. Quantitative trait loci regulating the fatty acid profile of acylsugars in tomato. Molecular Breeding 34, 1201–1213.

Leitch IJ, Hanson L, Lim KY, Kovarik A, Chase MW, Clarkson JJ, Leitch AR. 2008. The ups and downs of genome size evolution in polyploid species of

Nicotiana (Solanaceae). Annals of Botany 101, 805–814.

Leong BJ, Hurney SM, Fiesel PD, Moghe GD, Jones AD, Last RL. 2020. Specialized metabolism in a nonmodel nightshade: trichome acylinositol biosynthesis. Plant Physiology 183, 915–924.

Li J, Mutanda I, Wang K, Yang L, Wang J, Wang Y. 2019. Chloroplastic metabolic engineering coupled with isoprenoid pool enhancement for committed taxanes biosynthesis in *Nicotiana benthamiana*. Nature Communications 10, 4850.

Liu X, Enright M, Barry CS, Jones AD. 2017. Profiling, isolation and structure elucidation of specialized acylsucrose metabolites accumulating in trichomes of *Petunia* species. Metabolomics 13, 85.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2–ΔΔ*C*T method. Methods 25, 402–408.

Lou YR, Anthony TM, Fiesel PD, Arking RE, Christensen EM, Jones AD, Last RL. 2021. It happened again: convergent evolution of acylglucose specialized metabolism in black nightshade and wild tomato. Science Advances 7, eabj8726.

Luu VT, Weinhold A, Ullah C, Dressel S, Schoettner M, Gase K, Gaquerel E, Xu S, Baldwin IT. 2017. O-Acyl sugars protect a wild tobacco from both native fungal pathogens and a specialist herbivore. Plant Physiology 174, 370–386.

Maluf WR, Maciel GM, Gomes LAA, Cardoso MDG, Gonçalves LD, da Silva EC, Knapp M. 2010. Broad-spectrum arthropod resistance in hybrids between high- and low-acylsugar tomato lines. Crop Science 50, 439–450.

McKenzie CL, Weathersbee AA III, Puterka GJ. 2005. Toxicity of sucrose octanoate to egg, nymphal, and adult *Bemisia tabaci* (Hemiptera: Aleyrodidae) using a novel plant-based bioassay. Journal of Economic Entomology 98, 1242–1247.

Moghe GD, Last RL. 2015. Something old, something new: conserved enzymes and the evolution of novelty in plant specialized metabolism. Plant Physiology 169, 1512–1523.

Moghe GD, Leong BJ, Hurney SM, Jones AD, Last RL. 2017. Evolutionary routes to biochemical innovation revealed by integrative analysis of a plant-defense related specialized metabolic pathway. eLife 6, e28468.

Nadakuduti SS, Uebler JB, Liu X, Jones AD, Barry CS. 2017. Characterization of trichome-expressed BAHD acyltransferases in *Petunia axillaris* reveals distinct acylsugar assembly mechanisms within the Solanaceae. Plant Physiology 175, 36–50.

Ning J, Moghe GD, Leong B, Kim J, Ofner I, Wang Z, Adams C, Jones AD, Zamir D, Last RL. 2015. A feedback-insensitive isopropylmalate synthase affects acylsugar composition in cultivated and wild tomato. Plant Physiology 169, 1821–1835.

Pérez-Castorena AL, Martínez M, Maldonado E. 2010. Labdanes and sucrose esters from *Physalis sordida*. Journal of Natural Products 73, 1271–1276.

Pichersky E, Lewinsohn E. 2011. Convergent evolution in plant specialized metabolism. Annual Review of Plant Biology 62, 549–566.

Puterka GJ, Farone W, Palmer T, Barrington A. 2003. Structure–function relationships affecting the insecticidal and miticidal activity of sugar esters. Journal of Economic Entomology 96, 636–644.

Qu YF, Xu ML, Cao JM, Sun HQ, You XW, Wang GP, Jiang CH, Wang YY, Chang AX. 2017. Differential analysis of major glandular trichome secretion between different tobacco genotypes. Acta Tabacaria Sinica 24, 45–52. [In Chinese].

Rodríguez-López MJ, Garzo E, Bonani JP, Fernández-Muñoz R, Moriones E, Fereres A. 2012. Acylsucrose-producing tomato plants forces *Bemisia tabaci* to shift its preferred settling and feeding site. PLoS ONE 7, e33064.

Rodriguez AE, Tingey WM, Mutschler MA. 1993. Acylsugars of *Lycopersicon pennellii* deter settling and feeding of the green peach aphid (Homoptera: Aphididae). Journal of Economic Entomology 86, 34–39.

Savary S, Willocquet L, Pethybridge SJ, Esker P, McRoberts N, Nelson A. 2019. The global burden of pathogens and pests on major food crops. Nature Ecology & Evolution 3, 430–439.

Schenck CA, Anthony TM, Jacobs MK, Jones AD, Last RL. 2022. Natural variation meets synthetic biology: promiscuous trichome expressed acyltransferases from *Nicotiana acuminata*. BioRxiv doi: 10.1101/2022.02.25.482030. [Preprint].

Schilmiller AL, Charbonneau AL, Last RL. 2012. Identification of a BAHD acetyltransferase that produces protective acyl sugars in tomato trichomes. Proceedings of the National Academy of Sciences, USA 109, 16377–16382.

Schilmiller AL, Gilgallon K, Ghosh B, Jones AD, Last RL. 2016. Acylsugar acylhydrolases: carboxylesterase-catalyzed hydrolysis of acylsugars in tomato trichomes. Plant Physiology 170, 1331–1344.

Schilmiller AL, Moghe GD, Fan P, Ghosh B, Ning J, Jones AD, Last RL. 2015. Functionally divergent alleles and duplicated loci encoding an acyltransferase contribute to acylsugar metabolite diversity in *Solanum trichomes*. The Plant Cell 27, 1002–1017.

Severson RF, Arrendale RF, Chortyk OT, Green CR, Thome FA, Stewart JL, Johnson AW. 1985. Isolation and characterization of the sucrose esters of the cuticular waxes of green tobacco leaf. Journal of Agricultural and Food Chemistry 33, 870–875.

Severson RF, Arrendale RF, Chortyk OT, Johnson AW, Jackson DM, Gwynn GR, Chaplin JF, Stephenson MG. 1984. Quantitation of the major cuticular components from green leaf of different tobacco types. Journal of Agricultural Food Chemistry 32, 566–570.

Simmons AT, Gurr GM, McGrath D, Martin PM, Nicol HI. 2004. Entrapment of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) on glandular trichomes of *Lycopersicon* species. Australian Journal of Entomology 43, 196–200.

Slocombe SP, Schauvinhold I, McQuinn RP, *et al.* 2008. Transcriptomic and reverse genetic analyses of branched-chain fatty acid and acyl sugar production in *Solanum pennellii* and *Nicotiana benthamiana*. Plant Physiology 148, 1830–1846.

Smeda JR, Schilmiller AL, Anderson T, Ben-Mahmoud S, Ullman DE, Chappell TM, Kessler A, Mutschler MA. 2018. Combination of acylglucose QTL reveals additive and epistatic genetic interactions and impacts insect oviposition and virus infection. Molecular Breeding 38, 3.

Smeda JR, Schilmiller AL, Last RL, Mutschler MA. 2016. Introgression of acylsugar chemistry QTL modifies the composition and structure of acylsugars produced by high-accumulating tomato lines. Molecular Breeding 36, 160.

van der Hoeven RS, Steffens JC. 2000. Biosynthesis and elongation of short- and medium-chain-length fatty acids. Plant Physiology 122, 275–282.

Vontimitta V, Danehower DA, Steede T, Moon HS, Lewis RS. 2010. Analysis of a *Nicotiana tabacum* L. genomic region controlling two leaf surface chemistry traits. Journal of Agricultural and Food Chemistry 58, 294–300.

Wagner GJ. 1999. Leaf surface chemistry. In: Davis DL, Nielsen MT, eds. Tobacco: production, chemistry, and technology. Oxford, UK: Blackwell Publishing, 292–303.

Walters DS, Steffens JC. 1990. Branched chain amino acid metabolism in the biosynthesis of *Lycopersicon pennellii* glucose esters. Plant Physiology 93, 1544–1551.

Wang E, Gan S, Wagner GJ. 2002. Isolation and characterization of the *CYP71D16* trichome-specific promoter from *Nicotiana tabacum* L. Journal of Experimental Botany 53, 1891–1897.

Weinhold A, Baldwin IT. 2011. Trichome-derived *O*-acyl sugars are a first meal for caterpillars that tags them for predation. Proceedings of the National Academy of Sciences, USA 108, 7855–7859.

Weng JK. 2014. The evolutionary paths towards complexity: a metabolic perspective. New Phytologist 201, 1141–1149.

5

Conclution and Prospects

Chapter Five Conclusion and Prospects

1 Conclusion

In this thesis, the progress in research on acylsugars derived from *Solanaceae* family was reviewed. Based on previous reports, we put forward the necessity to study the genes responsible for the synthesis of acylsugars in tobacco and three genes were identified.

Acylsugars are a class of plant defense-related secondary metabolites that were first identified in and widely produced across the *Solanaceae* family (Arrendale et al., 1990; Haliński and Stepnowski, 2013; Ghosh et al., 2014; Kroumova et al., 2016; Liu et al., 2017; Moghe et al., 2017; Fan et al., 2019; Leong et al., 2020; Lou et al., 2021). In the early 1980s, researchers began to pay attention to the biological activities of solanaceous acylsugars. However, breakthroughs in the structure identification and the biosynthesis studies of *Solanaceae* acylsugars were only recorded in the past two decades due to the rapid developments in chromatographic detection techniques, molecular biology and bioinformatics. (Ghosh et al., 2014; Liu et al., 2017; Fan et al., 2016; Moghe et al., 2017; Nadakuduti et al., 2017;). In this thesis (Chapter two), we review and summarize the research progress on the chemical structures, biosynthetic mechanisms and bioactivities of the acylsugars. Reports have shown that solanaceous acylsugars generally consist of a sucrose, glucose, or inositol sugar backbone, as well as various acyl chains (C2-C12) attached to different positions of the sugar core. Consequently, hundreds of acylsugars exist across the *Solanaceae* family and even within a single species (Moghe et al., 2017). The biosynthetic pathway of these acylsugars primarily includes two parts; namely, acyl chain synthesis and the assembly of acyl chains with the corresponding sugar cores. Short acyl chains are mainly derived from branched-chain amino acid (BCAA) metabolic pathways; the chains are further extended to generate longer acyl chains through the α-ketoacid elongation (α-KAE) pathway in tobacco and petunia or through the fatty acid synthase (FAS) pathway in tomato and *Datura* (Kandra et al. 1990; Walters and Steffens 1990;Slocombe et al. 2008; Ning et al. 2015; Fan et al., 2020). Acylsugar acyltransferases are key enzymes that catalyze the attachment of acyl chains to sugar molecules (Schilmiller et al., 2012; Schilmiller et al., 2015; Fan et al., 2016; Fan et al., 2017; Moghe et al., 2017; Nadakuduti et al., 2017; Leong et al., 2020; Lou et al., 2021; Schenck et al., 2022). The occurrence of various genes involved in the synthesis and mechanisms of acylsugars are important reasons for the wide diversity of acylsugars in the *Solanaceae* family. For instance, the effects of gene duplication, divergence, loss and allelic variation on ASATs during evolution consequently altered the enzyme activity, thus, leading to substrate preferences and a degree of promiscuity, whicheventually contributes to the diversity of acylsugar phenotypes within the *Solanaceae* family (Kim et al., 2012; Schilmiller et al., 2015; Fan et al., 2016; Moghe et al., 2017). Furthermore, enzymes from core metabolism, including

isopropylmalate synthase (Ning et al., 2015) and invertase (Leong et al., 2019) which are involved in Leu and carbon metabolism, respectively, are recruited to specialized acylsugar metabolism through the evolution of trichome-specific expression and this also affects acyl chain or the diversity of sugar cores in tomato. In addition to the aforementioned enzymes that contribute to the biosynthesis and diverse networks of acylsugars, acylsugar acylhydrolases (Schilmiller et al., 2016), which have been demonstrated *in vitro* to remove acyl groups from specific acylsugar positions, may degrade or edit acylsugars *in vivo* and provide different acyl acceptor substrates for ASATs; thus, helping to generate acylsugar diversity. *Solanaceous* acylsugars possess a variety of biological activities, such as insect-resistance, antimicrobial, antidehydration, and antitumor activities. Their bioactivities often vary with their chemical structures (Chortyk et al., 1993; Chortyk et al., 1996;Leckie et al., 2016), and acylsugars with high or broad-spectrum activities (such as acylsugars in *Solanum pennellii and Nicotiana gossei*) have attracted extensive attention in the breeding of *Solanaceae* crops and the development of natural insecticides (Severson et al., 1994, Chortyk et al., 1997; Lawson et al., 1997; McKenzie et al., 2005; Maluf et al., 2010; Leckie et al., 2012; Leckie et al., 2014; Smeda et al., 2016; Smeda et al., 2018).

Tobacco is an important model plant for genetic transformations. It is considered an efficient green bioreactor because of its amenability to transient and stable genetic transformations, as well as its high leaf biomass. Furthermore, tobacco is an important cash crop globally, and the taxes generated from it are one of the main sources of fiscal revenue in tobacco-growing countries. In addition to defense-related features, tobacco-derived acylsugars are important precursors of flavor that contribute to the aroma quality of tobacco leaves. Acylsugars in *N. tabacum* are mainly tetraacylsucroses with an acetyl group at the R6 position and three C3 to C8 straight or branched acyl chains at the R2, R3, and R4 positions of the pyranose ring (Severson et al., 1985; Jia et al., 2013; Vontimitta et al., 2010).These acylsugarsare differ from the acylsugar profiles of *S. lycopersicum*, *P. axillaris*, and *S. sinuata*. Although significant progress has been made in mining key genes involved in the biosynthesis of acylsugars in these *Solanaceae* plants, the genes and biosynthetic mechanisms of acylsugars in tobacco remain unclear. In this study, through gene homology comparison and detection of transcriptional expression levels, we obtained three candidate genes, including *NtASL1*, *NtASAT1*, and *NtASAT2*, and then using a variety of functional identification methods, we demonstrated, for the first time, their roles in the synthesis of tobacco acylsugars.

1.1 The Role of NtALS1 in Acylsugar Biosynthesis in N.tabacum

Acetolactate synthase (ALS) is a key enzyme in the synthesis of BCAAs. It is a common target for many highly selective and low-toxicity herbicides, and previous studies have mainly focused on the development and utilization of herbicides. One study demonstrated that chlorsulfuron, a specific inhibitor of ALS, significantly reduced the content of acylsugar branched-chain fatty acids in tobacco GTs (Kandra and Wagner 1990). Previously, two ALS genes have been reported in *N. tabacum* (Lee et al., 1988; Keeler et al., 1993); however, the identity of the genes specifically

involved in the synthesis of acylsugar acyl chains remains unclear. In our first study (Chapter Three), we identified three ALS genes in tobacco using a homology-based gene prediction. Expression pattern analysis showed that in contrast to the two genes previously reported (these two genes are the same as *NtALS2* and *NtALS3* in our study), which are expressed in different tissues, *NtALS1* is trichome-specific and its activity was localized in the chloroplast. Knockout of *NtALS1* in the flue-cured tobacco cultivar K326 led to a significant reduction in acylsugars, but no obvious changes in trichome-specific diterpenes. Phylogenetic and expression analyses suggested that each acylsugar-containing *Solanaceae* species possessed an ortholog of *NtALS1*, whereas there is no *NtALS1* ortholog in the diploid ancestor *N. sylvestris*, which is a non-producer of acylsugars. This indicated that *NtALS1* may have originated from the diploid ancestor *N. tomentosiformis*, which produces acylsugars. Our findings suggest that *NtALS1* plays an essential role in the biosynthesis of acylsugars in tobacco plants. As the levels of acylsugars in *NtALS1* knockout mutants were only significantly reduced, but not completely missing, we hypothesized that *NtALS2* and *NtALS3* might also be involved in the biosynthesis of tobacco acylsugars; however, this position requires further research. Based on our results and those of previous studies (Ning et al., 2015; Leong et al., 2019), we speculate that most of the acylsugar biosynthesis genes (such as *SlIPMS3*, *SpASFF1*, and *NtALS1*) may originate in primary metabolism. Moreover, they may have experienced gene duplication and specialization via spatiotemporal expression to participate in tissue-specific biosynthesis of acylsugars, thereby helping plants to adapt to changes in external environmental conditions during long-term evolution.

1.2 The Roles of NtASAT1 and NtASAT2 in The Biosynthesis of Acylsugar in N. tabacum

Acylsugar acyltransferases have been reported to be responsible for the attachment of the acyl chains to sugar cores to form acylsugars in various *Solanaceae* species (Schilmiller et al., 2012; Schilmiller et al., 2015; Fan et al., 2016; Nadakuduti et al., 2017; Moghe et al., 2017; Fan et al., 2017). In our second study (Chapter Four), to mine the genes responsible for the assembly of *N. tabacum* acylsugars, we isolated three ASAT genes from the genome data of *N. tabacum* through homologous comparison, and we subsequently characterized two *ASATs* using a variety of methods. Analysis of expression patterns showed that *NtASAT1* and *NtASAT2* were trichome-specific and localized in the endoplasmic reticulum. *In vitro* assays for enzymatic activities showed that *NtASAT1* and *NtASAT2* sequentially catalyze two additive steps of acyl donors to sucrose to produce diacylsucrose. Knockout of *NtASAT1* or *NtASAT2* in Cigar tobacco BH and (or) flue-cured tobacco HD resulted in deficiency in acylsucrose. Analyses of genomic and acylsugar profiles suggested that both *NtASATs* originated from the ancestor *N. tomentosiformis*, which produced acylsugars, and the lack of *NtASAT1* ortholog as well as the non-functional *NtASAT2* ortholog may be responsible for the non-detection of acylsugars in *N. sylvestris*. These results suggest that NtASAT1 and NtASAT2 are the key enzymes involved in the assembly of acylsugars in tobacco. However, the results of overexpression indicated that neither NtASAT1 nor NtASAT2 is a limiting enzyme for the

biosynthesis of acylsugars, and the overexpression of a single *NtASAT* gene did not effectively increase the content of acylsugars in tobacco. Combining our *in vitro* findings with previous reports （Fan et al., 2016; Nadakuduti et al., 2017; Moghe et al., 2017), we found NtASAT1 and its reported orthologs could catalyze the attachment of acyl group to R2 position of sucrose. We deduce that the ASAT orthologs in *Solanaceae* may have the ability to catalyze the attachment of acyl chain to the same position of the sugar core, and that NtASAT2 may be similar to its orthologs to catalyze the attachment of acyl donors on the R4 position of sucrose. Moreover, we also found that there was no expected monoacylsucrose in NtASAT2 knockout mutant of *N. tabacum* cv. HD and in *NtASAT1* overexpressing plants of *N. sylvestris*. These results indicate that there are some unknown mechanism affecting the monoacylsucrose accumulation *in vivo*.

The study of this dissertation extends our understanding of acylsucrose biosynthesis in tobacco plants, and also provides new genes for further study on molecular regulatory mechanisms of acylsugar biosynthesis, for the molecular breeding of tobacco aroma quality, and engineering of pathogen- and insect-resistant crops.

2 Discussion and Prospects

Tobacco is a smokable leafy cash crop, moreover, the quality of the aroma of raw tobacco is a key factor in determining its industrial value. Tobacco acylsugars are not only important as precursors of flavor emanating from tobacco leaves, but they also possess various biological activities, such as insect resistance, antifungal, antibacterial, antidehydration, and antitumor activities. Therefore, increasing tobacco acylsugar content through genetic manipulations may effectively improve the aroma quality of tobacco leaves and accelerate the commercial development and utilization of tobacco acylsugars. Because acylsugars are specifically synthesized in tobacco glandular trichomes, genetic manipulations to increase its acylsugar content can be considered from two aspects: on the one hand, by modulating key genes affecting the development of glandular trichomes to increase the density of tobacco trichomes, i.e., increasing the sites of synthesis of acylsugars; and on the other hand, by upregulating key genes in the biosynthetic pathway of acylsugars to improve the synthetic capacity of glandular trichomes for sugar esters. In terms of increasing glandular trichome density, it has been shown that the tomato *woolly* (*Wo*) gene, a key gene in tomato epidermal hair formation, can interact with the cell cycle-related gene, *SlCycB2*, thereby inducing a cellular transition from G_2 to M phase and ultimately promoting the formation of multicellular epidermal hairs (Yang, et al., 2011). In *N. tabacum* cv. K326, editing of *NtCycB2*, a negative regulator gene of glandular trichome generation, using CRISPR-Cas9 technology, yielded mutant materials with significantly increased glandular trichome density (Pan et al., 2019). In addition, it has been shown that overexpression of MIXTA and MIXTA-LIKE 1 homologs of goldenseal in tobacco resulted in a significant increase in glandular trichome density (Payne et al., 1999; Perez-Rodriguez et al., 2005), suggesting that the presence of MIXTA and MIXTA-LIKE 1 homologs in tobacco may be involved

in the upregulation of epidermal hair genesis. In terms of regulating the ability of acylsugar synthesis in tobacco glandular trichomes, little is known about the key genes involved in the regulation of acylsugar synthesis in tobacco and no scientific reports are available. Although three genes (*NtASAT1*, *NtASAT2*, and *NtALS1*) involved in tobacco acylsugar synthesis were identified in this study, overexpression of *NtASAT1* or *NtASAT2* did not effectively improve the acylsugar content, and it is presently unknown if the overexpression of *NtALS1* will improve the acylsugar content of tobacco. Therefore, these genes cannot be effectively utilized for breeding. At present, regarding the synthesis and regulatory mechanisms of *N. tabacum* acylsugars, there are still many unanswered questions and unexplored genes need to be further investigated. Additionally, there are 76 species in *Nicotiana* genus; within and across species, there are tremendous acylsugar structural variations, and the bioactivities of acylsugars vary with their chemical structures. However, the mechanism of synthesis of different acylsugars in wild tobacco is poorly understood. Based on this study and the previous reports, it is necessary to sustain extensive and in-depth research related to tobacco acylsugars from the following aspects:

First, the function of *NtALSs* requires further investigation. In the BCAA metabolic pathway, ALS gene catalyzes the combination of two molecules of pyruvate to produce 2-acetolactate or pyruvate and α-ketobutyrate to produce 2-aceto-2-hydroxybutyrate. The two keto acids generated can be used as primers to produce elongated branched keto acids via the α-keto acid elongation pathway, and these keto acids of different chain lengths are catalyzed by BCKD to form acyl-CoAs, the active acyl chain substrate required for acylsugar synthesis. In our study, although the involvement of the *NtALS1* gene in tobacco acylsugar synthesis *in vivo* was initially verified by CRISPR-Cas9 technology, we only compared the levels of the end-products, acylsugars, in wild-type tobacco and the mutants, but did not test the content of the direct product catalyzed by NtALS1, other intermediates in the synthesis of acyl chains, and branched-chain amino acids. Therefore, the enzymatic activity of NtALS1 on different substrates and the effect of NtALS1 on the synthesis of different branched acyl chains remain unknown. Furthermore, it remains to be verified whether *NtALS1* overexpression can increase the content of tobacco acylsugars, and whether NtALS2 and NtALS3 are involved in tobacco acylsugar synthesis. Next, it is necessary to clarify these unanswered questions using gene function identification tools, such as gene overexpression, gene knockout, *in vitro* enzyme activity, and assays, such as GC/MS, LC/MS, and LC-Q-TOF to evaluate the value of *NtALSs* in genetically regulating tobacco acylsugar content or acylsugar types.

Second, some unknown mechanisms related to NtASAT1 and NtASAT2 require further exploration. In our study, the expected monoacylsucrose was not detected in the HD mutant knocking out *NtASAT2* or in *N. sylvestris* overexpressing *NtASAT1*. Although we discussed multiple possibilities for these phenomena in this study, the exact causes are not yet known. Next, using *NtASAT1*- and *NtASAT2*-knockout mutants with their wild-types as controls and through comparative analysis of the transcriptome, proteome, and metabolome of glandular trichomes, it is promising to further elucidate the unknown regulatory mechanism of monoacylsucrose synthesis

in tobacco. Differentially expressed candidate genes in different test materials will be mined and characterized using various functional identification tools, such as tissue and subcellular localization, overexpression, gene knockout, and *in vitro* enzymatic activities. Through these studies, it is expected that more key genes and regulatory factors involved in the synthesis of sugar esters can be obtained for tobacco breeding. Moreover, in our study, overexpression of *NtASAT1* or *NtASAT2* did not increase sugar ester content. According to the present study and previous reports (Slocombe et al., 2008; Chang et al., 2020), genes, such as *NtALS1*, which are involved in acylsugar acyl chain synthesis, were mainly localized in chloroplasts (or plastids), whereas genes, such as *NtASAT1* and *NtASAT2*, which are involved in acylsugar assembly, were localized in the endoplasmic reticulum. Therefore, we speculate that the overexpression of *NtASAT1* or *NtASAT2* may not effectively induce the accumulation of acyl chain precursors, failing to effectively increase the end-product sugar ester content. Next, it is necessary to test the acyl chain pools in *NtASAT1* or *NtASAT2* overexpressing plants and wild-type controls to make it clear. In addition, referring to the technique of synthesizing taxanes using chloroplast engineering in *N. benthamiana* (Li et al., 2019), it is necessary to explore whether the acylsugar content of tobacco can be improved by the co-expression of acyl chain synthesis and acylsugar assembly genes in chloroplast.

Third, more unknown genes involved in acylsugar synthesis in *N. tabacum* need to be further investigated. Our study identified the genes responsible for the first two steps in the assembly of acylsugar in *N. tabacum*; however, the genes involved in the third and fourth steps of acylsugar assembly remain unknown. According to previous reports and our study, the structures of tetra-acylsucroses in tobacco and petunia are relatively similar, and the identified *NtASAT1/2* are homologous and functionally similar to *PaxASAT1/2*. In the present study, a *PaxASAT3* homolog was obtained through a homolog comparison of *N. tabacum* genome data. It is predicted to regulate the third step of acylsugar assembly, but its function needs to be further elucidated. No *PaxASAT4* homologs were found in *N. tabacum.* Recently, four ASATs, *NacASAT1-NacASAT4*, involved in the assembly of acylsucrose in *N. acuminata* have been reported. Next, we used these four gene sequences to isolate their homologs from *N. tabacum* genome data by BLAST analysis, and then through tissue expression pattern analysis, gene knockout, overexpression, and *in vitro* enzyme activity assays to identify whether these isolated genes were involved in the third and fourth steps of acylsugar assembly in *N. tabacum.* In addition, apart from the three genes (*NtALS1*, *NtASAT1*, and *NtASAT2*) identified in this study, no other genes involved in acylsugar synthesis have been reported in *N. tabacum*. Next, a cDNA library of tobacco glandular trichomes can be constructed using the promoters of the three genes obtained as target sequences, screening the transcription factors using the yeast monohybrid method, and then characterizing the roles of the transcription factors screened using gene function identification tools, such as knockout and overexpression. For transcription factors with regulatory functions, other proteins interacting with these transcription factors can be explored using yeast double-hybrid, Co-immunoprecipitation (Co-IP), pull-down, and other techniques. Through these studies, the regulatory genes and networks of acylsugar synthesis will

be further elucidated, and the key genes or transcription factors regulating acylsugar synthesis can be screened for use in tobacco breeding.

Fourth, in the long run, it is necessary to elucidate the acylsugar biosynthetic mechanisms of different wild tobacco species with high acylsugar activities. Wild tobacco species are rich in acylsugar structural types (Arrendale et al. 1990; Moghe et al. 2017; Schenck et al., 2022), and many wild tobacco acylsugars have high biological activities, such as acylsugars from *N. gossei*, *N. benthimiana,* and *N. attenuate* have good defenses against insects. Because the chromosome number of wild tobacco is different from that of *N. tabacum*, it is difficult to transfer the acylsugar traits of wild tobacco to cultivated tobacco by hybridization. Therefore, elucidating the mechanism of sugar ester synthesis in wild tobacco and mining usable key genes for tobacco breeding or acylsugar biosynthesis may be an effective means to accelerate the utilization of beneficial acylsugars of wild tobacco in cultivated tobacco and in commercial development. In the future, for wild tobacco species containing acylsugars with high biological activities, genome sequencing can be used to obtain reference genomic data first and then conduct transcriptome sequence analysis of glandular trichomes and leaves without trichomes to mine genes that are differentially expressed in these two tissues. Based on all known *Solanaceae* genome databases, the function of the differentially expressed genes will bepredicted by homologous comparison analysis, phylogenetic tree analysis, and homologous protein motif analysis. Candidate genes that may participate in acylsugar synthesis will be screened for further functional identification. In addition, for different wild tobacco species with different acylsugar structures, the transcriptome, proteome, and metabolome of glandular trichomes can be compared and analyzed, while candidate genes that may participate in regulating the structural differences of acylsugars can be identified, and their functional identification can be carried out. Key genes verified to regulate acylsugar synthesis in wild tobacco can be utilized in cultivated tobacco breeding by transgenic means.

Furthermore, the identification of the bioactivity of acylsugars with different chemical structures needs to be further investigated. Previous studies have demonstrated that acylsugars play a rolein protecting plants against insects, fungi, and desiccation (Chortyk et al., 1997; Lawson et al., 1997; McKenzie et al., 2005; Maluf et al., 2010; Leckie et al., 2012; Leckie et al., 2014; Smeda et al., 2016; Luu et al., 2017; Smeda et al., 2018;Feng et al., 2021), and have many potential industrial applications (Hill and Rhode, 1999; Garti et al., 2000). Acylsugars with different structures have different biological activities, and acyl chain types and sugar cores contribute to these activity diversity (Chortyk et al., 1993; Chortyk et al., 1996;Leckie et al., 2016). For example, *Nicotiana* acylsugars with long acyl chains possess the highest antibiotic activities (Chortyk et al., 1993) and eight carbon long acyl chains were the most protective against white whiteflies (Chortyk et al., 1996). To date, more than 300 acylsugar structural variants have been identified in *Nicotiana* (Schenck et al., 2022), however, the biological activity of each acylsugar structure and the degree of its functional activity are still unclear. In future studies, it is necessary to further strengthen the identification of the functions of acylsugars with different structures in biological activities, such as antiinsect, antifungal,

antibacterial, antioxidant and antidesiccant, to clarify the differences in their activity and to screen acylsugars suitable for various uses, such as insecticides, cosmetic or food additives, and pharmaceutical ingredients. These studies are of great significance for the subsequent development and utilization of different types of *Nicotiana* acylsugars for crop breeding, industry, and commerce.

Finally, based on known biosynthetic genes and regulatory mechanisms of acylsugars, it is expected that an increasing number of new acylsugars beneficial to humans will be obtained through *in vitro* and *in vivo* biosynthesis. *In vitro* studies have shown that four acylsugar acyltransferases (NacASAT1-NacASAT4) together with structurally diverse acyl-CoAs and sucrose in a single enzyme assay can not only synthesize acylsugars detected in plants but also produce acylsugars not present in *Nicotiana* plants (Schenck et al., 2022). Moreover, SlASAT1 and NacASAT1 catalyze sucrose acceptor and acyl-CoA donors to produce different monoacylsucroses (Fan et al., 2016; Schenck et al., 2022); however, when they were placed in one pot with the same substrates, mono- and di-acylsucroses appeared (Schenck et al., 2022). Therefore, in the future, we can use *in vitro* reactions and the mixing of genes involved in acylsugar assembly from different species to synthesize different structural aclysugars that do not exist in plants but are beneficial to human beings and animals. In addition, A report on taxane biosynthesis in *N.benthamiana* by metabolic engineering highlights the great potential of tobacco as a platform for the production of various chemicals (Li et al. 2019). With the elucidation of acylsugar biosynthetic genes and mechanisms of *Nicotiana*, tobacco can be used as a platform for acylsugar synthesis by regulating genes involved in metabolic pathways, such as acyl chain synthesis and assembly, to develop various plant-derived acylsugars with diverse bioactivities and high safety for human beings. These synthetic acylsugars can be used as insecticides, the ingredients of antitumor medicines, and flavor additives in cigarette.

References

Arrendale RF, Severson RF, Sisson VA, Costello CE, Leary JA, Himmelsbach DS, Van Halbeek H (1990) Characterization of the sucrose ester fraction from Nicotiana glutinosa. Journal of Agricultural and Food Chemistry 38 (1):75-85. doi:10.1021/jf00091a015

Chang A-X, Chen B, Yang A-G, Hu R-S, Feng Q-F, Chen M, Yang X-N, Luo C-G, Li Y-Y, Wang Y-Y (2020) The trichome-specific acetolactate synthase NtALS1 gene, is involved in acylsugar biosynthesis in tobacco (Nicotiana tabacum L.). Planta 252 (1):13. doi:10.1007/s00425-020-03418-x

Chortyk OT, Kays SJ, Teng Q (1997) Characterization of insecticidal sugar esters of Petunia. Journal of Agricultural and Food Chemistry 45 (1):270-275. doi:10.1021/jf960322f

Chortyk OT, Severson RF, Cutler HC, Sisson VA (1993) Antibiotic Activities of Sugar Esters Isolated from Selected Nicotiana Species. Bioscience, Biotechnology, and Biochemistry 57 (8):1355-1356. doi:10.1271/bbb.57.1355

Fan P, Leong BJ, Last RL (2019) Tip of the trichome: evolution of acylsugar metabolic diversity in Solanaceae. Current Opinion in Plant Biology 49:8-16. doi:10.1016/j.pbi.2019.03.005

Fan P, Miller AM, Liu X, Jones AD, Last RL (2017) Evolution of a flipped pathway creates metabolic innovation in tomato trichomes through BAHD enzyme promiscuity. Nat Commun 8 (1):2080. doi:10.1038/s41467-017-02045-7

Fan P, Miller AM, Schilmiller AL, Liu X, Ofner I, Jones AD, Zamir D, Last RL (2016) In vitro reconstruction and analysis of evolutionary variation of the tomato acylsucrose metabolic network. Proc Natl Acad Sci U S A 113 (2):E239-248. doi:10.1073/pnas.1517930113Feng H, Acosta-Gamboa L, Kruse LH, Tracy JD, Chung SH, Nava Fereira AR, Shakir S, Xu H, Sunter G, Gore MA, Casteel CL, Moghe GD, Jander G (2021) Acylsugars protect Nicotiana benthamiana against insect herbivory and desiccation. Plant Mol Biol. doi:10.1007/s11103-021-01191-3

Garti N, Clement V, Fanun M, Leser ME (2000) Some Characteristics of Sugar Ester Nonionic Microemulsions in View of Possible Food Applications. Journal of Agricultural and Food Chemistry 48 (9):3945-3956. doi:10.1021/jf991393s

Ghosh B, Westbrook TC, Jones AD (2014) Comparative structural profiling of trichome specialized metabolites in tomato (Solanumlycopersicum) and S. habrochaites: acylsugar profiles revealed by UHPLC/MS and NMR. Metabolomics 10 (3):496-507. doi:10.1007/s11306-013-0585-y

Haliński Ł P, Stepnowski P (2013) GC-MS and MALDI-TOF MS profiling of sucrose esters from Nicotiana tabacum and N. rustica. Zeitschrift fur Naturforschung C, Journal of biosciences 68 (5-6):210-222. doi:10.1515/znc-2013-5-607

Hill K, Rhode O (1999) Sugar-based surfactants for consumer products and technical applications. Lipid / Fett, vol 101.

Jia C, Wang Y, Zhu Y, Xu C, Mao D (2013) Preparative isolation and structural characterization of sucrose ester isomers from oriental tobacco. Carbohydrate Research 372:73-77. doi:10.1016/j.carres.2013.01.021

Kandra L, Severson R, Wagner GJ (1990) Modified branched-chain amino acid pathways give rise to acyl acids of sucrose esters exuded from tobacco leaf trichomes. European journal of biochemistry 188 (2):385-391.

Keeler SJ, Sanders P, Smith JK, Mazur BJ (1993) Regulation of tobacco acetolactate synthase gene expression. Plant Physiol 102:1009–1018. https://doi.org/10.1104/pp.102.3.1009

Kim J, Kang K, Gonzales-Vigil E, Shi F, Jones AD, Barry CS, Last RL (2012) Striking Natural Diversity in Glandular Trichome Acylsugar Composition Is Shaped by Variation at the Acyltransferase2 Locus in the Wild Tomato Solanum habrochaites. Plant Physiology 160 (4):1854-1870. doi:10.1104/pp.112.204735

Kroumova ABM, Zaitlin D, Wagner GJ (2016) Natural variability in acyl moieties of sugar esters produced by certain tobacco and other Solanaceae species. Phytochemistry 130:218-227. doi:10.1016/j.phytochem.2016.05.008

Lawson DM, Lunde CF, Mutschler MA (1997) Marker-assisted transfer of

acylsugar-mediated pest resistance from the wild tomato, Lycopersicon pennellii, to the cultivated tomato, Lycopersicon esculentum. Molecular Breeding 3 (4):307-317. doi:10.1023/A:1009677412902

Leckie BM, D'Ambrosio DA, Chappell TM, Halitschke R, De Jong DM, Kessler A, Kennedy GG, Mutschler MA (2016) Differential and Synergistic Functionality of Acylsugars in Suppressing Oviposition by Insect Herbivores. Plos One 11 (4). doi:10.1371/journal.pone.0153345

Leckie BM, De Jong DM, Mutschler MA (2012) Quantitative trait loci increasing acylsugars in tomato breeding lines and their impacts on silverleaf whiteflies. Molecular Breeding 30 (4):1621-1634. doi:10.1007/s11032-012-9746-3

Leckie BM, Halitschke R, De Jong DM, Smeda JR, Kessler A, Mutschler MA (2014) Quantitative trait loci regulating the fatty acid profile of acylsugars in tomato. Molecular Breeding 34 (3):1201-1213. doi:10.1007/s11032-014-0110-7

Lee KY, Townsend J, Tepperman J, et al (1988) The molecular basis of sulfonylurea herbicide resistance in tobacco. EMBO J 7:1241–1248. https://doi.org/10.1002/j.1460-2075.1988.tb02937.x

Leong BJ, Hurney SM, Fiesel PD, Moghe GD, Jones AD, Last RL (2020) Specialized Metabolism in a Nonmodel Nightshade: Trichome Acylinositol Biosynthesis. Plant Physiol 183 (3):915-924. doi:10.1104/pp.20.00276

Leong BJ, Lybrand DB, Lou Y-R, Fan P, Schilmiller AL, Last RL (2019) Evolution of metabolic novelty: A trichome-expressed invertase creates specialized metabolic diversity in wild tomato. Science Advances 5 (4):eaaw3754. doi:10.1126/sciadv.aaw3754

Li J, Mutanda I, Wang K, Yang L, Wang J, Wang YJNC (2019) Chloroplastic metabolic engineering coupled with isoprenoid pool enhancement for committed taxanes biosynthesis in Nicotiana benthamiana. 10 (1):1-12.

Liu X, Enright M, Barry CS, Jones AD (2017) Profiling, isolation and structure elucidation of specialized acylsucrose metabolites accumulating in trichomes of Petunia species. Metabolomics 13 (7). doi:10.1007/s11306-017-1224-9

Lou YR, Anthony TM, Fiesel PD, Arking RE, Christensen EM, Jones AD, Last RL (2021) It happened again: Convergent evolution of acylglucose specialized metabolism in black nightshade and wild tomato. Sci Adv 7 (46):eabj8726. doi:10.1126/sciadv.abj8726

Luu VT, Weinhold A, Ullah C, Dressel S, Schoettner M, Gase K, Gaquerel E, Xu S, Baldwin IT (2017) O-Acyl Sugars Protect a Wild Tobacco from Both Native Fungal Pathogens and a Specialist Herbivore. Plant Physiol 174 (1):370-386. doi:10.1104/pp.16.01904

Maluf WR, Maciel GM, Gomes LAA, Cardoso MDG, Knapp M (2010) Broad-Spectrum Arthropod Resistance in Hybrids between High- and Low-Acylsugar Tomato Lines. Crop Science, vol 50.

Mckenzie CL, Weathersbee AA, Puterka GJ (2005) Toxicity of Sucrose Octanoate to Egg, Nymphal, and Adult Bemisia tabaci (Hemiptera: Aleyrodidae) Using a Novel Plant-Based Bioassay. Journal of Economic Entomology.

Moghe GD, Leong BJ, Hurney SM, Jones AD, Last RL (2017) Evolutionary routes to biochemical innovation revealed by integrative analysis of a plant-defense related specialized metabolic pathway. Elife 6. doi:10.7554/eLife.28468

Nadakuduti SS, Uebler JB, Liu X, Jones AD, Barry CS (2017) Characterization of Trichome-Expressed BAHD Acyltransferases in Petunia axillaris Reveals Distinct Acylsugar Assembly Mechanisms within the Solanaceae. Plant Physiology, vol 175.

Ning J, Moghe GD, Leong B, Kim J, Ofner I, Wang Z, Adams C, Jones AD, Zamir D, Last RL (2015) A Feedback-Insensitive Isopropylmalate Synthase Affects Acylsugar Composition in Cultivated and Wild Tomato. Plant Physiol 169 (3):1821-1835. doi:10.1104/pp.15.00474

Payne T, Clement J, Arnold D, Lloyd A (1999) Heterologous myb genes distinct from GL1 enhance trichome production when overexpressed in Nicotiana tabacum. Development 126 (4):671-682. doi:10.1242/dev.126.4.671

Pan Y, Wang Z, Li X, Yan X, Zhang H, Sun J, Cui H (2019) Creating a high trichome density variety, hairy K326, by CRISPR-Cas9 technique. Tobacco Science & Technology, vol 52.

Perez-Rodriguez M, Jaffe FW, Butelli E, Glover BJ, Martin C (2005) Development of three different cell types is associated with the activity of a specific MYB transcription factor in the ventral petal of Antirrhinum majus flowers. Development 132 (2):359-370. doi:10.1242/dev.01584Schenck CA, Anthony TM, Jacobs M, Jones AD, Last RL (2022) Natural Variation Meets Synthetic Biology: Promiscuous Trichome Expressed Acyltransferases from Nicotiana acuminata. bioRxiv.

Schilmiller AL, Charbonneau AL, Last RL (2012) Identification of a BAHD acetyltransferase that produces protective acyl sugars in tomato trichomes. Proc Natl Acad Sci U S A 109 (40):16377-16382. doi:10.1073/pnas.1207906109

Schilmiller AL, Gilgallon K, Ghosh B, Jones AD, Last RL (2016) Acylsugar Acylhydrolases: Carboxylesterase-Catalyzed Hydrolysis of Acylsugars in Tomato Trichomes. Plant Physiol 170 (3):1331-1344. doi:10.1104/pp.15.01348

Schilmiller AL, Moghe GD, Fan P, Ghosh B, Ning J, Jones AD, Last RL (2015) Functionally divergent alleles and duplicated Loci encoding an acyltransferase contribute to acylsugar metabolite diversity in Solanum trichomes. Plant Cell 27 (4):1002-1017. doi:10.1105/tpc.15.00087

Severson RF, Arrendale RF, Chortyk OT, Green CR, Thome FA, Stewart JL, Johnson AW (1985) Isolation and characterization of the sucrose esters of the cuticular waxes of green tobacco leaf. Journal of Agricultural and Food Chemistry 33 (5):870-875. doi:10.1021/jf00065a026

Severson RF, Chortyk OT, Stephenson MG, Akey DH, Neal Jr JW, Pittarelli GW, Jackson DM, Sisson VA (1994) Characterization of natural pesticide from Nicotiana gossei. In. ACS Publications,

Slocombe SP, Schauvinhold I, McQuinn RP, Besser K, Welsby NA, Harper A, Aziz N, Li Y, Larson TR, Giovannoni J (2008) Transcriptomic and reverse genetic analysesof branched-chain fatty acid and acyl sugar production in Solanum pennellii

and Nicotiana benthamiana. Plant physiology 148 (4):1830-1846

Smeda JR, Schilmiller AL, Anderson T, Ben-Mahmoud S, Ullman DE, Chappell TM, Kessler A, Mutschler MA (2018) Combination of Acylglucose QTL reveals additive and epistatic genetic interactions and impacts insect oviposition and virus infection. Molecular Breeding 38 (1). doi:10.1007/s11032-017-0756-z

Smeda JR, Schilmiller AL, Last RL, Mutschler MA (2016) Introgression of acylsugar chemistry QTL modifies the composition and structure of acylsugars produced by high-accumulating tomato lines. Molecular Breeding, vol 36.

Vontimitta V, Danehower DA, Steede T, Moon HS, Lewis RS (2010) Analysis of a Nicotiana tabacum L. Genomic Region Controlling Two Leaf Surface Chemistry Traits. Journal of Agricultural and Food Chemistry 58 (1):294-300. doi:10.1021/jf903256h

Walters DS, Steffens JC (1990) Branched chain amino acid metabolism in the biosynthesis of Lycopersicon pennellii glucose esters. Plant Physiology 93 (4):1544-1551.

Yang C, Li H, Zhang J, Luo Z, Gong P, Zhang C, Li J, Wang T, Zhang Y, Lu Ye, Ye Z (2011) A regulatory gene induces trichome formation and embryo lethality in tomato. Proceedings of the National Academy of Sciences, vol 108.

Appendix – Publications and Projects

Publications

1. Aixia Chang[*†], Zhongyi Hu[*], Biao Chen, Herve Vanderschuren, Ming Chen, Yafang Qu, Weisong Yu, Yangyang Li, Huiqing Sun, Jianmin Cao, Kumar Vasudevan , Chenying Li, Yanan Cao, Jianye Zhang, Yeming Shen, Aiguo Yang[†],Yuanying Wang[†]. Characterization of trichome-specific BAHD acyltransferases involved in acylsugar biosynthesis in *Nicotiana tabacum*. *Journal of Experimental Botany* (2022), https://doi.org/10.1093/jxb/erac095.

2. Ai-Xia Chang[†], Biao Chen[†], Ai-Guo Yang, Ri-Sheng Hu, Quan-Fu Feng, Ming Chen, Xiao-Ning Yang, Cheng-Gang Luo, Yang-Yang Li[*], Yuan-Ying Wang[*]. The trichome-specific acetolactate synthase NtALS1 gene, is involved in acylsugar biosynthesis in tobacco (Nicotiana tabacum L.). *Planta* (2020) 252:13.

3. CHEN Biao, CHEN Ming, QU Yafang, CHENG Lirui, LI Yangyang, YANG Aiguo, HU Risheng, LIU Dan, LUO Chenggang, XIANG Shipeng, FENG Quanfu*, **CHANG Aixia***. Screening and Assisted Breeding Applications of SSR Molecular Markers of Sugar Esters in Tobacco. Chinese Tobacco Science. 2019 Vol. 24 (3) ：8-15 (In Chinese).

4. CHANG Aixia, QU YaFang, WANG GuoPing, LUO ChengGang, FENG QuanFu, CHEN ZhiQiang, FU XianKui,WANG YuanYing*. Research on Key Component and Molecular Basis Responsible for the Specific Aroma Traits of Flue-cured Tobacco Dabaijin599. Chinese Tobacco Science. 2018 Vol. 24 (3) (In Chinese).

5. QuYafang, Xu Minglu, Cao Jianmin, Sun Huiqing, You Xiangwei, Wang Guoping, Jiang Caihong, Wang Yuanying, **Chang Aixia***. Comparative analysis of major glandular trichome secretions of different tobacco types. Acta Tabacaria Sinica. 2018 Vol. 24 (1): 45-52 (In Chinese).

6. CHANG Aixia , GUO Lijie, LIU Dan, LUO Chenggang, FENG Quanfu,WEI Kai, WANG Lan*.Transgenic Tobacco Coker371 Gold Harboring the Arabidopsis Flowering Gene FT and its Early Blooming Properties. Chinese Tobacco Science. 2016, Vol. 37 (1): 1-7.(In Chinese).

7. WANG Guoping, WANG Haibo, LI Yanli, LUO Chenggang, CAO Jianmin, ZHANG Yu, FENG Quanfu, **CHANG Aixia***.Comparison of Multiple Organic Acid Contents in Different Flue-cured Tobacco Genotypes.Chinese Tobacco Science. 2016 Vol. 37 (1): 45-50. (In Chinese).

8. WANG Guo-ping, LI Yan-li, LUO Cheng-gang[1], REN Ming[1], CAO Jian-min[1],

ZHANG Yu[1], FENG Quan-fu[1], **CHANG Ai-xia***.The Association Analysis of Aroma Components with SSR Markers in Flue-cured Tobacco(Nicotiana tabacum). Journal of Plant Genetic Resources. 2016 Vol. 17 (4): 671-682. (In Chinese)

9. Caiyun Liu*, **Aixia Chang** and Chuanyin Du. Genetic, Physiological and Biochemical Analysis of the Formation of Yellow-green Leaf Color of Burley Tobacco (*Nicotiana tabacum*). Int. J. Agric. Biol., 2015,17: 767772.

10. **CHANG Ai-xia**, GUO Li-jie, LIU Dan, LUO Cheng-gang, WANG Lin-song, FENG Quan-fu, WANG Lan*. Tobacco Rapid Backcross Improvement Mediated by Arabidopsis Flowering Gene FT. Scientia Agricultura Sinica, 2015,48(13):2508-2517. (In Chinese).

Projects

Based on this doctoral research, four research projects have been funded.

1. “Mining and functional identification of genes involved in tobacco sucrose ester biosynthesis” (1610232017006). 2017~2019. Fundamental Research Funds for Central Non-profit Scientific Institution. Project host.

2. “Creating tobacco materials containing molecular modules for surface aroma precursors and directional improvement of aroma characters of main tobacco cultivars in Hunan province” (18-20Aa02). 2018~2020, funded by Hunan tobacco company. Project host.

3. “Discovery and identification of key functional genes involved in the synthesis of important tobacco aroma components” (1610232020001). 2020. Fundamental Research Funds for Central Non-profit Scientific Institution. Project host.

4. “Molecular control and directional improvement of surface chemical composition of flue-cured tobacco” (110202101005（JY-05）). 2021~2022. Funded by China National Tobacco Corporation. Project host.

.