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This manuscript contains seven tables, one color figure, and three supplementary figures.

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Patterns of inheritance of acylsugar acyl groups in selected interspecific hybrids of genus *Nicotiana*

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Abstract

Glandular trichomes on the surface of Solanaceae species produce acyl sugars that are species-, and cultivar-specific. Acyl sugars are known to possess insecticidal, antibiotic, and hormone-like properties, and as such have great potential as a class of naturally occurring pesticides and antibiotics. The objective of this work was to analyze the acyl composition of acyl sugars in the leaf trichome exudate from selected *Nicotiana* species and to follow the inheritance of acyl content in their hybrids. Trichome exudates were collected, and the acyl profiles of acyl sugars were identified via GC-MS. The variations in acyl group inheritance in the hybrids (a single parent resemblance, missing, complementary, and novel groups) matched the patterns described in the literature for a variety of secondary metabolites. However, we did not find a complementation of major parental acyl groups. Instead, in some hybrids we observed a dynamic change in the proportions of acyl groups, distinguishing the acyl group profiles as novel. We observed paternal (i.e. *N. tabacum* cv. Turkish Samsun × *N. benthamiana* hybrids) and maternal (i.e. *N. tabacum* cv. Samsun-nn × *N. otophora*) inheritance patterns, novel acyl profiles (*N. excelsior* hybrids), and missing acyl groups (*N. excelsiana*). Selective inheritance of some acyl groups in the hybrids of *N. benthamiana* (4- and 5-methylheptanoic isomers) or *N. alata* (octanoate) was found. Suggestions are given to explain certain patterns of inheritance. The data presented here contribute to the body of knowledge about the effect of interspecific hybridization on the secondary metabolites by including acylsugar acyl groups that have not been studied previously.

Keywords

Acylsugar acyl groups Genus *Nicotiana* Inheritance *Nicotiana* hybrids Trichomes

Introduction

Acyl sugars (ASs) are naturally-occurring secondary metabolites that are synthesized mainly by enzymes in the glandular trichomes on the surface of plants from the Solanaceae family. Much of the tobacco literature uses the term sugar esters (SEs) to describe acyl sugars. ASs are known to have important roles in plant defense against numerous pests and pathogens such as insects, aphids, whiteflies, budworms, and hornworms, bacteria and fungi (Cesio et al. 2006; Chortyk et al. 1993; Cutler et al. 1992; Leckie et al. 2012; Liu et al. 1996; Luu et al. 2017; Puterka et al. 2003; Severson 1985a). ASs belong to the group of glycosides in which sucrose, glucose, or fructose moieties are esterified to fatty acids having short ($\leq C_6$) and/or medium carbon chain length (C_{7-12}), and either straight or branched chain. The structures of ASs and their acyl substitutions have been elucidated and presented in numerous publications (Arrendale et al. 1990; Kaiser et al. 2018; Luu et al. 2017; Severson et al. 1991). Species from the genus *Nicotiana* in the Solanaceae family include tobacco (*Nicotiana tabacum*) and wild tobacco relatives (Goodspeed 1954). The ASs of *N. tabacum* vary from those of the other *Nicotiana* species with respect to sugar moiety and acyl groups attached to them (Ashraf-Kohorassani et al. 2008; Jia et al. 2013; Leffingwell 1999; Severson et al. 1985a). Tobacco produces sucrose esters, where the esterified acyl groups are short-length ($\leq C_6$) branched-chain groups with a minor presence of C_7 groups. Three acyl groups are esterified to the glucose moiety, and an acetyl group may be attached to the glucose and/or fructose. The major acyl groups in burley, Virginia, and flue-cured tobaccos are 2-methyl butyrate (2-MeBut), 3-methyl butyrate (3-MeBut), and acetate (Ac), and in oriental tobaccos the major groups are 3-methyl valerate (3-MeVal) and Ac (Ashraf-Kohorassani et al. 2008; Jia et al. 2013; Leffingwell 1999; Severson et al. 1985a). In some *Nicotiana* species such as *N. hesperis*, *N. occidentalis*, *N. alata*, *N. gosseii*, and *N. amplexicaulis*, glucose esters predominate (Jackson et al. 1998) or are present together with sucrose esters (Matsuzaki et al. 1989). Acyl groups in some wild *Nicotiana* species such as *N. glutinosa*, *N. occidentalis*, *N. obtusifolia*, and *N. alata* have up to 10 carbons, which form branched and/or straight chains (Chortyk et al. 1993; Cutler et al. 1992; Haliński and Stepnowski, 2013; Kroumova et al. 2016; Nottingham et al. 1996).

ASs with medium-chain acyl groups (C_{7-12}) are more toxic than those with short-chain groups (C_{2-6}) to soft bodied arthropods such as budworms, hornworms, aphids, and whitefly (Chortyk et al. 1996; Jackson and Danehower 1996; Jackson et al. 1998; Nottingham et al. 1996; Severson et al. 1991; Simonovska et al. 2006; Song et al. 2006) and bacteria (Chortyk et al. 1993; Cutler et al. 1992). Similarly, the medium-chain acyl groups 9-methyl nonanoate (9-MeNon) and decanoate (Dec) from wild tomatoes play an important role in insect resistance (Escobar-Bravo et al. 2016). Synthetic sucrose esters with octanoic acid (Oct) (C_8) have been successfully used against a range of arthropod species in tobacco (Song et al. 2006) and Florida citrus (Michaud and McKenzie 2004; Puterka et al. 2003).

Wild *Nicotiana* species are a great resource for genetic variability of the ASs. If the medium-chain acids can be introduced into the SEs of tobacco crops, they might enhance pest and insect resistance and consequently contribute to better quality and yield of tobacco leaves. One way of introducing new secondary metabolites is via hybridization. Plant hybridization is a known phenomenon. It is thought that around 70% of flowering plants originated via hybridization (Arnold 1994; Whitham et al. 1999). Hybridization can result in the introgression of traits, development of new species, and can influence the relationships between plants and their environment (Orians 2000). The effect of hybridization on the secondary product chemistry of hybrids and the impact on herbivore resistance has been the subject of several reviews (Cheng et al. 2011; Lopez-Caamal and Tovar-Sanchez 2014; Orians 2000; Rieseberg and Ellstrand 1993) summarizing 62 studies. The overall conclusion was that hybridization contributes to secondary metabolite diversity and affects herbivore resistance.

The genus *Nicotiana* comprises about 80 reported species, 57 of which have been shown to hybridize between themselves and with *N. tabacum* (Barbeć 2015; Lewis 2011). The interspecific crosses thus far have been estimated to number ~300 (Barbeć 2015). The abundance of successful interspecific crosses in this genus may be due to “slower evolution of genetic blocks to hybridization than the gene and chromosome alterations” (Goodspeed 1954). *Nicotiana* hybrids have been explored for a variety of reasons: (1) Initially they were used to study the phylogenetic relationships within *Nicotiana* (Barbeć 2015; Lewis 2011); (2) Hybridization is used to transfer useful genes from wild, undomesticated relatives into tobacco. These non-*tabacum* species have been explored for crop

improvement due to their genetic variability and because some of them harbor novel disease-resistance genes (see Lewis 2011 and the references therein). (3) The cytoplasmic and organelle genomes of wild species have been successfully used as a source of male-sterile cytoplasm (CMS) to increase the efficiency of hybrid seed production of commercial cultivars (Lewis 2011 and the references therein). (4) One important application of *Nicotiana* hybrids is that they can be advantageous platforms for the production of animal vaccines (Ling et al. 2012; Rice et al. 2013). (5) Intensive hybridization is being conducted within *N. tabacum* cultivars and introductions to “develop new tobacco hybrid cultivars bearing high yield, good quality, multiple disease resistance, and broad industry acceptability” (Miller, R., <https://reeis.usda.gov/web/crisprojectpages/0227012-tobacco-breeding-and-genetics.html>). (6) Tobacco hybrids have also been created as sources of genetic variations for trichome-produced secondary metabolites (Nielsen 1989).

Regardless of the broad-spectrum exploration of the *Nicotiana* hybrids, little attention had been paid to the inheritance of secondary metabolites produced by glandular trichomes, mainly diterpenes and ASs. As an exception, Nielsen (1989) quantitatively compared trichome-produced AS, together with terpenoids [*cis*-abienol, α - and β -cembratriene diols (α - and β -CBT-diols)] in tobacco hybrids and their parental species. In contrast, in the genus *Lycopersicon* from the same family, intense research has been conducted on acylsugars in the hybrids of tomato. The role of ASs against mites and aphids was observed in tomato hybrids after introducing type IV trichomes, a major producer of ASs having 10 carbon acyl groups, from wild tomatoes (Escobar-Bravo et al. 2016; Goffreda et al. 1990; McDowell et al. 2011; Snyder and Carter 1984). Variation in Type IV trichome density, and respectively the abundance of SE, among the individual hybrids correlated with the variation in mite response. Furthermore, hybrids between *L. esculentum* and *L. pennellii* were less infested with aphids (*Macrosiphum euphorbiae*) than the common tomato, due to the increased number of AS-producing trichomes and increased amount of ASs (Carter and Snyder 1985).

We were not able to find accessible literature wherein the acyl composition of acyl sugars in the interspecific *Nicotiana* hybrids has been reported. Moreover, to our knowledge, introduction of trichome-based pest and insect resistance (due to synthesis of variable ASs) from non-*tabacum* species into tobacco has not yet been reported.

Majority of the studies related to ASs and AS acyl groups of *Nicotiana* (and other solanaceous) species were done on leaves (Ashraf-Khorasani et al 2008; Chortyk et al, 1993; Leffingwell 1999; Matsuzaki et al. 1989, 1992; Severson et al. 1985b, 1985a, 1991; Simonovska et al., 2006;). Historically, leaves of *Nicotiana* species have been explored because of their commercial use and high-level accumulation of trichome exudate (5-30% of dry weight of leaves) playing important role in plant-insect, –microbe interaction (Wagner 1991). Here we analyzed trichome exudates from leaves of selected artificial *Nicotiana* hybrids and their parents for the presence of novel and variable ASs having medium chain-length, branched and straight-chain acyl groups along with short-chain groups to allow further tests for insecticidal potential. The ASs showing insecticidal properties can be used as templates for biotechnological design of acyl sugars that will improve pest resistance of *Nicotiana* crops. The variation of AS acyl group inheritance in the hybrids, such as complementary group inheritance, unidirectional inheritance from one of the parents, novel AS acyl group profile, and missing groups, were similar to the patterns described in the literature for a variety of secondary metabolites, including essential oils and phenolic glucosides (Cheng et al. 2011). An interesting phenomenon observed was the selective inheritance of certain acyl groups in the hybrids of *N. benthamiana* or *N. alata*. The data accumulated here contributes to the body of knowledge of the effect of hybridization on the secondary metabolites by including AS acyl groups that have not been previously studied.

Materials and methods

Plant materials

In 2000-2003, numerous interspecific crosses between commercial tobacco (maternal/paternal) and non-*tabacum* species (maternal/paternal) were performed by Kentucky Tobacco Research and Development Center (KTRDC) (Lexington, KY, USA) personnel to identify interspecific hybrids that could be appropriate for the large-scale production of economically important proteins and that had resistance to common tobacco diseases. Hybrid seeds were created via cross pollination, as described in detail in Zaitlin and Mundell (2006). Hybrid #s H110, H114, 37,

87, and H75, were selected due to their potential to have diverse set of acyl groups coming from both parents (Table 1). The horticultural *Nicotiana* hybrids 'Baby Bella', 'Tinkerbelle' and 'Whisper' were purchased from Swallowtail Garden Seeds, CA. Seeds from *N. excelsior* (KTRDC accession numbers 11-S-19-1 and 90-S-19-2), *N. benthamiana* (accession number S-6-5), and dark-type tobacco KY 171 were obtained from the KTRDC germplasm collection. *N. excelsiana* hybrid seeds were provided by Kentucky BioProcessing, Owensboro, KY, USA. *N. tabacum* cv. Samsun-nn (PI 552748), and *N. tomentosiformis* (PI 555572) seeds were obtained from the NPGS/GRIN collection. Flue cured Virginia K326 tobacco seeds were kindly provided by F.W. Rickard Seeds, Inc. (Winchester, KY, USA). Seeds of *Alatae* hybrids were a gift from Dr. Catherine Poisson (Bergerac Seed & Breeding, France).

Hybrid seeds' germination

Hybrid seeds from the KTRDC hybrid seed collection were sterilized by incubation in 70% ethanol for one minute, followed by a 10-minute incubation in 20% household bleach, (0.3% sodium hypochlorite aqueous solution) followed by rinsing five times in sterile water to remove all traces of bleach and ethanol. The seeds were then germinated in 95x15mm Petri dishes on agar medium containing Murashige and Skoog (MS) salts and micro-elements (PlantMedia), Gamborg vitamins (1 ml) (Sigma-Aldrich Co. Ltd.) and high-strength agar (0.56%) (RPI Corp). Approximately one month later, rooted plantlets were transplanted to Styrofoam float trays containing a medium specially formulated for tobacco (Carolina Choice Peat-Lite Mix). Ten days later fertilizer (Peters 20-10-20, 1.5 g l⁻¹) was added, and the solution was replaced weekly thereafter. Plants were grown there for 3 to 4 weeks, whereupon those with well-established roots were transplanted individually into 4-inch pots. Seeds of the horticultural hybrids were sown on Pro-Mix medium (Premier Horticulture Inc., Canada) in 4-inch pots that were then covered with plastic until germination occurred in a growth chamber. All plants were grown at 22.8 °C with a 16 h/8 h light/dark regime and 23% humidity. After germination, the plastic covers were removed and plants were allowed to grow under the same conditions.

Collection of trichome exudate, butyl ester preparation, and GC-MS analysis

Trichome exudate was collected from the mid-stem leaves. Collection was done when plants were at a vegetative stage, usually three months post-sowing. The maturation time (the time of flower production) of hybrid plants of different crosses varied, but that of the F1 hybrid plants from the same cross were similar. We have continuously observed that around three months post-sowing the AS acyl composition is stable. Extract collection and AS acyl derivatization with butanol were conducted as described in Severson et al. (1985b) with some modifications. Abundance of SEs varied among species. Four or 8 discs (1 cm radius) were washed for 1 min with CH₃CN, with these washes vacuum-evaporated to dryness, dissolved in CHCl₃ (1 ml), transferred to 1.5 ml GC vials and dried. Where SEs were in low abundance (e.g. H37 and *N. excelsiana*), whole leaves were washed with CH₃CN for 1 min, the washes were vacuum-evaporated to dryness and dissolved in CHCl₃ (10 ml). Polar impurities were removed by twice partitioning with H₂O. Chloroform fractions were evaporated to dryness in GC vials (1.5 ml) (Kroumova et al. 2016). Further saponification, butyl ester derivatization of acyl groups, GC-MS analysis and acyl identification were completed as described previously (Kroumova et al. 2016). AS acyl groups were analyzed as butyl esters. The linear structure of acyl groups detected from all species and hybrids, their full and abbreviated names are given in Fig. S1. For the comparison of major acyl groups between hybrids and their parental species, peak areas were converted to moles using the linear regression equations derived from the calibration curves of standards (Kroumova et al. 2016). In the tables and in the graph the relative abundance of AS acyl groups of the individual plant types was calculated as mol% of total acyl groups.

Results

Selection of artificial hybrids

Hybrids from the *Nicotiana* collection KTRDC were selected on the basis of parental origin. *N. tabacum* was a preferred maternal line, in order to have hybrids with good biomass (Ling et al. 2012; Zaitlin and Mundell 2006).

The other parent was selected because of its biosynthesis of medium length, branched, and straight-chain acyl groups (Table 1). We hypothesized that ASs produced by these hybrids may incorporate acyl groups from both parents. The major acyl groups in hybrids were postulated on the basis of the reviews showing that typically the F_1 hybrids express parental secondary metabolites (Cheng et al. 2011; Orians 2000). The non-*tabacum* interspecific hybrids *N. excelsior* × *N. bigelovii* were selected for the potential to combine 3-MeVal with straight-chain Oct and heptanoic acid (Hept), and medium-length branched-chain methyl heptanoic isomers (Table 1). Hybrid seeds H110 and H114 were produced by two independent hybridization events using the same parents. The same applies to the hybrids H75 and H87.

AS acyl group profiles

N. tabacum as an example of natural hybrid between *N. sylvestris* and *N. tomentosiformis*

An example of natural hybridization, the species *N. tabacum* originated from an interspecific hybrid between an ancestor of *N. sylvestris* and a particular ancestral lineage of *N. tomentosiformis* (and possibly *N. otophora*), that evolved hundreds of thousands of years after polyploidization (Gray et al. 1974; Lewis 2011). Table 2 compares the acyl composition of the modern parental species and two modern varieties of tobacco – oriental tobacco cv. Samsun-nn and the flue-cured Virginia tobacco cultivar K326. In *N. sylvestris* the major acyl group is acetate, followed by 4-methyl hexanoate (4-MeHex); 3-MeVal is a minor acyl group. *N. tomentosiformis* SEs contain high amounts of 3-MeVal, followed by acetate and 2-MeBut. The acyl composition data for *N. tomentosiformis* is very similar to that already published by Severson et al. (1985b). Samsun-nn has the typical oriental-type profile, where 3-MeVal is the major group, followed by Ac and 2- and 3-MeBut, while Virginia K326 (air-cured type) tobacco has mostly Ac and 2- and 3-MeBut. AS acyl composition data for Samsun-nn and Virginia K326 presented here are in agreement with the literature (Kallianos 1976; Leffingwell 1999; Severson et al. 1985b). With respect to the presence of 3-MeVal as a major acyl group and absence of MeHex isomers, Samsun-nn resembles *N. tomentosiformis*. In Virginia K326, 3-MeVal is substantially reduced, but 2- and 3-MeBut that are present at lower abundances in the parental SEs became second and third major groups, after the Ac (Table 2). We suggest that a mutation in the isopropylmalate synthase gene (IPMS) (reaction 1, Fig. S3) in Virginia causes accumulation of precursor molecules and increased synthesis of 2-MeBut. Other changes are involved as well, leading to the final acyl profile. The presence of 3-MeVal as a major group in both *N. tomentosiformis* and *N. tabacum* Samsun-nn, and the lack of MeHex isomers favors the paternity of *N. tomentosiformis* vs. *N. otophora*, where MeHex isomers are the major groups (See *N. otophora* data in Table 4).

Artificial *N. tabacum* hybrids

N. tabacum cv. Samsun-nn × *N. benthamiana* (H110 and H114)

These hybrids were generated from two independent hybridization events using the same parents. The phenotypes of the hybrids are shown in Fig. 1a, b. Three plants from each hybrid were analyzed, and the data are presented in Table 3. The acyl compositions among individual plants of each cross were consistent, with some variation detected in the relative amounts. The acyl profiles consisted mainly of 6- and 5-MeHept, with small amounts of 5- and 4-MeHex. The other groups were minor. The similarity among AS acyl profiles of individual plants from either hybrid is not surprising because the F_1 plants were derived from the same hybridization event. What is more interesting is that the acyl composition from the second, independent cross of the same species gave a very similar qualitative acyl profile. We would expect some variations, due to the stochastic separation of chromosomes during the meiosis in both parental species, and respectively different combination of allelic chromosomes. The data from the three individual hybrid plants from each cross are compared with the parental compositions data (Table 3). Parental AS acyl group profiles reported here are in agreement with the published literature (Chortyk et al. 1993; Matsuzaki et al. 1992; Severson et al. 1991). The maternal donor – *N. tabacum* cv. Samsun-nn synthesizes predominantly 3-MeVal and 3-MeBut, while *N. benthamiana* (paternal donor) makes predominantly 5- and 6-MeHept and the minor acyl groups 4-MeHex and 5-methyl hexanoate (5-MeHex) (Table 3). The hybrids' AS acyl group profiles matched the paternal *N. benthamiana* profile – with a prevalence of 5- and 6-MeHept acids (90%) and small amounts of 4- and 5-MeHex. All parental groups were present in the hybrids, but no

combination of the major acyl groups 3-MeVal and MeHept isomers occurred. 3-MeVal was present as a minor group.

***N. tabacum* × *N. otophora* hybrids (H75 and H87)**

The maternal parent of hybrid H75 is a 'dark type' tobacco cv. SN2108, and the maternal parent of hybrid H87 is the flue-cured tobacco cv. NC 297 (Table 1). The phenotype of the hybrids is shown in Fig. 1c, d. The AS acyl composition of the hybrids and the parental species is presented in Table 4. *N. tabacum* KY171 (dark type) and *N. tabacum* K326 (flue cured) shown in Table 4 are not the exact maternal species, but they are representatives of the dark and flue-cured class of tobaccos. For each hybrid, three individual plants were analyzed. Compositionally, the acyl profile was very similar among the F₁ plants of the same hybrid and between the individuals of the two hybrids. 3-MeVal was the predominant group, followed by 3- and 2-MeBut. There was some variability in the relative abundance among the individual plants of each hybrid. Still, when comparing the average values of individual acyl groups and their total distribution it is clear that there is a great similarity in ASs acyl group composition between the two hybrids (Table 4). The acyl profiles of the maternal species – dark tobacco (for H75) and flue-cured (for H87) tobaccos are also similar. Dark and flue-cured tobaccos were previously shown to produce ASs containing mostly 2- and 3-MeBut and Ac (Leffingwell 1999; Severson et al. 1985a). The major AS acyl groups of *N. otophora* are 4- and 5-MeHex and 3-MeVal (in declining order) (Severson et al. 1991). All parental acyl groups were inherited in the hybrids. 3-MeVal became a major group followed by small amounts of 2- and 3-MeBut. Some F₁ plants showed elevated abundances of MeHept isomers that otherwise were minor groups in N.t. K326 or were not detected in N.t. KY171. Thus, by crossing dark or flue-cured tobacco with *N. otophora*, the hybrids acquired novel AS acyl chemistry resembling oriental-type tobacco profile, but with additional eight-carbon branched-chain groups and missing Ac groups (Table 4).

Artificial hybrids of *N. excelsior*

Hybrid H37

This hybrid is a cross between *N. excelsior* and *N. bigelovii*. The maternal parent, *N. excelsior*, is a leafy herb with mostly glabrous leaves and stems (http://www.flora.sa.gov.au/efsa/lucid/Solanaceae/Nicotiana%20species/key/Australian%20Nicotiana%20species/Media/Html/Nicotiana_excelsior.htm). The paternal species, *N. bigelovii* (or *N. quadrivalvis*) (Knapp et al. 2004) is a bushy herb, with small-lamina leaves and numerous glandular trichomes (nathistoc.bio.uci.edu/plants/Solanaceae/Nicotiana%20quadrivalvis.htm). The hybrid plants were small in size with mostly glabrous leaves, resembling the maternal parent (Fig. 1e). The hybrids produced very little exudate as judged by the slight leaf stickiness and by the low relative abundance as revealed by GC-MS. The acyl composition was similar among the individual F₁ plants, comprising mostly 5- and 6-MeHept, followed by MeHex isomers and 3-MeVal. Comparison of the AS acyl groups between the parents and hybrids is shown in Table 5. We possessed two accession of *N. excelsior*. The precise maternal accession of Hybrid 37 was not given. That's why we analyzed both accession. High similarity of the acyl profile was observed between them with some differences in the proportion of groups (Table 5). *N. excelsior* SEs contained mostly Oct, 5-MeHept, Hept, and 2-MeBut while *N. bigelovii* synthesized mostly 3-MeVal, 2-MeBut, and Ac (Matsuzaki et al. 1989). Synthesis of the straight-chain group Oct and Hept was not transferred maternally to the hybrids. Qualitatively, the acyl composition of F1 plants was closer to the paternal species *N. bigelovii*. However, the relative abundance of individual acyl groups varied substantially from *N. bigelovii*, which resulted in a novel acyl profile (Table 5). This profile showed substantial presence of medium length, branched-chain groups (~75% of all acyl groups). Variability was observed among the individual hybrid plants. More study is needed to address this variability. Regardless of the extract's insecticidal potential due to the predominance of medium-length, branched-chain (MeHept and MeHex) isomers, the scarce yield makes the extract unsuitable for further examination. However, the exudate of the maternal species, *N. excelsior*, can be explored further as the acyl profile consists mostly of MeHex isomers and medium-length straight-chain groups (>72% of total acyl groups).

N. excelsiana

N. excelsiana is a fertile tetraploid hybrid derived from crossing *N. excelsior* and *N. benthamiana* that was created for the purpose of vaccine production (i.e. Ling et al. 2012). Like the maternal species *N. excelsior*, the hybrid is leafy with mostly glabrous leaves (Smith et al. 2009). The only AS acyl groups observed were 5- and 6-MeHept (Table 5). The comparison between parental species and *N. excelsiana* showed that the 6-MeHept group was inherited paternally from *N. benthamiana*, but 5-MeHept may have come from either parent. The straight-chain groups Hept and Oct from *N. excelsior* were not inherited in the hybrid. The acyl profile of ASs from *N. excelsiana* can be considered novel, consisting only of two acyl groups. The *N. excelsiana* AS acyl profile shows an example of missing secondary metabolites (Table 5). The trichome extract would be a good source for testing the insecticidal potential of MeHept isomers provided that a sufficient amount of SEs could be collected.

Artificial interspecific hybrids from section *Alatae*

N. × sanderae

N. × sanderae originated from a cross between *N. alata* and *N. forgetiana* (Chase et al. 2003). Images of this hybrid can be found on the web (<https://www.alamy.com/flowering-tobacco-nicotiana-x-sanderae-nicotiana-sanderae-potted-plants-image9257554.html>). *N. alata* is the species from which most of the modern horticulture hybrids are derived (<http://portlandnursery.com/plants/annuals/Nicotiana.shtml>). *N. alata* synthesizes straight-chain, short and medium length acyl groups with up to eight carbons, and also short length, branched-chain, acyl groups with up to six-carbons (Table 6). In contrast, *N. forgetiana* has predominantly branched-chain groups with up to seven carbons (Severson et al. 1991). The comparison of *N. × sanderae* and its parents is shown in Table 6. The hybrid's major groups are Oct, 2-MeBut, and Ac, followed by Hex. The maternal species *N. alata* produces the same major groups. Straight-chain groups Hex and Oct are inherited from *N. alata*, but Ac and 2-MeBut may have been inherited from either parent. MeHex isomers that are the second and third major acyl groups of *N. forgetiana* were not detected in the hybrid. The *N. × sanderae* AS acyl group composition was confirmed, and AS structures were elucidated by British American Tobacco, Inc., Souza Cruz, Brazil (Guilherme Sabin, personal communication).

Alatae hybrids

The *Alatae* hybrids were created by Catherine Poisson (Bergerac Seed & Breeding SC, France) by crossing two inbred lines. These lines originated from a breeding program that started by crossing wild species from section *Alatae* (*N. langsdorffii*, *N. alata*, *N. forgetiana*, *N. × sanderae*), but for some of them commercial varieties were additionally involved. However, the hybrids are not direct progenies of the original wild species and commercial varieties, as many crosses and backcrosses have been done through the years (Poisson Catherine, personal communication). We analyzed 15 hybrid lines (Fig. S2) and found their AS acyl group compositions to be similar. In hybrids PO# 67, 15, 69, 70, 81, 101, and 120, Oct was the predominant group (> 50 % of total acyl groups) (Fig. S2, Table 6) and the other groups were ≤10% each. In hybrids PO# 5, 33, 71, 72, 94, and 96, Oct was the major group followed by 2-MeBut. In all hybrids there was a presence of other than C₈ straight-chain groups (C₄, C₆, C₇, and C₉), and the total relative abundance of straight-chain groups in all hybrids was 60-90%. 2- and 3-Mebut were absent in POs 70, 111 and 112. There was variation in the relative acyl abundance between individual plants of each PO hybrid, but nevertheless the acyl profiles of the lines were well defined. The composition of PO5, 33, 71, 72, 94, and 96 hybrids was closer to that of *N. sanderae* and *N. langsdorffii* (Table 6). In section *Alatae* only *N. alata* and *N. langsdorffii* synthesize Oct, and therefore this acyl group could have been inherited from either of the species. MeHex isomers from *N. forgetiana* were not inherited during the breeding process. Acetate was not inherited from *N. alata* or *N. × sanderae* as well. Hept and Non groups were not present in the parental species, so they may be novel chemicals in the hybrids. The ASs of hybrids synthesizing mostly octanoic acid can be tested for insecticidal activity.

“Tinkerbelle”, “Baby Bella” and “Whisper”

The ornamental hybrids “Tinkerbelle” (*Nicotiana alata* “Tinkerbelle”), *Nicotiana × hybrida* “Baby Bella” (*Nicotiana × sanderae* “Baby Bella”) and “Whisper” are highly disease-tolerant hybrids that have a notable pleasant aroma and attractive petal colors (<https://www.gardenersworld.com/plants/nicotiana-alata-tinkerbelle/>;

<http://www.robsplants.com/plants/NicotSande>; <http://floranova.com/products/Nicotiana/>; <https://portlandnursery.com/annuals/nicotiana/>). These hybrids possess the most diverse AS acyl profiles compared to the other studied hybrids. Seventeen acyl groups were detected (Table 7). The hybrids' AS acyl composition was qualitatively similar, consisting mostly of Oct, and 2-MeBut (40 to 50% of total acyl groups) along with numerous but minor (<10% each) straight-chain acids (C₃, 4, 6-12) and branched-chain acids (MePro, 3-MeBut, MeVal, 4-MeHex and MeHept isomers). The acyl composition was similar to that of *N. alata*, and *N. × sanderae*, but it was enriched with six additional groups (Table 7). Hept, Non, Dec and Laur acyl groups may be novel for all the hybrids. There was variability in the relative proportions of AS acids among individual plants and among the hybrids. Oct was more prominent in "Baby Bella", while in "Tinkerbell" 2-MeBut was a predominant group followed by Oct and Ac. In "Whisper" the proportions between Oct and 2-MeBut varied from plant to plant (Table 7). MeHex isomers were found in the "Whisper" plants, but not in "Thinkerbell". In "Baby Bella" MePro varied from 0.5 to 13.8%. Oct varied from 7.2 to 34.5% in "Thinkerbell", and from 12 to 45% in "Whispers" (Table 7). Regarding the abundance of major acyl groups, the acyl compositions of "Tinkerbell" was more similar to *N. alata*, while "Baby Bella" acyl composition was more similar to *N. × sanderae*.

Discussion

In this work, we studied the inheritance of the trichome-produced AS acyl groups in interspecific hybrids in the genus *Nicotiana* for the purpose of finding unique acyl compositions, combining diverse acyl groups from both parents. Of great interest was the inheritance of medium-length, straight or branched-chain groups as major groups in the ASs because they have been shown to be more toxic to arthropod pests (Escobar-Bravo et al. 2016; Jackson and Daneshmand 1996; Jackson et al. 1998; Severson et al. 1991b; Simonovska et al. 2006; Song et al. 2006).

The parental species of *Nicotiana* hybrids have diverse acyl profiles. The combined genomes in the hybrids would carry all the genes necessary for synthesis of ASs and AS acyl groups featured in both parents and the interaction between the alleles from both genomes will determine the final profile in the hybrids. There is not sufficient knowledge about the genes and the regulatory factors participating in the synthesis of ASs in the genus *Nicotiana* to satisfactorily explain the observed patterns of inheritance. Figure S3 shows suggested α -KAE pathways for the synthesis of acyl-CoA groups (Kroumova et al. 1994) in some species of genus *Nicotiana* and in petunia. The pathways for the acyl groups' synthesis were concluded for *N. glutinosa*, *N. gossii*, and *N. benthamiana* (Kroumova et al. 2003). Numerous acyltransferase genes that play roles in acyl sugar assembly have been discovered among the species of genus *Solanum* and in petunia (Moghe et al. 2017; Nadakuduti et al. 2017) but orthologous acyltransferase genes have not been reported in *Nicotiana* species. We hypothesize that IPMS enzyme(s) (reaction 1, Fig. S3), branched-chain α -keto acid dehydrogenase (BCKD) enzyme(s) (reaction 3, Fig. S3), and putative acyltransferases (reaction 4, Fig. S3) play roles in determination of the uniqueness of acyl groups in individual species, and for the variability in acyl sugar compositions commonly observed. Trichome-specific IPMS (reaction 1, Fig. S3) from *N. tabacum* has been characterized and was shown to be essential for the synthesis of 3-MeVal (Gwynn et al. 1985; https://www.lens.org/lens/patent/EP_2565265_A1), while the tomato E1- β subunit of BCKD (reaction 3, fig. S3) was shown to affect branched-chain acyl groups in *N. benthamiana* (Slocum et al. 2008). It is clear however, that many more genes are potentially relevant to AS acyl chain synthesis and ASs assembly, keeping in mind the great variability of ASs in the numerous *Nicotiana* species, and taking into consideration the voluminous work on the synthesis of ASs in the genus *Solanum* in recent years (Fan et al. 2016; Ning et al., 2015; Schillmiller et al. 2015, 2016). Genes involved in the metabolism of ASs have been studied extensively in solanaceous crops because of their important role in plant defense and also for the purpose of increasing AS-mediated pest resistance (Glas et al. 2012; Mandal et al. 2018; Moghe et al. 2017). Acyl sugar chemistry modification has been achieved in tomato by introducing individually, or in combination, several quantitative trait loci (QTLs) from *S. pennellii*. The introduction of more than one trait altered the abundance of total SE, the acyl group profile, and the diversity of SE due to the additive and epistatic interactions between the QTLs featuring certain genes (Leckie et al. 2012; Smeda et al. 2017).

Regardless of the potential participation of numerous genes in the synthesis of acyl groups and ASs assembly, it has been proposed that in the hybrids, in general, one or few genes exhibiting dominance/recessivity regulate the

expression of secondary compounds (Gwynn et al. 1985; Lopes-Caamal and Tovar-Sanches 2014). Inheritance of some of the acyl groups in *Nicotiana* hybrids fits such a proposition, and is discussed below.

We have observed several patterns of AS acyl group inheritance (chemistry resembling that of a single parent, complementary acyl groups, missing acyl groups, and novel groups) that matched the patterns described by others for a variety of secondary metabolites, including essential oils, phenolic glucosides, cembratriene diols (CBT-diols), alkaloids, fatty acids, and phenolic constituents (Cheng et al. 2011; Court et al. 1992; Orians 2000). In addition, we observed variability in the expression of individual acyl groups. According to the literature, the first-generation hybrids (F_1) usually express both parental secondary metabolites (complementary expression) (Cheng et al. 2011; Rieseberg and Ellstrand 1993; Orians 2000), which is why our expectations were for the hybrid ASs to combine a variety of acyl groups in substantial abundance. In this study, complementary inheritance was observed but the expected abundance was not. In hybrids 110 and 114, all acyl groups were present, but Ac and MePro were not synthesized in the abundance that corresponded to that of the parental species. The overall acyl group profile of H110 and H114 resembled that of *N. benthamiana* (paternal inheritance) (Table 3). Similarly, it has been shown that in some *Nicotiana* hybrids the flower color was sometimes closer to one of the parents (Ling et al. 2012; Patel et al. 2011; Pental et al. 1984). When dark or flue-cured tobacco was crossed with *N. otophthora*, the F_1 hybrids (H75 and H87) also had the acyl profile of both parents. However, the ratio of acyl groups and the major acyl groups did not resemble the profile of either parent (Table 4) but were similar to that of the oriental tobacco. We suggest that the strong reduction in 4-MeHex and the robust synthesis of 3-MeVal (already present in low abundance in the parents) in the hybrids may be caused by an isoform of IPMS (reaction 1, Fig. S3). Both acyl groups are products of the ante-iso branch of the α -ketoacid elongation pathway (α -KAE) (Fig. S3), where one cycle of elongation produces 3-MeVal, and two cycles produce 4-MeHex (Kroumova and Wagner 2003). The IPMS gene for synthesis of 4-MeHex in *N. otophthora* may be in a heterozygous state, in which the dominant allele was not inherited in both hybrids, thus leading to the accumulation of the precursor 2-oxo-4 MeHex for synthesis of 3-MeVal.

The 5- and 6-MeHept groups produced by *N. benthamiana* were also inherited in *N. excelsiana* (Table 5). We hypothesize that in *N. benthamiana* both alleles of the putative gene(s) responsible for synthesis of MeHept isomers are in a dominant state, causing synthesis to occur in the hybrid. We think that one candidate gene important for the presence of MeHept in ASs is BCKD that is involved in branched-chain amino acid degradation and is potentially relevant to AS branched-chain acid synthesis (Slocumbe et al. 2008). After BCKD E1- β silencing (reaction 3, Fig. S3) in *N. benthamiana*, a significant reduction in 5- and 6-MeHept was observed, while in *S. pennellii*, reduction was observed in the synthesis of MePro and 8-MeNon (Slocumbe et al. 2008). Acyltransferase genes can be involved as well. Acyltransferases were shown to contribute to acylsugar diversity in the genus *Solanum* (Schillmiller et al. 2015). We suggest that in the genus *Nicotiana*, genes orthologous to those in *Solanum* may participate in the assembly of acylsugars.

In hybrid H37 (*N. excelsior* \times *N. bigelovii*), Hept and Oct were not inherited, while 6-MeHept became a second major group in contrast to both parents where it had a minor presence. MeHex isomers were also elevated compared to their minimal presence in both parents (and 6-MeHept). We suggest that in this particular hybrid, ASAT enzymes may be involved (reaction 4, Fig. S3).

The Oct group from *N. alata* was inherited in the hybrids regardless of the SE acyl composition of the other parent. We hypothesize that, similar to MeHept in *N. benthamia*, the gene(s) important for Oct synthesis is in a homozygous dominant state and the inheritance is manifest in the hybrids. In contrast, Oct and Hept from *N. excelsior*, and 4- and 5-MeHex from *N. forgetiana* or *N. otophthora*, were not inherited. It is possible that the genes involved in the synthesis of these groups are in a heterozygous state, and that the hybrids inherited the recessive allele (or the allele that is not functional). Acetate was not inherited in the *Alatae* hybrids. Most likely this may be related to acetyl-specific acyltransferase.

The appearance of novel acyl groups was observed, interestingly, only in *Alatae* hybrids and in the horticulture hybrids. The horticulture hybrids are also related to *Alatae* parents, and we may suggest that the novel groups are features in *Alatae* hybrids. Orians (2000) indicated that novelty may be more common for plants that produce chemicals in specialized cells, i.e. glandular trichomes. These novel acyl groups (together with Hex and Oct that are

present in the parental species) may have come from the straight-chain branch of the α -KAE pathway (Fig. S3) as in petunia and *N. gossei* (Kroumova and Wagner 2003). It is also possible to be synthesized by fatty acid elongation path, via two-carbon elongation, characteristic of fatty acid biosynthesis (Kroumova and Wagner 2003; Walters and Steffens 1990).

The data presented in this paper show variability in acyl group inheritance in *Nicotiana* F₁ hybrids, and thus acyl profile prediction based on the chemotypes of the parental species could be inaccurate. Hybrids may inherit the acyl groups from both parents, but it is important also which acyl groups are in abundance and which groups will contribute to the pest resistance or will improve the hybrid fitness in the environment. In *N. tabacum* cv. Samsun-nn \times *N. benthamiana* hybrids, 3-MeVal and MeBut isomers became minor groups. Similar changes were observed for Ac, MeBut isomers, and MeHex isomers in hybrids 75 and 87, while MeHept isomers were slightly increased (Table 4). Straight-chain groups from *N. excelsior* were not inherited. In contrast, in *N. \times sanderae*, the straight-chain acyl groups Hex and Oct were inherited maternally from *N. alata*. The horticulture hybrids are said to be highly disease-tolerant (www.plant-world-seeds.com), and we assume that Oct contributes to this resistance. Synthetic sucrose octanoate esters have strong pesticidal and insecticidal effects (Michaud and McKenzie 2004; Technical Evaluation Report Sucrose Octanoate Esters 2005). The natural octanoate-containing ASs are synthesized in the species from section *Alatae* (Chortyk et al. 1993; Cutler et al. 1992; Kroumova et al. 2016, and current work). Thus, octanoic acid could be a good candidate for introgression into tobacco.

We did not find any correlation between the parental origins of individual acyl groups and their inheritance in hybrids. In *N. tabacum* cv. Samsun-nn, hybrids 110/114, and *N. excelsiana*, the inheritance of the major acyl groups was paternal. In *N. \times sanderae* inheritance was maternal, while in H75 and H87 (*N. tabacum* \times *N. otophora*) and in H37 the acyl profiles did not match either parent with respect to the major acyl groups.

This study shows different patterns of inheritance of AS acyl groups, and additional work is needed to further guide preparation of interspecific hybrids to provide sufficient amounts of unusual ASs in order to test their insect and disease resistance potential. Knowledge of the way that particular acyl groups function in plant protection could help increase trichome-based resistance in *N. tabacum*.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Table 1 Selected interspecific hybrids and possible major AS acyl group inheritance from both parents.

| Hybrid number | Maternal species | Major acyl groups | Pollen from: | Major acyl groups | Hypothesized major acyl groups in the hybrids' ASs |
|---------------|--------------------------------------|-------------------------------------|-------------------------|--|--|
| H110/ H114 | <i>N. t.</i> cv. Samsun- nn | 3-MeVal ^{1,2} | × <i>N. benthamiana</i> | MeHept ^{a,3,4,5} | 3-MeVal + MeHept |
| H75 | <i>N. t.</i> SN 2108 (dark) | MeBut ^{b,1} | × <i>N. otophora</i> | MeHex ^c , 3-MeVal ⁶ | MeBut, MeHex, 3-MeVal |
| H87 | <i>N. t.</i> NC 297 (flue- cured) | MeBut ¹ | × <i>N. otophora</i> | MeHex, 3-MeVal ⁶ | MeBut, MeHex, 3-MeVal |
| H37 | <i>N. excelsior</i> | Oct, 5-MeHept, Hept ⁷ | × <i>N. bigelovii</i> | 3-MeVal ^{3,6} | Oct, 5-MeHept, 3-MeVal, |

¹Severson et al. 1985a; ²Leffingwell 1999; ³Severson et al. 1991b; ⁴Matsuzaki et al. 1992; ⁵Chortyk et al. 1993; ⁶Matsuzaki et al. 1989;; ⁷This work; ^aMeHept includes both - 5- and 6- MeHept isomers; ^bMeBut includes both 2- and 3- MetBut; ^cMeHex includes both 4-and 5-MeHex

Table 2 AS acyl group compositions of two commercial tobacco cultivars and their progenitor species. The data in each column represent average of three individually analyzed plants. The underlined acyl groups are the most abundant. The other acyl groups are expressed as mol% if total.

| Acyl group | <i>N. sylvestris</i> ¹ ♀ | <i>N. tomento- siformis</i> ♂ | <i>N. t.</i> Samsun nn (oriental type) | <i>N. t.</i> K326 (flue-cured) |
|---------------|-------------------------------------|-----------------------------------|---|-----------------------------------|
| mol% of total | | | | |
| Ac | <u>64.3</u> | 25.0 | 30.9 | <u>36.5</u> |
| MePro | - ^a | 1.0 | 9.0 | 6.6 |
| But | 6.6 | 1.9 | 4.5 | 2.0 |
| 2-MeBut | 4.8 | 5.9 | 11.2 | 24.6 |
| 3-MeBut | 1.0 | 3.4 | 10.5 | 19.2 |
| 3-MeVal | 1.9 | <u>61.3</u> | <u>33.9</u> | 2.6 |
| 4-MeVal | 1.0 | 1.3 | tr ^b | 1.2 |
| 4-MeHex | 15.6 | - | - | 2.8 |
| 5-MeHex | 4.9 | - | - | 1.3 |
| 5-MeHept | - | - | - | 1.5 |
| 6-MeHept | - | - | - | 1.6 |

^a – Not observed, ^btr – Trace amount ¹ The data is from Kroumova et al. 2016

Table 3 Comparison of the major AS acyl groups in two interspecific hybrids and the parental species. The data of the parental species represent average from three plants. Three individual F1 plants are shown for each hybrid. Underlined acyl groups are the most abundant. The numbers represent mol% of total.

| Acyl group | <i>N.t.</i> Samsun-nn | <i>N. benth.</i> | <u>H110</u> | | | <u>H114</u> | | |
|---------------|-----------------------|------------------|-------------|-------------|-------------|-------------|-----------------|-------------|
| | ♀ | ♂ | 1 | 2 | 3 | 1 | 2 | 3 |
| mol% of total | | | | | | | | |
| Ac | 32.4 | 16.2 | 0.4 | - | 1.3 | - | - | - |
| MePro | 9.4 | 7.7 | 0.8 | - | 1.6 | 0.9 | - | - |
| 2-MeBut | 11.7 | 0.6 | 0.3 | - | 0.5 | 0.5 | tr ^b | tr |
| 3-MeBut | 11.0 | 0.2 | 0.6 | - | 1.2 | 1.2 | 0.6 | tr |
| 3-MeVal | <u>35.5</u> | 1.1 | 2.2 | - | 2.4 | 4.0 | 1.5 | 1.3 |
| 4-MeHex | - ^a | 1.8 | 2.8 | 1.9 | 2.7 | 2.5 | 2.2 | 1.8 |
| 5-MeHex | - | 4.5 | 6.5 | 5.6 | 6.3 | 8.1 | 8.7 | 6.8 |
| 5-MeHept | - | 31.4 | 30.6 | 27.7 | 29.2 | 25.1 | 23.8 | 22.7 |
| 6-MeHept | - | <u>36.6</u> | <u>55.6</u> | <u>64.7</u> | <u>54.9</u> | <u>57.6</u> | <u>63.2</u> | <u>67.4</u> |

^a - Not observed; ^b - Traces

Table 4 AS acyl composition of Hybrids 75 and 87 and their parental species. The data for the dark tobacco are averaged from two plants. The data for the flue-cured tobacco are averaged from three plants. Three individual F1 plants are shown for each hybrid. Underlined acyl groups are the most abundant. The numbers represent mol% of total.

| Acyl group | <i>N.t.</i> KY171* | <i>N. otophora</i> ¹ | <u>H75</u> | | | <u>H87</u> | | | <i>N.t.</i> K326** |
|---------------|--------------------|---------------------------------|-------------|-------------|-------------|-------------|-------------|-------------|--------------------|
| | ♀ (dark type) | ♂ | 1 | 2 | 3 | 1 | 2 | 3 | ♀ (flue cured) |
| mol% of total | | | | | | | | | |
| Ac | <u>47.5</u> | | - | - | 6.7 | - | - | - | <u>36.5</u> |
| MePro | 3.8 | | 1.1 | - | 0.7 | 3.3 | - | 2.2 | 6.6 |
| But | 0.9 | | 0.5 | - | 0.3 | 0.5 | - | - | 2.0 |
| 2-MeBut | 24.3 | | 9.5 | 4.3 | 5.3 | 14.4 | 4.8 | 9.0 | 24.6 |
| 3-MeBut | 15.0 | | 11.8 | 6.4 | 7.4 | 12.5 | 4.9 | 8.0 | 19.2 |
| 3-MeVal | 5.8 | + ^b | <u>70.9</u> | <u>67.2</u> | <u>67.5</u> | <u>58.9</u> | <u>82.1</u> | <u>44.6</u> | 2.6 |
| 4-MeVal | - ^c | | 2.8 | 2.6 | 3.4 | 2.0 | 2.5 | 1.6 | 1.2 ^a |
| 4-MeHex | 1.4 ^a | +++ ^b | 0.8 | 1.0 | 0.9 | 0.9 | 1.0 | 1.7 | 1.3 ^a |
| 5-MeHex | 0.5 ^a | ++ ^b | - | 1.4 | 1.1 | 0.8 | - | 2.5 | 2.8 ^a |
| 5-MeHept | - | | 0.9 | 4.7 | 4.0 | 2.4 | 1.8 | 7.8 | 1.6 ^a |
| 6-MeHept | - | | 1.6 | 12.4 | 2.7 | 4.4 | 3.0 | 22.6 | 1.5 ^a |

* *N.t.* KY171 is not the exact maternal parent for H75, but it is representative of dark type tobaccos; ** *N.t.* K326 is not the exact maternal parent for H87, but it is representative of flue-cured tobaccos. ^a Peak abundance is low and may be missed if small samples are taken for GC analysis; ^c -Not detected ; ^{1, b} The data is from Severson et al. (1991) where acyl groups are not quantified but the order of abundance is given.

Table 5 AS acyl compositions of *N. excelsior* interspecific hybrids and their parental species. The data for the parental species are averaged from three plants. Three individual H37 F1 plants are shown. The underlined acyl groups are the most abundant. The numbers represent mol% of total.

| Acyl group | <i>N. excelsior</i> ♀ | | <i>N. bigelovii</i> ¹ ♂ | Hybrid 37 | | | <i>N. benthamiana</i> ♂ | <i>N. excelsiana</i> * |
|---------------|-----------------------|-------------|---------------------------------------|-------------|-------------|-------------|----------------------------|------------------------|
| | 11-S-19-1 | 90-S-19-2 | | 1 | 2 | 3 | | |
| mol% of total | | | | | | | | |
| Ac | 1.3 | 8.6 | 17.0 | 9.6 | 6.5 | 1.5 | 16.0 | - |
| MePro | 1.6 | 2.2 | 9.6 | 3.8 | 4.6 | 3.4 | 7.6 | - |
| But | 1.0 | 1.3 | 0.1 | 1.1 | 3.1 | 2.2 | 0.1 | - |
| 2-MeBut | 7.3 | 13.6 | 19.5 | 1.6 | 7.6 | 4.3 | 0.6 | - |
| 3-MeBut | 0.4 | 1.7 | 5.4 | 2.1 | 6.0 | + | 0.2 | - |
| 3-MeVal | 0.7 | - | <u>45.9</u> | 5.1 | <u>20.5</u> | 5.2 | 1.1 | - |
| 4-MeVal | - ^a | - | - | 1.4 | 1.6 | 2.1 | 0.9 | - |
| 4-MeHex | tr ^b | tr | 0.1 | 4.0 | 7.7 | 10.0 | 1.8 | - |
| 5-MeHex | tr | tr | 0.3 | 7.1 | 8.0 | 11.8 | 4.4 | - |
| 5-MeHept | 31.3 | 15.9 | 0.6 | 21.8 | 19.8 | <u>30.8</u> | 31.1 | 36.9 |
| 6-MeHept | 3.7 | 1.2 | 1.6 | <u>42.5</u> | 14.5 | 28.6 | <u>36.2</u> | <u>63.1</u> |
| Hept | 14.5 | 6.8 | - | - | - | - | - | - |
| Oct | <u>38.2</u> | <u>48.7</u> | - | - | - | - | - | - |

^a - Not observed; ^b tr-traces; -; *The data was generated from pooled leaf extract from several plants; ¹The data is from Matsuzaki et al. (1989)

Table 6 AS acyl composition of *Alatae* hybrids and their parental species. The data for *N. alata* and *N. × sanderae* are averaged from three plants. Three individual plants from two representative hybrids are shown. All species in the table were involved in the creation of the PO hybrids. The underlined acyl groups are the most abundant. The numbers represent mol% of total.

| Acyl group | <i>N. alata</i> ¹ ♀ | <i>N. forgetiana</i> ² ♂ | <i>N. × sanderae</i> ¹ | <u>PO67*</u> | | | <u>PO72**</u> | | | <i>N. langsdorfii</i> ³ |
|---------------|-----------------------------------|--|-----------------------------------|--------------|-----|-----|---------------|------|------|------------------------------------|
| | | | | 1 | 2 | 3 | 1 | 2 | 3 | |
| mol% of total | | | | | | | | | | |
| Ac | <u>23.9</u> | | 21.5 | - | - | - | - | - | - | |
| MePro | 14.3 | | 3.8 | 4.2 | 3.4 | 5.0 | 6.6 | 7.1 | 5.7 | + |
| But | 7.4 | | 3.6 | - | - | - | 4.0 | 4.8 | 4.2 | |
| 2-MeBut | 20.4 | <u>+++</u> ^b | 30.2 | 4.3 | 5.5 | 4.9 | 23.2 | 35.9 | 23.1 | + |
| 3-MeBut | 6.9 | | 0.9 | 3.7 | 3.4 | - | 4.2 | 2.4 | - | |
| 3-MeVal | 3.9 | | 1.9 | - | - | - | - | - | - | |
| Hex | 6.2 | | 3.5 | - | 5.0 | - | 6.8 | 5.4 | 5.7 | |

| | | | | | | | | | | |
|---------|----------------|---|-------------|-------------|-------------|-------------|-------------|-------------|------------------------|-----|
| 4-MeHex | - ^a | + | - | - | - | - | - | - | - | - |
| 5-MeHex | - | + | - | - | - | - | - | - | - | - |
| Hept | - | | - | - | 4.7 | 6.7 | 5.8 | 2.9 | 3.6 | |
| Oct | 16.8 | | <u>34.7</u> | <u>87.8</u> | <u>73.0</u> | <u>83.4</u> | <u>44.0</u> | <u>38.9</u> | <u>54.</u> <u>4</u> | +++ |
| Non | - | | - | 4.2 | 5.0 | - | 5.2 | 2.6 | 3.3 | |

^a - not observed; ^b + -not quantified, but 2-MeBut in *N. forgetiana* and Oct in *N. langsdorfii* are designated as major groups; *SE acyl composition of PO67 is similar to that of POs 15, 69, 70, 81, 101, and 120. **SE acyl composition of PO72 is similar to that of POs 5, 33, 71, 80, 94, and 96; ¹ Kroumova et al. 2016; ^{2,b} Severson et al. 1991; ^{3,b} Cutler et al. 1992

Table 7 AS acyl composition of horticulture hybrids “Thinkerbell”, “Baby Bella” and “Whisper”. *N. alata* and *N. × sanderae* are some of the parental species (on the basis of the commercial providers’ information <https://www.gardenersworld.com/plants/nicotiana-alata-tinkerbell/>; <http://www.robsplants.com/plants/NicotSande>). The data for *N. alata* and *N. × sanderae* are averaged from three plants. Individual F1 plants from each hybrid are shown. The underlined acyl groups are the most abundant. The numbers represent mol% of total.

| Acyl group | <i>N. alata</i> ¹ | <i>N. × sanderae</i> ¹ | <u>"Thinkerbell"</u> | | | <u>"Baby Bella"</u> | | | <u>"Whisper"</u> | | |
|---------------|------------------------------|-----------------------------------|----------------------|-------------|-------------|---------------------|-------------|-------------|------------------|-------------|-------------|
| | * | * | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| mol% of total | | | | | | | | | | | |
| Ac | <u>23.9</u> | 21.5 | 32.0 | - | 13.0 | 21.5 | - | 4.4 | 3.3 | 3.0 | 1.4 |
| Pro | - | - | 5.4 | - | 3.7 | 5.3 | - | - | - | - | - |
| MePro | 14.3 | 3.8 | 13.3 | 3.1 | 12.2 | 13.8 | 0.5 | 4.7 | 6.3 | 5.5 | 1.5 |
| But | 7.4 | 3.6 | 0.9 | 1.5 | 2.0 | 9.7 | 1.1 | 4.4 | 3.7 | 2.5 | 1.0 |
| 2-MeBut | 20.4 | 30.2 | <u>32.9</u> | <u>38.4</u> | <u>37.3</u> | - | 14.7 | 22.0 | <u>23.8</u> | 20.8 | 21.3 |
| 3-MeBut | 6.9 | 0.9 | 2.7 | 2.3 | 3.2 | - | 1.3 | 2.6 | 3.8 | 3.5 | 3.2 |
| 3-MeVal | 3.9 | 1.9 | 1.2 | 3.6 | 4.8 | 6.8 | 0.7 | - | 6.2 | 4.6 | 2.1 |
| 4-MeVal | - | - | tr | 1.2 | tr | 0.7 | 0.8 | - | 3.0 | 2.2 | 0.6 |
| Hex | 6.2 | 3.5 | 0.8 | 2.6 | 1.5 | 4.6 | 6.3 | 3.1 | 3.5 | 2.9 | 3.0 |
| 4-MeHex | - | - | 0.5 | - | 0.3 | - | - | - | 5.6 | 3.4 | 1.0 |
| 5-MeHex | - | - | 0.4 | - | 0.3 | - | - | - | - | - | 0.8 |
| Hept | - | - | 0.3 | 2.0 | 0.7 | 1.7 | 4.5 | 1.9 | 4.1 | 2.7 | 1.7 |
| 5-MeHept | - | - | - | - | - | - | 0.4 | - | 4.2 | 2.4 | 1.5 |
| 6-MeHept | - | - | - | - | - | - | 0.5 | - | 3.5 | 2.7 | 1.5 |
| Oct | 16.8 | <u>34.7</u> | 7.2 | 34.5 | 16.3 | <u>31.2</u> | <u>57.9</u> | <u>37.8</u> | 12.7 | <u>30.8</u> | <u>45.1</u> |
| Non | - | - | 1.0 | 5.7 | 2.2 | 3.5 | 9.6 | 5.0 | 4.2 | 3.8 | 5.0 |
| Dec | - | - | 1.1 | 4.0 | 2.1 | 0.9 | 1.3 | 13.1 | 8.0 | 5.8 | 7.1 |
| Laur | - | - | 0.3 | 1.0 | 0.4 | 0.3 | 0.4 | 0.9 | 3.9 | 3.3 | 2.9 |

^a - Not observed; ¹ Kroumova et al. 2016

Figure legends

Fig. 1. Phenotypes of two *N. tabacum* cv. Samsun-nn \times *N. benthamiana* F1 hybrids, of two *N. tabacum* \times *N. otophora* F1 hybrids, and of *N. excelsior* \times *N. bigelovii* F1 hybrid.

Electronic supplementary materials

Title:

Patterns of inheritance of acylsugar acyl groups in selected interspecific hybrids of genus *Nicotiana*

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Journal:

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Content:

Figs. S1-S6

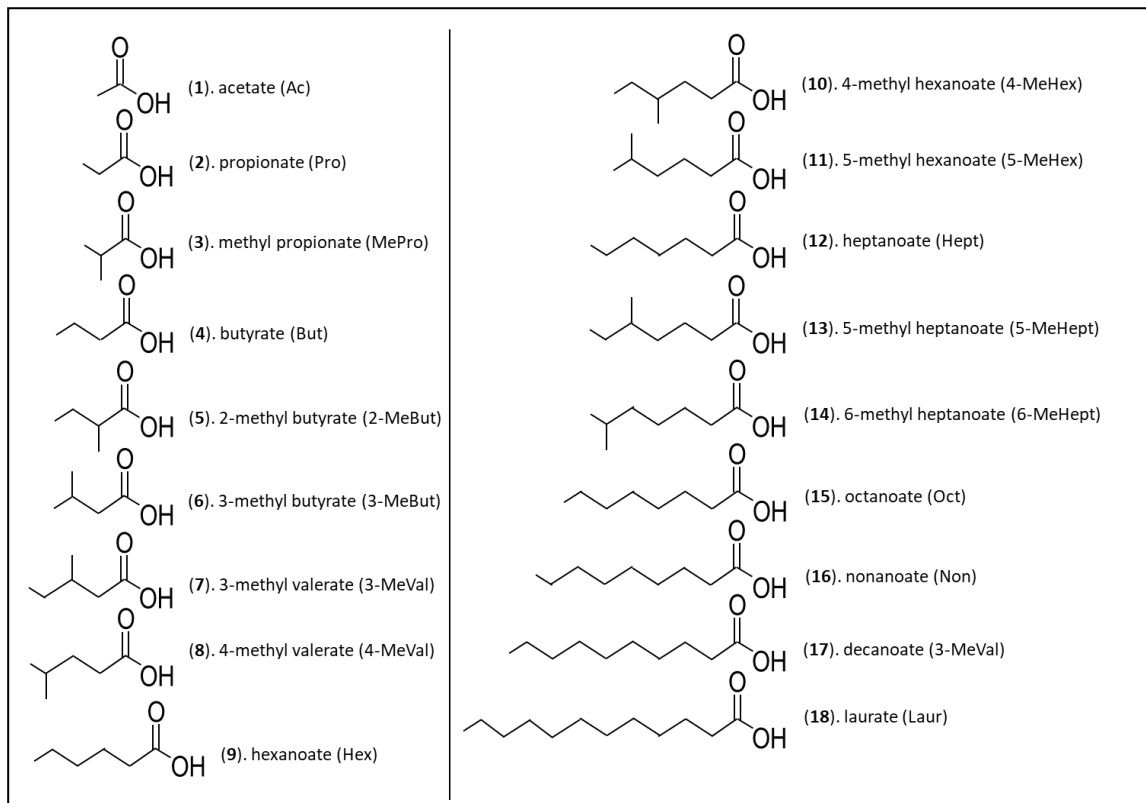


Figure S1 Linear structure of all acyl groups found.

An abbreviation for each group is given in parenthesis. The figure is modified from Kroumova et al. (2016).

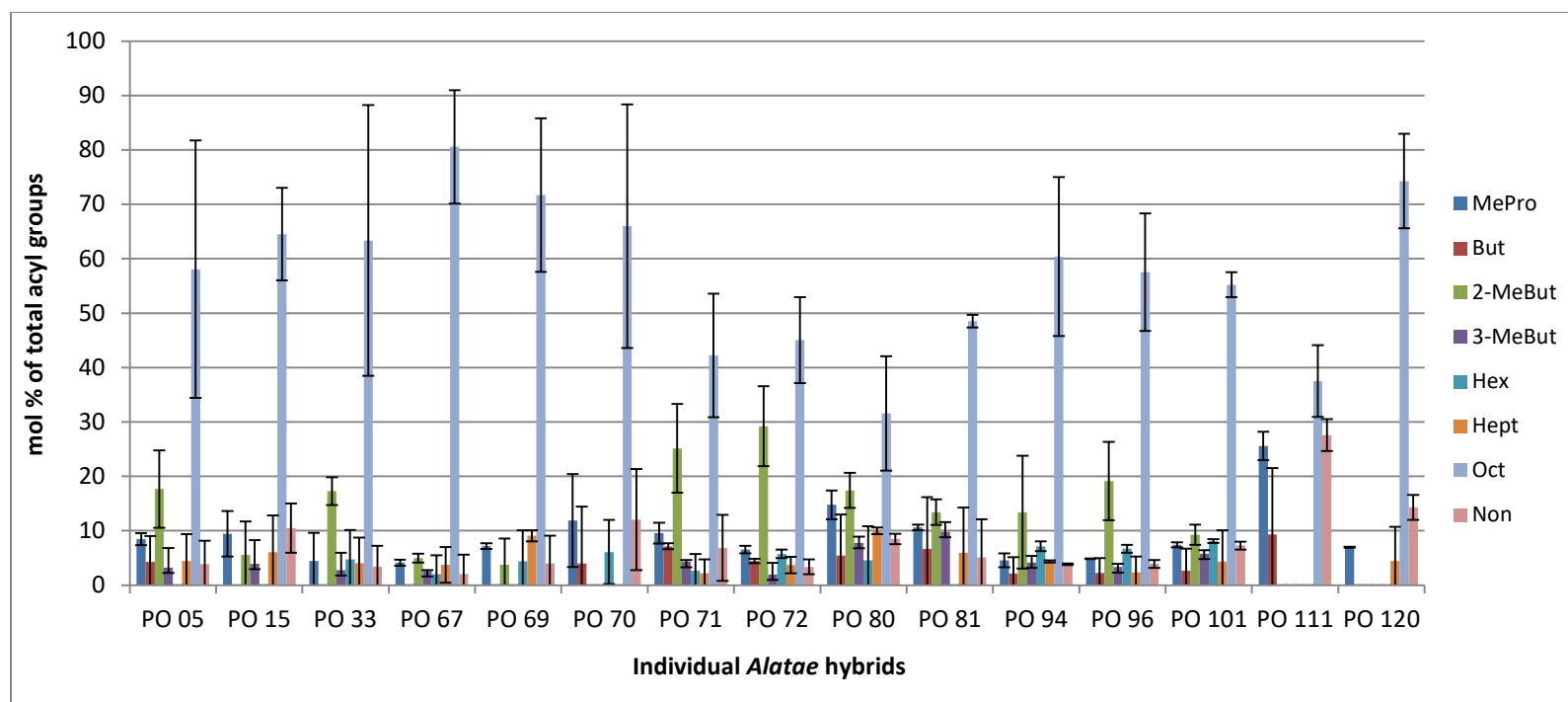


Figure S2 AS acyl group composition of *Alatae* hybrids

Peak areas of acyl butyrates were converted to mols. The values are expressed as mol% of total. Three separate plants from each hybrid line were analyzed. The columns present the average value, and the error bars- standard deviation.

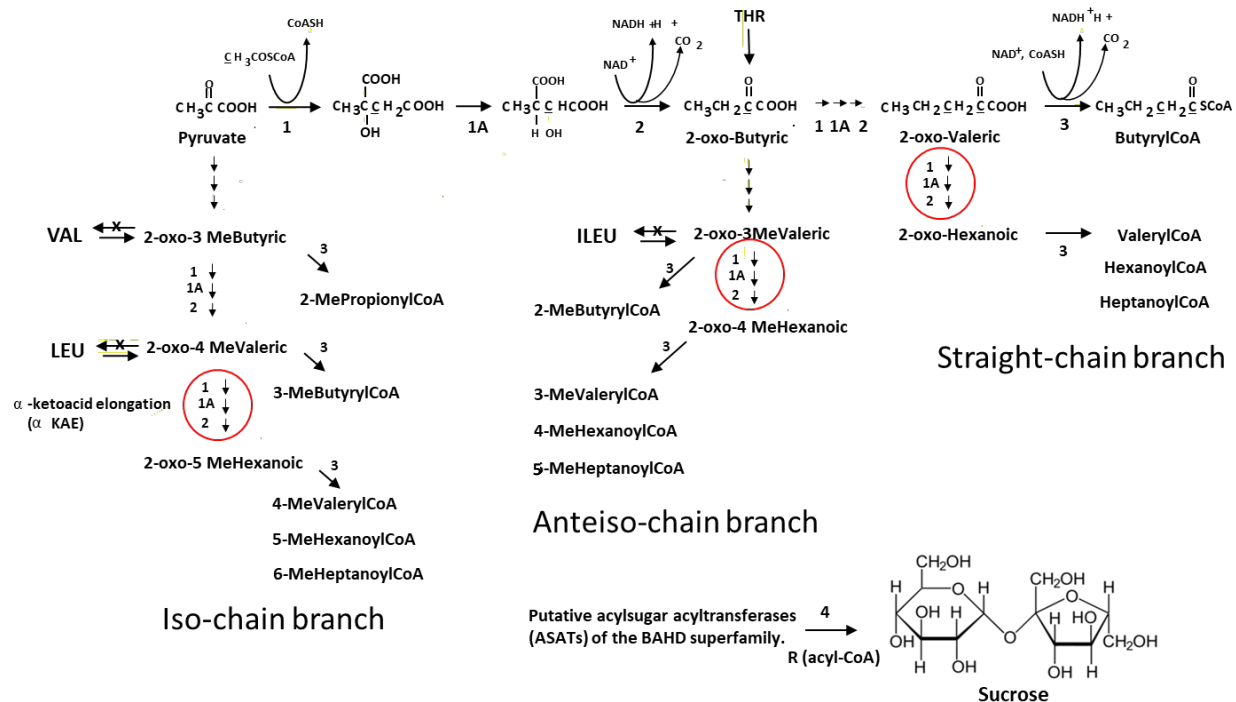


Figure S3 Suggested pathways for synthesis of acyl-CoA groups

This figure is modified from Kroumova et al. (1994). Structurally, acyl groups possess either iso-branched carbon chain, anteiso-branched carbon chain or straight carbon chain. Acyl groups with iso-chain carbon skeleton come from modified valine (Val) and leucine (LEU) metabolism/catabolism; acyl groups with anteiso-chain carbon skeleton come from modified isoleucine (ILEU) metabolism/catabolism, and acyl groups with straight-chain carbon skeleton come from pyruvate- or threonine (THR)-derived 2-oxo-butyrate (Kandra et al. 1990). Synthesis of the acyl groups shown is proved to occur via one-carbon chain elongation called alpha-ketoacid elongation (α-KAE –circled in red)(Kroumova et al., 1994) in *N. benthamiana*, *N. glutinosa*, *N. gossii* and petunia (Kroumova et al. 1994, 2009). Enzymes catalyzing reaction 1, 1A, 2 and 3 are assumed to be **1**: 2-isopropylmalate synthase (IPMS) (condensation), **1A**: 3-isopropylmalate dehydratase (isomerization), **2**: 3-isopropylmalate dehydrogenase (oxidative decarboxylation), **3**: 2-oxo-isovalerate dehydrogenase (oxidative decarboxylation) or branched-chain ketoacid dehydrogenase (BCKD) (Kandra et al, 1990; Mooney et al., 2002). **4**: In genus *Solanum* and in petunia the assembly of acyl sugars was shown to involve numerous acyltransferases (reaction 4) that attach the acyl-CoAs to the sucrose or glucose moieties (Moghe et al, 2017, Nadakuduti et al., 2017, references are given in the manuscript), but orthologous acyltransferase genes have not been reported for *Nicotiana* species. In *Nicotiana* species enzymes catalyzing acyl sugar assembly (reaction 4) are assumed to be also acyltransferases.

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