# Generation and Characterization of Tomato Lines with Modified Acylsugar and Fatty Acid Profiles

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John Reid Smeda

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# Generation and Characterization of Tomato Lines with Modified Acylsugar and Fatty Acid Profiles

John Reid Smeda PhD

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Acylsugars are accumulated by several wild relatives of tomato and are implicated in mediating a variety of defensive plant-insect interactions. Acylsugars are produced at high levels by glandular trichomes of *Solanum pennellii* and the ability to produce acylsucrose acylsugars at elevated levels was previously bred into tomato using *S. pennellii* (Correll) D'Arcy accession LA716, leading to the creation of the benchmark line CU071026, which exhibits an acylsugar chemotype profile distinct from that of *S. pennellii* LA716.

Four QTL previously associated with changes in fatty acid profile (FA2, FA5, FA7, and FA8), were individually combined with the five introgressions of CU071026. Characterization of the resulting lines indicated alterations to acylsugar level and acylsugar chemotype by modulating the length, orientation and/or relative proportion of fatty acid acyl groups, leading to changes in overall acylsugar composition.

The combinatory effect of three of the QTL (FA2, FA7, and FA8) on acylsugar level and chemotype was evaluated by combining these QTL. Characterization of the resulting lines demonstrated that the fatty acid QTL interacted additively and epistatically to alter acylsugar level and chemistry, increasing the diversity of acylsugars accumulated. The acylsugar level and chemotype traits of the lines generally displayed high heritability and minimal environmental interaction.

Three acylglucose QTL (AG3, AG4, AG11), previously associated with control of acylsugar moiety, were utilized to generate lines with acylglucose accumulation. Two approaches were taken to generate these lines and characterization demonstrated that epistatic interactions among the three acylglucose QTL controlled acylsugar level and acylglucose

accumulation and that the three QTL can result in moderate levels of acylglucoses, but that an additional QTL might be needed for high acylglucose accumulation. Additionally, characterization of the acylsugar chemistry revealed impact of the acylglucose QTL on the composition of accumulated acylsucroses, and potential impact on the fatty acid composition.

The knowledge and the germplasm from this dissertation will support the generation of high acylsugar-accumulating tomato lines and hybrids with increased acylsugar profile diversity. This germplasm can also contribute to elucidation of the mechanism of insect resistance mediated by acylsugars, and assist with identification of yet-unknown acylsugar synthesis genes.

## BIOGRAPHICAL SKETCH

John Reid Smeda was born in Lafayette Indiana and raised in Leland Mississippi and Columbia Missouri. His parents, Reid and Corinne, played a large role in his education and John was homeschooled until 8th grade, particularly by his mother, Corinne, who was an elementary school teacher. John's parents continued their involvement in his education by helping start a private Christian school, Heritage Academy, in Columbia MO, and both taught classes there. John's father was also a professor at the University of Missouri in Weed Science, and played a large role in stimulating John's interest in agriculture from a young age. Reid managed a large vegetable and herb garden in both MS and MO, and John and his two sisters, Rachel and Robin, helped maintain the garden. Later when John was older he took a larger role in the garden, including picking and selling produce to local stores and restaurants. In particular, John enjoyed working with the various vegetable crops, such as tomatoes, peppers, eggplant and watermelons. During his childhood in Missouri, John also developed a fascination with insects, many of which he collected from around the 42 acres they lived on and raised them. Swallowtail butterflies and giant silk moths were some of his favorite pets.

In 2007 John received scholarships to attend the University of Missouri and study Plant Sciences. During his time at Missouri, John worked in the campus greenhouses as a pest scouter and pesticide applicator. During this time John began to dream of opportunities to combine his passion for plants and insects. In his Junior and Senior years John worked in a nematology lab with Dr. Melissa Mitchum and her PhD student Amy Replogle, and used molecular breeding approaches to generate and evaluate the efficacy of Arabidopsis receptor mutants to inhibit beet cyst nematode infection as a proxy for understanding how to generate resistance to the soybean cyst nematode in soybean. After 4 years of study he graduated summa cum laude with a Bachelor of Science degree in Plant Sciences with an emphasis in Plant Breeding. John's desire to pursue a graduate degree in Plant Breeding was cemented by

his interactions with Dr. Anne McKendry, who taught several plant breeding classes and provided guidance in applying for the NSF GRFP fellowship.

John received an NSF GRFP in Spring of 2011 and chose Cornell over NC State University to study plant breeding because he was thrilled about an opportunity to pursue a plant breeding project that involved entomology. John desired to utilize plant breeding and genetics to improve the resistance of vegetable crops, like tomato to insect pests and diseases and viruses they vectored. Additionally, John wanted to utilize extensive collaboration to help elucidate how the insects interacted with the tomato plants and functionally how to generate durable insect resistance. John hopes to generate varieties of tomatoes that are resistant to insects and viruses to diminish pesticide use and that can be utilized in the context of integrated pest management by smallholder farmers in developing countries where pesticide usage is cost-prohibitive.

To my parents Reid and Corinne

And to my beautiful wife, Karen. I could not have done this without you.

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## LIST OF ABBREVIATIONS

ai-C5: 2-Methylbutanoate (anteiso branched 5-carbon acyl group)

i-C4: 2-Methylpropanoate (iso branched 4-carbon acyl group)

i-C5: 3-Methylbutanoate (iso branched 5-carbon acyl group)

i-C9: likely 7-Methyloctanoate (iso branched 9-carbon acyl group)

i-C10: 8-Methylnonanoate (iso branched 10-carbon acyl group)

i-C11: 9-Methyldecanoate (iso branched 11-carbon acyl group)

i-C12: likely 10-Methylundecanoate (iso branched 12-carbon acyl group)

i-C13: likely 11-Methyldodecanoate (iso branched 13-carbon acyl group)

i-C14: likely 12-methyltridecanoate (iso branched 14-carbon acyl group)

n-C10: n-Decanoate (straight chain 10-carbon acyl group)

n-C12: n-Dodecanoate (straight chain 12-carbon acyl group)

# CHAPTER 1

## Introgression of Acylsugar Chemistry QTL Modifies the Composition and Structure of Acylsugars Produced by High-accumulating Tomato Lines


## ABSTRACT

Acylsugars are important insect defense compounds produced at high levels by glandular trichomes of the wild tomato, *Solanum pennellii*. The ability to produce acylsugars at elevated levels was bred into the tomato line CU071026. This study utilized a marker-assisted backcross approach to individually introgress into CU071026 and to fine map the three quantitative trait loci (QTL) FA5QTL, FA7QTL, and FA8QTL, which were previously associated with changes in acylsugar chemistry. Additional breeding with, and fine-mapping, the previously introgressed QTL FA2QTL was also conducted. The effect of these four QTL on acylsugar quality and quantity in the presence of the five introgressions of CU071026 was evaluated. Incorporation of the QTL altered acylsugar chemotype by modulating the length, orientation and/or relative proportion of fatty acid acyl groups. The resulting quantities of acylsugar produced in most of the new lines were similar to that of CU071026; however, introgression of FA5QTL reduced acylsugar levels. The acylsugar lines containing each QTL were characterized for acylsugar level, trichome abundance, and acylsugar chemistry through gas chromatography/mass spectrometry and liquid chromatography/mass spectrometry. The novel acylsugar chemotype lines created can contribute to elucidation of the mechanism of insect resistance mediated by acylsugars, and help with identification of yet-unknown genes contributing to acylsugar synthesis and diversity.

## INTRODUCTION

Human population growth, dietary needs, and a more variable climate demand increased, sustainable food production. Phytophagous insects and the viruses they vector are among the most significant challenges to maintaining and increasing crop quality and yield. Plants have evolved myriad ways to combat threats from herbivores and pathogens, including the production of specialized metabolites that facilitate mediation of physiological stresses and also function as defenses. Breeding for metabolites that contribute to plant defense has not been a major focus of plant breeders since use of synthetic pesticides provided cheap and relatively effective control of insects. However, increased awareness of economic and environmental costs of chemical control, and the desire to support sustainable agriculture, demand novel plant breeding strategies including utilization of natural defensive traits to control plant pests. The tremendous diversity of secondary metabolites implies ecological functions in stress mediation (Leckie et al. 2016), and provides opportunities and valuable tools to employ plant chemistry to improve host-plant resistance and advance food security (Dixon 2001; Schilmiller et al. 2008; Wink 2010).

One promising class of secondary metabolites receiving increased attention is a family of sugar polyesters known as acylsugars. Acylsugars are accumulated by numerous species in the nightshade family (Solanaceae), such as wild potato (*Solanum berthaultii*), tobacco (*Nicotiana tabacum*), Petunia (*Petunia hybrida*), and several wild relatives of tomato, such as *Solanum pennellii*, *S. galapagense,* and *S. habrochaites* (Fobes et al. 1985; Burke et al. 1987; King et al. 1986, 1988; King and Calhoun 1988; Ohya et al. 1996; Kim et al. 2012; Schilmiller et al. 2015). Acylsugars are implicated in mediating a variety of plant-insect interactions, including feeding deterrence and oviposition preference (Severson et al. 1985, Goffreda and Mutschler 1989; Hawthorne et al. 1992; Rodriguez et al. 1993; Juvik et al. 1994, Liedl et al. 1995, Fancelli et al. 2005; Leckie et al. 2016). In *Nicotiana attenuata* acylsugars play a role in indirect defense against *Manduca sexta* by serving as a volatile attractant to a ground hunting ant,

*Pogonomyrmex rugosus*, after undergoing hydrolysis in the caterpillar midgut (Weinhold and Baldwin 2011). In tomato species, the acylsugars produced and secreted from glandular trichomes are composed of a sugar backbone (sucrose or glucose) to which several short to medium chain aliphatic acids are esterified. These fatty acids can be either straight-chained or branched (Fobes et al. 1985; Burke et al. 1987; Shapiro et al. 1994; Schilmiller et al. 2010, 2012, 2016; Fan et al. 2016).

*Solanum pennellii* (Correll) D'Arcy accession LA716 accumulates substantial amounts of acylsugars which have been shown to effectively control many insects and provides a promising source of direct insect resistance (Goffreda and Mutschler 1989; Hawthorne et al. 1992; Rodriguez et al. 1993; Juvik et al. 1994; Shapiro et al. 1994; Liedl et al. 1995 that can be transferred to tomato (Mutschler and Wintermantel 2006, Leckie et al. 2012). Efforts to transfer acylsugar production from *S. pennellii* LA716 into tomato led to the creation of the Cornell benchmark line, CU071026, which accumulates *ca.* 15% the level of acylsugar of *S. pennellii* LA716 (Leckie et al. 2012). CU071026 contains five introgressions from LA716 on chromosomes 2, 3, 7 and 10 (called AS2, AS3, AS7, AS10.1, and AS10.2, respectively; see Supplementary Table S1 of Leckie et al. 2012 for markers and map positions of the *S. pennellii* LA716 introgressions in CU071026). Although the level of acylsugars accumulated by CU071026 and its sister lines is lower than the extremely high levels of *S. pennellii* LA716, the level of acylsugars produced by these tomato lines is sufficient to significantly reduce *Bemisia tabaci* oviposition on lines grown in field cages (Leckie et al. 2012) and acylsugar producing hybrids also reduced the incidence of tomato infectious chlorosis virus in fields with heavy pressure from the whitefly *Trialeurodes vaporariorum* (Mutschler and Wintermantel 2006).

*Solanum pennellii* accessions have various acylsugar chemotypes that vary with geographical location (Shapiro et al. 1994; Ning et al. 2015), which suggests the possibility of adaptation and selection of specific metabolic profiles in response to local herbivore pressures. The acylsugars of *S. pennellii* LA716 are predominantly acylglucoses with a characteristic array

of fatty acids including 2-Methylpropanoate (i-C4), 2-Methylbutanoate (ai-C5), 3-Methylbutanoate (i-C5), 8-Methylnonanoate (i-C10), n-Decanoate (n-C10), and n-Dodecanoate (n-C12) (Burke et al. 1987; Shapiro et al. 1994; Blauth et al. 1999). In contrast, the profile of CU071026, which was bred using *S. pennellii* LA716, is exclusively acylsucroses with predominantly ai-C5, i-C5 and n-C12 fatty acids, and only trace or undetectable levels of i-C4, i-C10 and n-C10 (Leckie et al. 2014). The fatty acid profile of CU071026 is similar to that of cultivated tomato, which predominantly accumulates i-C5, ai-C5 and n-C12 fatty acids as well (Schilmiller et al. 2010 and Ghosh et al. 2014). Work with purified acylsugars from CU071026 and several *S. pennellii* accessions, including *S. pennellii* LA716, indicates that purified acylsugars of CU071026 are less effective at equimolar levels than purified acylsugars of some *S. pennellii* accessions at controlling whitefly (*Bemisia tabaci*) and western flower thrips (*Frankliniella occidentalis*) feeding and oviposition in laboratory assays (Leckie et al. 2016), suggesting that insect control of CU071026 or derived lines could be improved by altering their acylsugar chemotypes. QTL that affect acylsugar chemistry have been identified (Blauth et al. 1998, 1999; Schilmiller et al. 2010, 2012, 2015; Leckie et al. 2013, 2014; Fan et al. 2016) and shown to alter the chemotype of acylsugars accumulated in tomato lines (Schilmiller et al. 2010) such as the mono-introgression lines (ILs) created by Eshed and Zamir (1994, 1995). Addition of QTL that alter acylsugar chemotype into CU071026 could provide a means of generating acylsugars with stronger or broader insect resistance than that of CU071026.

The objectives of this study were to individually introgress several previously identified acylsugar chemotype QTL into CU071026 to create a set of tomato sister lines and extensively characterize these lines for alterations in the acylsugars accumulated through gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS). The ILs were identified as an optimum source of the desired *S. pennellii* LA716 acylsugar chemotype QTL for transfer to CU070126. Three QTL: fatty acid 5 (FA5QTL) and fatty acid 7 (FA7QTL) (identified in Leckie et al. 2014), and fatty acid 8 (FA8QTL) (identified in

Blauth et al. 1999; Schilmiller et al. 2010; and Leckie et al. 2014), previously shown to alter acylsugar chemistry, were introgressed into CU071026 to test the effect of these QTL in an acylsugar-producing tomato line, both to confirm function, and to create tomato lines for testing against insects. Identification of plants with recombinations within these introgressions during transfer allowed fine mapping of the QTL within them. An additional QTL, fatty acid 2, (FA2QTL), on chromosome 2, was previously introgressed into CU071026 (Leckie et al. 2014); additional selection was performed to create a FA2 acylsugar line (FA2/AS). The acylsugars accumulated by the altered acylsugar chemotype lines were characterized by acylsugar assay, which measures acylsugar level, by GC-MS analysis, which determines relative proportions of the fatty acids present, and by LC-MS characterization, which determines the relative proportions of acylsugar molecules accumulated with information concerning the number and length of fatty acids esterified to the sugar backbone. The implications of these data are discussed, including whether adding the acylsugar chemotype QTL affects levels of acylsugars accumulated, leads to greater diversity in the fatty acids esterified to the sugar molecules, and/or also in changes in the specific acylsugars accumulated.

## MATERIALS AND METHODS

### Plant materials

CU071026 is an acylsugar-producing tomato line bred using *S. pennellii* LA716 by the Cornell University tomato breeding program, which is the source of the seed used. Seeds from *S. pennellii* LA716 were produced by the Cornell University tomato breeding program, derived from seed originally obtained from the Tomato Genetics Resource Center (TGRC) at the University of California at Davis. The modified acylsugar tomato line FA2/AS was developed from CU071026 by the Cornell University tomato breeding program, which is the source of FA2/AS seed.

A series of tomato lines with individual introgressions of *S. pennellii* LA716 DNA in the processing tomato M82 (a sub-selection of UC82-B) were produced by Eshed and Zamir (1994, 1995). Based on prior QTL analysis the introgression line IL2-4 was used by the Cornell University tomato breeding program as the source of FA2QTL (Leckie et al. 2014). Similarly, we chose the introgression lines IL5-3, IL7-4-1 and IL8-1-1 to use as sources of the FA5, FA7 and FA8 QTLs for transfer. The seed of IL5-3, IL7-4-1 and IL8-1-1 were produced at Cornell University, derived from seed obtained from D. Zamir (Hebrew University of Jerusalem, Rehovot, Israel). Seed of M82 was produced by the Cornell University tomato breeding program, derived from seed originally obtained from the Tomato Genetics Resource Center (TGRC) at the University of California at Davis.

**Plant growth conditions**

Seed were germinated in 32 cell flat cups with LM1 (Lambert, Rivière-Ouelle, Quebec, Canada) mix until *ca.* five weeks of age, during which time any necessary marker based genetic analysis could be completed. Selected plants were transplanted to eight in. clay pots of LM111 (Lambert, Rivière-Ouelle, Quebec, Canada) mixed with turface (Turface Athletics, Buffalo Grove, IL) in a 1:1.8 ratio, with 0.3% unimix (10-5-10) and calcium sulfate additive. Plants for all populations and experiments were grown in a greenhouse in the Guterman Bioclimatic Laboratory and Greenhouse Complex at Cornell University in Ithaca NY, and were typically maintained at 29°C: 20°C day night temperatures with a 16:8 hr light:dark photoperiod.

**Breeding scheme for transfer of QTL**

For transfer of each QTL an IL line was selected that putatively contained the QTL of interest. Selection of IL line was based on marker data from IL lines compared to QTL marker mapping intervals identified in Leckie et al. (2014). The selected IL line was crossed as the female parent to CU071026, and the resulting F1 plant backcrossed to CU071026 to create the BC1F1

populations (CU071026 x (IL line x CU071026). Selection of plants from the BC1F1 populations were based on markers within the five *S. pennellii* LA716 introgressions possessed by CU071026 and markers within the additional introgression being introduced into CU071026. The original introgressions of CU071026 were selected for homozygosity, using markers, to maintain the CU071026 introgressions in the new line. The markers within the new IL introgression were utilized to select for both presence of the new introgressions, and for plants with recombinations within the new introgressions, to reduce introgression sizes and fine map the new QTL within these introgressions.

**Genotypic screening**

Molecular markers utilized in all populations to select for CU071026 regions are provided in Table 1.1. Identity and location of markers used to introgress FA5QTL, FA7QTL, and FA8QTL into the presence of the five CU071026 introgressions are provided in Table 1.2.

Table 1.1 Markers and map locations delineating CU071026 introgressions based on Tomato-EXPEN SL2.50 ITAG2.4

| Marker[a] | Chromosome | Start Position (Bp) | End Position (Bp) |
|---|---|---|---|
| C2_At4g37300 | 2 | 53,834,679 | 53,835,655 |
| C2_At3g26900 | 2 | 54,947,728 | 54,950,298 |
| solcap_snp_sl_63290[b] | 3 | 1,390,271 | 1,390,304 |
| TG130 | 3 | 1,755,716 | 1,756,224 |
| C2_At5g24120 | 3 | 1,914,316 | 1,920,336 |
| C2_At3g02420 | 3 | 11,509,743 | 11,514,318 |
| C2_At5g23060 | 3 | 64,448,262 | 64,451,474 |
| C2_At3g15430 | 7 | 65,800,017 | 65,803,497 |
| TG303 | 10 | 1,773,625 | 1,774,114 |
| C2_At5g60990 | 10 | 1,853,562 | 1,864,123 |
| SSR85 | 10 | 61,580,912 | 61,581,577 |
| C2_At3g12290 | 10 | 62,141,901 | 62,147,562 |

[a] Full marker information provided by The Sol Genomics Network (SGN; http://solgenomics.net/)

[b] A cleaved amplified polymorphic sequence (CAPS) marker was designed from this SNP

Table 1.2. Markers and map locations used to introgress FAQTL based on Tomato-EXPEN SL2.50 ITAG2.4

| Marker[a] | Chromosome | Start Position (Bp) | End Position (Bp) |
|---|---|---|---|
| solcap_snp_sl_51242[b] | 5 | 16,840,699 | 16,840,733 |
| T1601 | 5 | 41,511,439 | 41,512,805 |
| solcap_snp_sl_69382[b] | 5 | 54,980,534 | 54,980,568 |
| solcap_snp_sl_29[b] | 5 | 60,842,571 | 60,842,605 |
| C2_At1g10500 | 5 | 62,107,578 | 62,107,796 |
| solcap_snp_sl_25859[b] | 5 | 62,738,979 | 62,739,013 |
| TG23 | 5 | 63,347,293 | 63,348,190 |
| C2_At2g26590 | 7 | 6,878,227 | 6,899,018 |
| C2_At4g29490 | 7 | 32,758,106 | 32,807,954 |
| solcap_snp_sl_52215[b] | 7 | 38,290,705 | 38,290,739 |
| solcap_snp_sl_70150[b] | 7 | 51,197,900 | 51,197,934 |
| solcap_snp_sl_100893[b] | 7 | 54,044,220 | 54,044,254 |
| C2_At2g30520 | 7 | 56,723,902 | 56,725,342 |
| U221657 | 8 | 3,279,725 | 3,283,085 |
| solcap_snp_sl_51919[b] | 8 | 3,781,348 | 3,781,382 |
| solcap_snp_sl_51931[b] | 8 | 4,103,545 | 4,103,579 |
| solcap_snp_sl_51969[b] | 8 | 4,744,567 | 4,744,601 |
| solcap_snp_sl_69286[b] | 8 | 5,597,495 | 5,597,529 |
| solcap_snp_sl_69336[b] | 8 | 9,125,184 | 9,125,218 |
| C2_At5g27390 | 8 | 22,850,567 | 22,857,802 |
| C2_At1g30360 | 8 | 27,897,600 | 27,905,822 |
| C2_At4g33030 | 8 | 52,633,704 | 52,637,369 |

[a] Full marker information provided by The Sol Genomics Network (SGN; http://solgenomics.net/).
[b] A cleaved amplified polymorphic sequence (CAPS) marker was designed from this SNP

**Phenotypic screening**

Acylsugar level: Levels of acylsugar for plants of the controls and of populations in the development of the FA/AS lines were measured on 9-10 weeks of age plants using the method of Leckie et al. (2012), which is a modification of the prior method described by Goffreda et al. (1990), replacing the Nelson reaction originally used to measure sugar (Goffreda et al. 1990) with a modified peroxidase/glucose oxidase assay (Setter et al. 2001) that measures glucose. For these replicated screens, four plants of each genotype were sampled, collecting four samples of two lateral leaflets from leaves that were two to three nodes from the apex of stems. Each two leaflet sample was each placed in wide mouth plastic scintillation vials and completely

dried in a seed dryer at $29^0$ C. Fully dried leaflets were rinsed with 3 ml of methanol containing methyl heptanoate (30 mg $L^{-1}$), an internal standard for fatty acid analysis. The assay uses 100ul of each rinsate. Leaflets were redried immediately after rinsing and weighed, so that acylsugar level could be expressed per weight dried leaf. Dried leaf weights ranged from about 50 to 90 mg. Acylsugar level data were analyzed using ANOVA in JMP Pro 11 (SAS Institute Inc. 2014), and means were separated by Tukey-Kramer HSD ($p < 0.05$). Prior to analysis, acylsugar level data were $Ln_{(x)}$ transformed to improve normality.

Fatty acid characterization: Percentages of each type of fatty acids from each sample were ascertained by collecting pairs of young, fully expanded primary lateral leaflets, rinsing leaflets with 3 ml of methanol containing methyl heptanoate (30 mg $L^{-1}$) as an internal standard, and then utilizing transmethylation/GC-MS analysis, as described in Leckie et al. (2014). Peak areas of the resulting chromatograms were calculated using Varian MS Workstation Version 6.9.1 (Agilent Technologies, Santa Clara, CA) and levels of respective fatty acids were determined through comparison with levels of the internal standard to generate relative proportions of each fatty acid. Percent fatty acid GC data was analyzed using ANOVA in JMP Pro 11 (SAS Institute Inc. 2014), and means separated by Tukey-Kramer HSD ($p < 0.05$). Prior to analysis, data for i-C4, n-C10 and i-C11 (9-methyldecanoate) aliphatic acids were cube root transformed and the data for ai-C5 and i-C13 (11-methyldodecanoate) were $\log_{10(x+1)}$ transformed to improve normality.

Heritability estimation: Broad sense heritability estimates were generated by using acylsugar level and fatty acid data from 2014 and 2015 over several environments. Heritability for acylsugar level and the major fatty acids was calculated according to Holland et al. (2003) using variances obtained from the lmer function in the lme4 package in R (Bates et al. 2014) where genotype, location, year, genotype by location, and genotype by year were treated as random effects.

Acylsugar composition characterization: LC-MS was utilized to analyze the composition of acylsugars accumulated in each line. Three samples of a single primary lateral leaflet per genotype were taken and extracted with a buffer consisting of isopropanol:acetonitrile:water (3:3:2 v/v/v) containing 0.1% formic acid and 10 uM of propyl-4- hydroxybenzoate, an internal standard, and processed as described in Schilmiller et al. (2015). Acylsugar results from the LC-MS analysis are described using the nomenclature of Schilmiller et al. (2010), in which the acylsugar name S4:17 indicates a sucrose backbone sugar, with four fatty acid acyl chains that have a total of 17 carbons. LC-MS data were analyzed by hierarchical clustering with a Pearson correlation using a pairwise average-linkage clustering method for both genotypes and acylsugars using the hierarchical clustering tools provided by GenePattern Reich et al. (2006).

Trichome density: Since acylsugars are secreted by glandular trichomes, the density of type IV and type VI trichomes were evaluated for each fatty acid line to provide further characterization of factors related to acylsugar production and defense. Four plants of each fatty acid line were simultaneously grown in a greenhouse and sampled at 9-10 weeks of age for trichome counts. Two young, expanding, primary lateral leaf samples were taken from each plant; two interveinal areas on the abaxial side of the leaflet from each sample were used to count trichomes. Trichomes were visualized with a Carl Zeiss 475003, 9901 microscope using 63x power. An eye piece grid was utilized to facilitate counting, and a 5x5 section of the grid, where each square measured 0.0256mm$^2$ was used to count trichomes, for a total counting area of 0.64mm$^2$. Only those trichomes whose base fell in the 5x5 grid were counted. Trichome counts were later adjusted to number of trichomes per mm$^2$ and trichome density data was analyzed using ANOVA in JMP Pro 11 (SAS Institute Inc. 2014), and means separated by Tukey-Kramer HSD ($p < 0.05$). Prior to analysis, data for density of Type IV and VI trichomes were $\log_{10(x+1)}$ transformed to improve normality.

## RESULTS AND DISCUSSION

### Introgressing FA7QTL

FA7QTL was readily transferred into CU071026 and fine-mapped due to the dominant nature of this QTL. FA7QTL was putatively localized in and introgressed from IL7-4-1, which possesses a 56.3 Mbp chromosome 7 introgression from *S. pennellii* LA716 in the M82 background (Long et al. 2013). A 380 individual (CU071026 x (CU071026 x IL7-4-1)) BC1F1 population was used to identify, through marker assisted selection (MAS), those plants that were homozygous for the majority of the five CU071026 *S. pennellii* introgressed regions, and were also heterozygous for the IL7-4-1 introgression. In addition, one recombinant BC1F1 plant (131285-176) was identified that was homozygous for the CU071026 introgressions AS3, AS10.1 and AS10.2 and heterozygous for the CU071026 introgressions AS2 and AS7, as well as heterozygous for a *ca* 3.2 Mb sub-region of the IL7-4-1 introgression.

BC1F1 plant 131285-176 and sibling plants containing the entire IL7-4-1 introgression and homozygous for AS2, accumulated acylsugar levels at least as high as that of the CU071026 control and selections homozygous for AS2 but lacking the entire IL7-4-1 introgression (data not shown). Additionally, 131285-176 and plants containing the entire IL7-4-1 region produced acylsugars with an increase in n-C10 and a decrease in n-C12 fatty acids compared to selections lacking the IL7-4-1 introgression (Figure 1.1) which is consistent with the impact of FA7QTL on acylsugar fatty acid profile as described by Leckie et al. (2014). Specifically, BC1F1 plants that did not possess the IL7-4-1 introgression averaged 1.2% n-C10 and 45.6% n-C12, while BC1F1 plants possessing the IL7-4-1 introgression accumulated on average 5.3% n-C10 and 38.0% n-C12. The GC fatty acid profile of these BC1F1 plants confirms that the full IL7-4-1 introgression contains FA7QTL, that the subintrogression in 131285-176 still possesses FA7QTL, and that FA7QTL functions in a background that contains the five introgressions of CU071026. These results also support those of Leckie et al. (2014),

which indicate that FA7QTL reduces the extension fatty acid products by two carbons. The level of acylsugars accumulated was not compared among the selections because the CU071026 introgressions (AS2 and AS3) are recessive, and were segregating, thus confounding any effect of the IL7-4-1 introgression on acylsugar level in this generation.

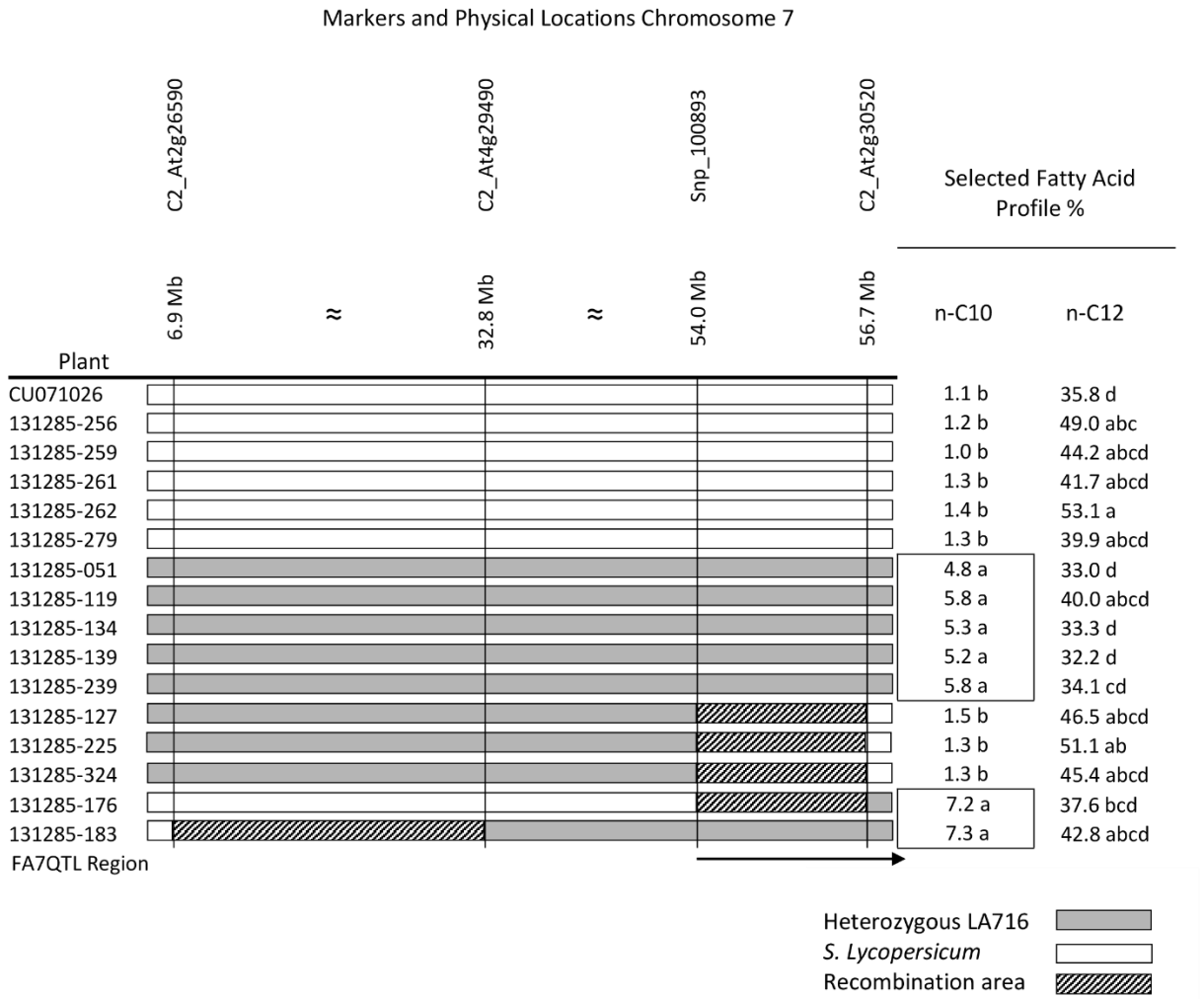



Figure 1.1. Markers, genotypes and selected fatty acid data, diagnostic for the presence of FA7QTL, in CU071026 and selected individuals out of a BC1F1 population showing the relative location of FA7QTL as past snp_100893 (solcap_snp_sl_100893) and tightly linked to C2_At2g30520. N-C10 fatty acid cube root transformed prior to analysis. ≈ represents a large physical distance. Means followed by different letters within a column are significantly different at $P < 0.05$.

The purpose of the BC1F2 population was to obtain plants homozygous for FA7QTL and to observe the effect of this QTL in the homozygous condition in an acylsugar background, which has not previously been reported. The 131285-176 individual that was homozygous for the AS3 and AS10 CU071026 introgressions, and heterozygous for the AS2 and AS7 introgressions of CU071026 was chosen for production of a BC1F2 population. In this BC1F1 selection, the AS7 introgression and sub IL7-4-1 introgression were in trans configuration, so a recombination between them was necessary to get both regions homozygous in the BC1F2. Individuals from a 188 plant BC1F2 population were selected by MAS and grown to obtain plants that were homozygous for the AS2 and AS7 CU071026 regions as well as for the *ca.* 3.2 Mbp subsection of the IL7-4-1 introgression. We screened for plants in which both of the parental gametes were recombinant between AS7 and FA7 so that the plants were homozygous for both AS7 and the IL7-4-1 sub-region; five of the 188 plants had the desired recombinations so that the AS7 and sub IL7-4-1 regions were both homozygous and 2 of these plants were also homozygous for AS-2. These two individuals, as well as a plant that was homozygous for the AS2 and sub IL7-4-1 regions but had lost AS7, all produced high levels of acylsugar and accumulated increased n-C10 and decreased n-C12 fatty acids, which suggests the AS7 introgression is not necessary to maintain acylsugar level and has no impact on the fatty acid profile (Table 1.3). The GC fatty acid profile of tomato plants homozygous for the *S. pennellii* LA716 introgressions within CU071026, and also for the *ca* 3.2 Mbp sub IL7-4-1 region is largely the same for plants heterozygous for the entire or sub IL7-4-1 introgression, which suggests that FA7QTL is largely dominant in its impacts (Figure 1.1 and Table 1.3). The two plants homozygous for the *ca.* 3.2 Mbp FA7QTL sub-region containing both AS7 and FA7QTL as well as for the other four introgressions of CU071026 were observed in the greenhouse and one with higher seed set was selected as the initial plant to establish the line FA7/AS.

Table 1.3. Haplotype summary of the homozygous effect of FA7QTL on acylsugar level and fatty acid profile in a BC1F2 population

| QTL[ab] | | n[c] | Acylsugar as percent of CU071026 (%) | Selected fatty acid profile (%) | | | | |
|---|---|---|---|---|---|---|---|---|
| FA7 | AS7 | | | i-C4 | ai-C5 | i-C5 | n-C10 | n-C12 |
| 1 | 3 | 2 | 101.5 | 1.6 | 12.3 | 56.5 | 2.7 | 26.5 |
| 3 | 1 | 1 | 126.5 | 1.6 | 14.5 | 50.0 | 10.8 | 22.8 |
| 3 | 3 | 2 | 116.6 | 1.1 | 15.4 | 56.3 | 9.5 | 17.4 |

[a] 1: locus homozygous for *S. lycopersicum* alleles; 3: locus homozygous for *S. pennellii* LA716 alleles

[b] Haplotype 1 3 are selections lacking the IL7-4-1 introgression

[c] Number of plants identified/tested with respective haplotype

GC data from additional recombinant plants in FA7QTL BC1F1 fine-mapped the location of FA7QTL within the original *ca.* 56.3 Mbp IL7-4-1 introgression and facilitated selection of a *ca* 3.2 Mbp sub-region carrying FA7QTL. All recombinant plants that were heterozygous for at least marker C2_At2g30520 (Tomato SL2.50 ITAG2.4 Solgenomics.net) (56,723,010 - 56,725,124 bp), near the bottom of the IL7-4-1 introgression, accumulated acylsugar with increased n-C10 (Figure 1.1). Three recombinant plants that were heterozygous for most of the introgression, but that had lost the region near C2_At2g30520, accumulated low levels of n-C10 and higher levels of n-C12; averages of 1.4% and 47.7% respectively. Together these data indicated FA7QTL was tightly linked with the C2_At2g30520 marker, and that FA7QTL was between dCAPS_100893 (derived from solcap_snp_sl_100893) (*ca.* 54,043,669 - 54,044,558) and at or extending slightly past C2_At2g30520. Later genotyping utilizing genotyping by sequencing indicated the sub FA7QTL introgression ranged from a single nucleotide polymorphism (snp) at 55,977,484 bp to a snp at 59,210,920 bp, or *ca.* 3.2 Mbp in length.

**Introgressing FA8QTL**

Introgressing FA8QTL was more challenging due to the recessive nature of this QTL. FA8QTL was putatively localized in and transferred from IL8-1-1 which possesses a *ca.* 50 Mbp chromosome 8 introgression from *S. pennellii* LA716 (Long et al. 2013). A 184 individual BC1F1 population (CU071026 x (CU071026 x IL8-1-1)) was used to identify, through MAS,

plants homozygous for the CU071026 regions and heterozygous for the IL8-1-1 introgression. While the BC1F1 population provided a number of recombinants for the IL8-1-1 introgression, we could not use GC data from BC1F1 plants to select recombinants that possessed FA8QTL, since the location of FA8QTL within the IL8-1-1 introgression was unknown and the effect of FA8QTL on the GC profile largely recessive (Leckie et al. 2014). Therefore, to ensure maintenance of FA8QTL, we selected a plant, 121225-111, which was heterozygous for the full length IL8-1-1 introgression and homozygous for all five CU071026 regions, to create a BC1F2 population and test QTL impact.

The purpose of the BC1F2 populations was to confirm that FA8QTL was contained within IL8-1-1 and observe its effect on acylsugar level and fatty acid profile in the presence of the five CU071026 introgressions. Seeds from the BC1F1 selection, 121225-111, were used to create a 67 plant FA8QTL BC1F2-(A) population, from which 16 plants were identified that were homozygous for the entire IL8-1-1 introgression. The five of those homozygous IL8-1-1 selections that were tested by GC analysis accumulated an average of 21.2% i-C4 and 12.0% i-C5 fatty acids (Figure 1.2). Conversely, CU071026 accumulated 1.4% i-C4 and 36.6% i-C5; similarly four BC1F2-(A) plants that lost the IL8-1-1 region accumulated 1.2% i-C4 and 35.0% i-C5. These results demonstrate the principle effect of FA8QTL, which is a large increase in i-C4 and a simultaneous decrease in i-C5 fatty acids. Four plants heterozygous for the IL8-1-1 introgression accumulated 1.9% i-C4 and 33.8% i-C5 fatty acids, further confirming the function of FA8QTL is largely recessive. BC1F2-(A) selections homozygous for the full length FA8/QTL introgression appeared to accumulate slightly lower levels of acylsugars than that of CU071026 (Figure 1.2), which could either be due to linkage drag in the *ca.* 50 Mbp IL8-1-1 introgression or to the impact of the QTL itself. Data from a BC1F2 (A) population confirm that IL8-1-1 contains FA8QTL, and that it functions in the presence of the five CU071026 introgressions.

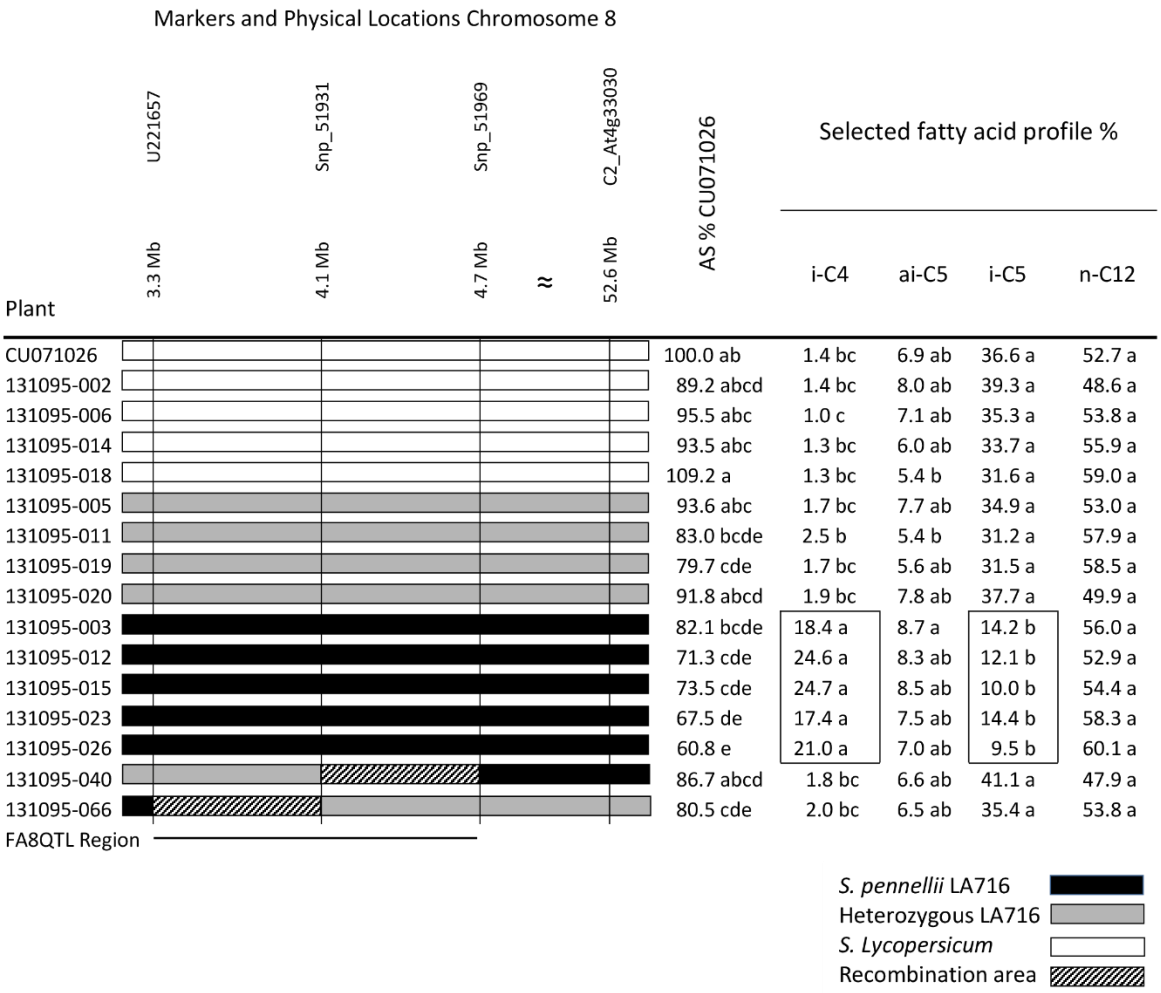



Figure 1.2. Markers, genotypes and selected fatty acid data, diagnostic for the presence of FA8QTL, in CU071026 and selected individuals out of a BC1F2 population showing the relative location of FA8QTL between U221657 and snp_51969 (solcap_snp_sl_51969). Two additional markers run on all plants, (C2_ At5g27390 at 22.8 Mbp and C2_ At1g30360 at 27.9 Mbp) revealed no recombinations between snp_51969 and C2_At4g33030. i-C4 data cube root transformed prior to analysis to improve normality. ≈ represents a large physical distance. Means followed by different letters within a column are significantly different at $P < 0.05$.

Recombinant plants from the BC1F2 (A) population also allowed mapping of FA8QTL to a *ca.* 1.5 Mbp region (Figure 1.2). The average i-C4 and i-C5 fatty acid proportion for recombinant plants 131095-040 and 131095-066 was 1.9% and 38.2% respectively, which was very similar to individuals heterozygous for the entire introgression, indicating FA8QTL was located in the heterozygous region both plants shared. This shared heterozygous region containing FA8QTL was *ca.* 1.5 Mbp from marker U221657 (3,280,372 - 3,283,363 bp) to solcap_snp_sl_51969 (4,744,567 - 4,744,601 bp) (Tomato SL2.50 ITAG2.4 Solgenomics.net). This location is consistent with the QTL on chromosome 8 identified in Blauth et al. (1999) for presence of iso-C4 fatty acids in acylsugars. One of the BC1F2 (A) individuals homozygous for the entire introgression that also set seed well in the greenhouse was selected to establish the new line designated FA8/AS.

Recent work by Ning et al. (2015) identified a non-functional isopropylmalate synthase (Solyc08g014230) in the *S. pennellii* LA716 introgression contained in the IL8-1-1 line, and demonstrated that this gene is responsible for an increase in i-C4 fatty acids in the acylsugars of this IL line. This gene, originally integral in leucine biosynthesis, is critical for production of i-C5 Coenzyme A (CoA), a precursor of i-C5 fatty acids. A non-functional copy of this enzyme leads to an increase in i-C4 fatty acids, through an increase in i-C4 CoA, the precursor of i-C4 fatty acids. The location of Solyc08g014230 (Tomato SL2.50 ITAG2.4 Solgenomics.net) (3,945,013 - 3,955,435 bp), falls within our proposed region for FA8QTL, and is likely the gene responsible for this QTL.

With the success in fine-mapping FA8QTL, we attempted to use this information to decrease the size of the IL8-1-1 introgression in an acylsugar breeding line. Because neither recombinant plant from FA8QTL BC1F2 (A) had the type of subintrogression needed to produce the desired plant, we returned to a recombinant individual, 121225-31, from FA8QTL BC1F1 which had a recombination such that it had lost at least 40 Mbp of the IL8-1-1 introgression, but still possessed the top *ca.* 6 Mbp of the IL8-1-1 introgression with FA8QTL. Plant 121225-31

was still heterozygous for the AS3 and AS10.1 regions of CU071026. Seeds from 121225-31 were sown to generate a 377 individual FA8QTL BC1F2 (B) population. Through MAS we identified individuals that were homozygous for both CU071026 regions, but we observed extreme segregation distortion in this population for the sub IL8-1-1 region, specifically 282 plants heterozygous for the sub IL8-1-1 region, 92 plants that lost the region, and a complete lack of plants homozygous for the *S. pennellii* copy of the sub IL8-1-1 region. This distortion was contrary to the segregation we observed in the BC1F2 (A) population, where the full length IL8-1-1 region segregated normally, however it is consistent with a segregation distortion trait previously noted for chromosome 8 from *S. pennellii* (Eshed and Zamir 1994). These results suggests that some genetic characteristic of BC1F1 plant 121225-111, which was the parent of FA8QTL BC1F2 (A) population, allowed the normal segregation of full IL8-1-1 chromosome 8 introgression in this population, but the genetic characteristic was not present in BC1F1 plant 121225-31, the parent of FA8QTL BC1F2 (B) population. This pattern is further supported by results of additional progenies derived from BC1F2 (B) population. The self-progeny of one BC1F2 (B) plant (131254-066), which was homozygous for the CU071026 regions and heterozygous for only the sub IL8-1-1 region, produced a 189 plant BC1F3 population with extreme distortion for the sub IL8-1-1 region (54 plants homozygous for tomato alleles, 134 heterozygous, but no plants homozygous for *S. pennellii* alleles within the sub IL8-1-1 region). Furthermore, a BC1F3 plant (131544-003) heterozygous for the IL8-1-1 sub-region, and homozygous for all CU071026 regions, was crossed as a female to a line with the full length IL8-1-1 introgression selection from the FA8QTL BC1F2 (A) population. In this cross, both parental plants were homozygous for the CU071026 regions therefore the only *S. pennellii* LA716 region segregating was the sub IL8-1-1 introgression. The 64 plant F1 progeny from this cross also showed observed extreme distortion, such that all individuals were found to be heterozygous for the sub IL8-1-1 region, with a complete lack of individuals homozygous for *S. pennellii* LA716 from IL8-1-1.

In creating the IL lines, Eshed and Zamir (1994) noted the male gametes possessing the chromosome 8 *S. pennellii* LA716 alleles were eliminated in the original line IL8-1; the original introgression in line IL8-1 was maintained by selecting and self-pollinating plants heterozygous for the introgression. Later, line IL8-1-1, was released, in which the chromosome 8 introgression was homozygous and which was used in our work. It was initially thought that the IL8-1-1 introgression was reduced in size from that of IL8-1, perhaps eliminating a region responsible for the segregation distortion. However more recent characterization by SSR markers indicates that IL8-1 and IL8-1-1 contain the same length *S. pennellii* LA716 introgression for chromosome 8 (Long et al. 2013). Additionally, recent genotyping of the original Zamir ILs revealed that IL8-1 and IL8-1-1 were identical for 3503 snps and in particular shared 85 snps in common on chromosome 8 from S. pennellii LA716 (Sim et al. 2012). The fact that IL8-1-1 introgression can be homozygous but is putatively the same length as IL8-1, which cannot be homozygous, might suggest the line IL8-1-1 contains a gene elsewhere in its genome which is necessary to allow normal segregation and homozygosity for the IL8-1/IL8-1-1 introgression. The loss of the normal segregation in the BC1F2 population (B) that was derived from IL8-1-1 could suggest that the factor is not within region of the IL8-1-1 introgression.

**Introgressing FA5QTL**

The transfer of FA5QTL led to complex results due to its interaction with acylsugar level. Schilmiller et al. (2010, 2016) through LC-MS identified a dominant QTL in IL5-3 that lowered acylsugar levels compared to M82. Additionally, Leckie et al. (2012, 2014) identified QTL on chromosome 5 with overlapping genetic intervals that affected fatty acid profile and also reduced acylsugar level. It was not possible in these studies to determine if the results were due to two linked QTL or due to the same QTL. We introgressed FA5QTL into CU071026 using the line IL5-3, which possesses a 52.6 Mbp chromosome 5 introgression from *S. pennellii* LA716 (Long et al. 2013). Plants that were homozygous for the majority of the five *S. pennellii*

introgressed regions of CU071026, and also heterozygous for the IL5-3 introgression were selected by MAS from a 380 plant (CU071026 x (IL-5-3 x CU071026)) BC1F1 population. The recombinant BC1F1 plant, 131075-252, was homozygous for *S. pennellii* introgressions AS2, AS7 and AS10.1, and heterozygous for AS10.2, the top of AS3 from CU071026, and a *ca.* 3.5 Mbp sub-region of the IL5-3 introgression. 131075-252 and plants heterozygous for the entire IL5-3 introgression produced acylsugars with an increase in n-C12 and decrease in i-C5 fatty acids (data not shown), which is largely consistent with the impact of FA5QTL on acylsugar fatty acid profile as described in Leckie et al. (2014). BC1F1 plants containing the IL5-3 introgression generally accumulated far less acylsugar than plants that lost the introgression, but it was difficult to measure the effect of FA5QTL on acylsugar level since the CU071026 regions, which also affect total acylsugar, were segregating in the population, and so plants differ for presence/homozygosity of those region. The GC profile of the BC1F1 plants confirms that IL5-3 possesses FA5QTL, that the subintrogression in 131075-252 still contains FA5QTL, and that the QTL functions to alter the fatty acid profile in the presence of the five introgressions of CU071026.

Acylsugar and GC data from selected BC1F1 plants indicated that marker C2_At1g10500, on chromosome 5, from 62,107,578 - 62,107,796 bp in the 2.5 tomato genomic build (Tomato SL2.50 ITAG2.4), is strongly associated with both decreased acylsugar level and also the change in the fatty acid profile, supporting the idea that either two very closely linked QTL or pleiotropy of one QTL is responsible for both of these traits. It was important to progress to the BC1F2 to obtain lines with further reduction in introgression size, to attempt to separate the QTL for acylsugar level and fatty acid profile, if two linked QTL were controlling these traits.

The purpose of the BC1F2 population was to observe the effect of FA5QTL when homozygous both on acylsugar level and fatty acid profile, which has not previously been reported in an acylsugar accumulating background, as well as to identify recombinants that could indicate whether the acylsugar level and fatty acid phenotypes were governed by linked

QTL or pleiotropy. Seeds from the 131075-252 BC1F1 plant were used to produce a BC1F2 population; this BC1F1 plant was heterozygous for *ca.* 3.5 Mbp of the original *ca.* 52.6 Mbp IL5-3 introgression, extending from *ca.* solcap_snp_sl_29 (60,842,588 - 60,842,588 bp) to TG23 (63,347,423 - 63,348,074 bp) (Tomato SL2.50 ITAG2.4 Solgenomics.net), and was also heterozygous for AS10.2 and the top of AS3. Individuals from a 278 plant BC1F2 population were selected by MAS to obtain plants that were homozygous for the AS10.2 and top of AS3, and heterozygous or homozygous for presence or absence of the *ca.* 3.5 Mbp IL5-3 sub-region. The acylsugar level of plants homozygous for the 3.5 Mbp IL5-3 sub-region is largely the same for plants heterozygous for the sub IL5-3 introgression, which suggests that FA5QTL is largely dominant in its impacts on total acylsugar level (Figure 1.3). Specifically, individuals that had lost the IL5-3 sub-region (131245-008, 131245-045, and 131245-072) accumulated acylsugars about the level of CU071026, while plants that were heterozygous (131245-12, 131245-97, and 131245-105) and homozygous (131245-17, 131245-32, 131245-78) for the IL5-3 sub-region accumulated acylsugars at *ca.* 17 and 10 % of CU071026, respectively.

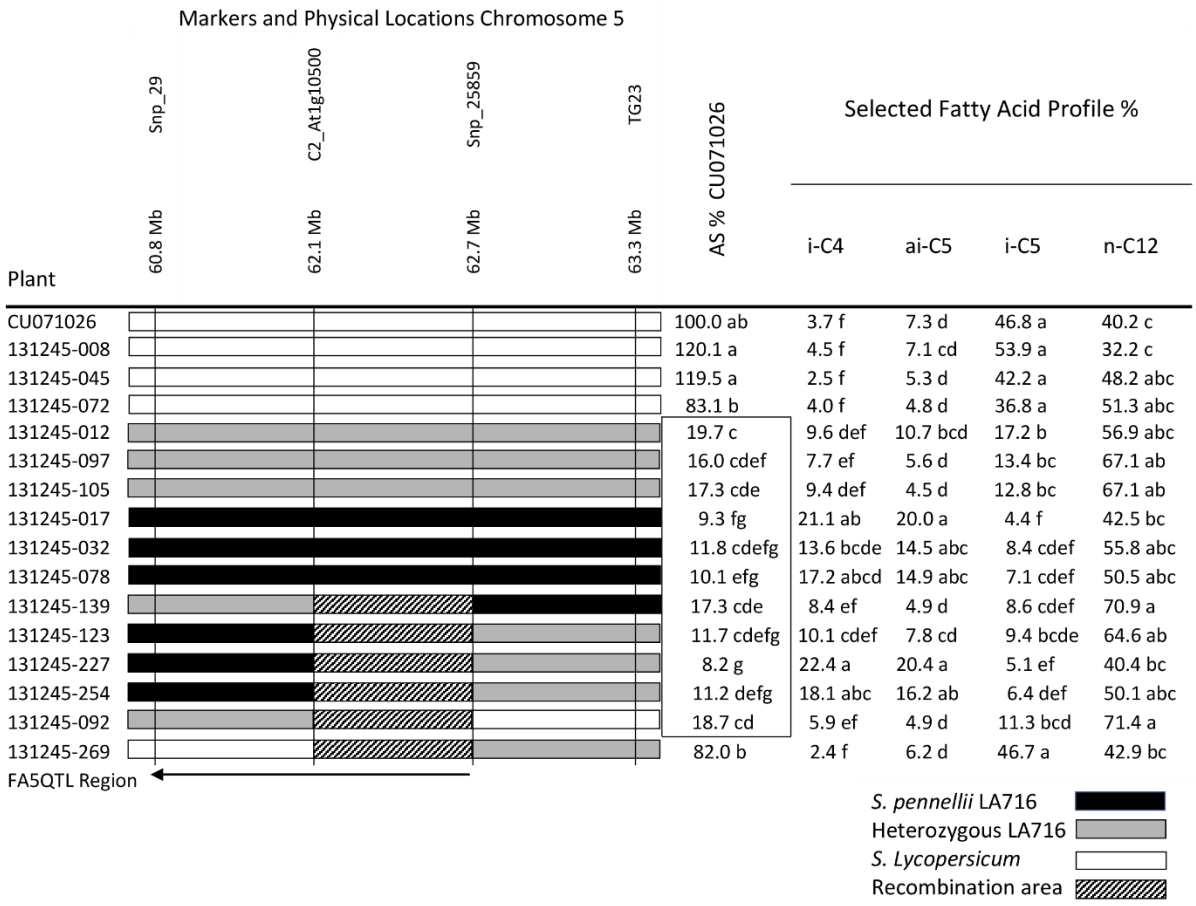



Figure 1.3. Markers, genotypes, acylsugar level and selected fatty acid data, diagnostic for the presence of FA5QTL. Plants include CU071026 and selected individuals, representative of a haplotype, out of a BC1F2 population indicating the relative location of FA5QTL at or before snp_29 and up to snp_25859 (solcap_snp_sl_25859). Plant 131245-227 had a recombination such that it lost a small region of AS3 from CU071026 on the top of chromosome 3. Plant 131245-092 was heterozygous for a small region at the top of AS3. i-C5 data Ln(x) transformed and Percent CU071026 data cube root transformed prior to analysis to improve normality. Means followed by different letters within a column are significantly different at $P < 0.05$.

The GC-measured fatty acid profiles of the same selected BC1F2 plants differed, depending on the genotype of FA5QTL (Figure 1.3). The BC1F2 plants lacking the IL5-3 sub-region exhibited an average of 44.4% i-C5, and 44.1% n-C12, which is very similar to that of the control CU071026, which averaged 46.8% i-C5 and 40.2% n-C12. Selected plants heterozygous for the IL5-3 sub-region in the FA5QTL BC1F2 averaged 13.3% i-C5 and 64.9% n-C12 fatty acids; this increase in n-C12 and decrease in i-C5 was comparable to that observed in the BC1F1 plants heterozygous for FA5QTL. The fatty acid profile of selections homozygous for the IL5-3 sub-region did not fully match that of the heterozygous plants (Figure 1.3). Specifically, while the homozygous plants were similar to the heterozygous siblings in having decreased i-C5 fatty acids, they did not show the large increase in n-C12. Six selections homozygous for the IL5-3 sub-region averaged 6.5% i-C5, and 47.7% n-C12. In addition, in selections homozygous for the IL5-3 sub-region, we saw a rise in the proportion of i-C4 and ai-C5 fatty acids, (18.6% and 17.4%), versus selections heterozygous for the IL5-3 sub-region which accumulated 8.8% and 7.0% i-C4 and ai-C5 fatty acids, respectively. Selections that lost the IL5-3 sub-region accumulated low levels of i-C4 (3.2%) and ai-C5 (6.0%), similar to CU071026, which accumulated 3.7% i-C4 and 7.3% ai-C5. These data could indicate that FA5QTL is not fully dominant, and or that there is a linked recessive QTL(s) in the IL5-3 sub-region that affects the accumulation of i-C4 and or ai-C5 fatty acids. We were unable to identify any recombinant plants in the BC1F2 that appeared to maintain FA5QTL affecting fatty acid profile while losing the negative acylsugar phenotype, which suggests very tight linkage, or more likely, pleiotropy for these two effects.

Utilizing plants recombinant for the IL5-3 introgression and GC data from the BC1F2 plants, we were able to fine map FA5QTL within the original *ca.* 52.6 Mbp IL5-3 introgression to a region of *ca.* 1.9 Mbp (Figure 1.3). Two selections, 131245-227 and 131245-254 both had low acylsugar levels and fatty acid profiles characteristic of plants homozygous for the IL5-3 sub-region. If 131245-227 and 131245-254 are homozygous for FA5, then the putative region is at

or before solcap_snp_sl_29 (snp_29), and extending to solcap_snp_sl_25859 (snp_25859) (62,738,979 – 62,739,013 bp) (Tomato SL2.50 ITAG2.4 Solgenomics.net). Two other recombinant plants, 131245-92 and 269, provide further support for this region. 131245-92 has low levels of acylsugar and a fatty acid profile like that of plants heterozygous for the IL5-3 sub-region. 131245-269 has levels of acylsugar and a fatty acid profile like that of CU071026 and selections that lost the sub-region. Again this suggests the location of FA5QTL is at or before snp_29 and up to snp_25859. Plant 131245-139 also supports the proposed region for FA5QTL. Plant 131245-139 had low acylsugar level (17.3% of CU071026), low levels of ai-C5 (4.9%) and high levels of n-C12 (70.9%), consistent with the profile of plants heterozygous for the IL5-3 sub region, which implies FA5QTL is before snp_25859. A final recombinant could provide greater delineation. Plant 131245-123 had very low acylsugar level (11.7% of CU071026), consistent with plants homozygous for the region, but a fatty acid profile more consistent with plants heterozygous for the region. If plant 131245-123 is heterozygous for FA5QTL, it would imply the location is between C2_At1g10500 and snp_25859, but most likely 131245-123 is homozygous for the QTL, which suggests, like the other recombinants, that the location of FA5QTL is at or before snp_29 and up to snp_25859 (*ca*. 1.9 Mbp).

The dominant QTL identified in Schilmiller et al. (2010) that greatly diminished the acylsugar level of IL5-3 was further elucidate by Schilmiller et al. (2016), where they identified a pair of acylhydrolases on chromosome 5 (Sopen05g030120 and Sopen05g030130) from *S. pennellii* LA716 and demonstrated that they function *in vitro* to cleave specific acyl groups from certain acylsucrose molecules. In cultivated tomato, these acylhydrolases are within the IL5-3 full introgression and located at *ca.* 62.05 Mbp, which is *ca.* 50,000 bp before C2_At1g10500, and consistent with our FA5QTL mapping interval. Schilmiller et al. (2016) showed that these acylhydrolases greatly contribute to diminished acylsugar levels in the line IL5-3, and provided *in vitro* evidence that *S. pennellii* LA716 acylhydrolases preferentially cleave acyl groups from acylsucroses accumulated by cultivated tomatoes, such as M82, rather than acylsucroses from

LA716, which accumulates structurally different acylsugars. The results of Leckie et al. (2012) support this idea, since their negative acylsugar level QTL TA5, in the vicinity of FA5QTL, only reduced acylsugar levels to *ca.* 44% of haplotypes lacking FA5QTL region. The lesser degree of reduction in acylsugar level in the Leckie et al. (2012) BC1F1 population could be partly due to FA5QTL being heterozygous, but also, since that population was segregating for most of the *S. pennellii* LA716 genome, it is likely that some of the SpASAT genes (Schilmiller et al. 2012, 2015; Fan et al. 2016) could have been present, leading to production of acylsucroses that are more like those in LA716 and therefore less subject to acyl cleavage.

The low acylsugar-producing IL5-3 line was also shown to accumulate mostly acylsugars with a long chain fatty acid, and only very low amounts of acylsugars with all short acyl chains, such as i-C5 (Schilmiller et al. 2016). This is largely consistent with GC-MS data from the FA5QTL BC1F2 population (Figure 1.3), which showed the presence of FA5QTL led to a reduction in i-C5 fatty acids and an increase in n-C12 fatty acids. The effect of FA5QTL in the homozygous condition on fatty acid profile in a higher acylsugar level line, rather than the M82 background, had not previously been reported, and it was intriguing to find different effects on the profile when FA5QTL was homozygous versus heterozygous in the lines bred using CU071026. In particular, the positive effect of FA5QTL, when homozygous, on i-C4 and ai-C5 was unexpected. Leckie et al. (2014) did find a weak association between FA5QTL and ai-C5, moderated by an epistatic interaction with another fatty acid QTL identified in that paper, FA11 but since they were only working in a BC1F1 population, FA5QTL would have been heterozygous, and the effect of FA5QTL in the homozygous condition would not have been seen.

We selected a plant out of the BC1F2 (131245-032) that was homozygous for the *ca.* 3.5 Mbp IL5-3 sub-region and all CU071026 regions and that set seed well in the greenhouse and designated the resulting tomato line FA5/AS; despite its severely reduced level of acylsugar, as the FA5/AS line contains the CU071026 regions necessary for acylsugar production. Taken

together, these data support the hypothesis of pleiotropy in that the same gene or genes, likely the *S. pennellii* acylhydrolases, which affect the fatty acid profile, also lead to a reduction in acylsugar level, and it is highly likely that these genes are responsible for the FA5QTL phenotypes. The SpASAT genes are not present in the FA5/AS line, and while these are good candidates for ameliorating the negative acylsugar phenotype of FA5QTL, there presumably are additional genes in *S. pennellii* that raise acylsugar levels, given that the level of acylsugars produced by CU071026 is only *ca.* 15% the level of the wild species (Leckie et al. 2012). Whether some or all of the SpASAT genes, or other classes of acylsugar level QTL, are sufficient to recover the CU071026 level of acylsugar in lines that also possess FA5QTL remains to be seen, but until the necessary regions from *S. pennellii* LA716 are identified and combined with FA5QTL, it is unlikely the current FA5/AS line containing FA5QTL can be utilized against insects due to very low acylsugar accumulation.

**Simultaneous characterization of all modified fatty acid lines**

Previous studies investigating the broad sense heritability of secondary metabolites in tomato, including some acylsugar compounds, suggest that heritability of secondary metabolites in tomato is often quite high (Alseekh et al. 2015). In agreement with that study, total acylsugar level displays high broad sense heritability in our acylsugar lines (0.76) (Table 1.4). Despite high heritability, acylsugar-accumulating tomatoes produce different quantities of acylsugar in different environments (Shapiro et al. 1994), indicating that the amount of acylsugar accumulated exhibits significant genotype by environment interaction. This variability makes it difficult to compare the new modified fatty acids lines for acylsugar related traits using data collected at different times and in different greenhouses as the lines were developed. Similarly, it was possible that environmental factors could impact fatty acid profiles of the acylsugars produced. Therefore, a final experiment growing all modified fatty acid acylsugar lines and controls in a replicated trial under greenhouse conditions was necessary to simultaneous

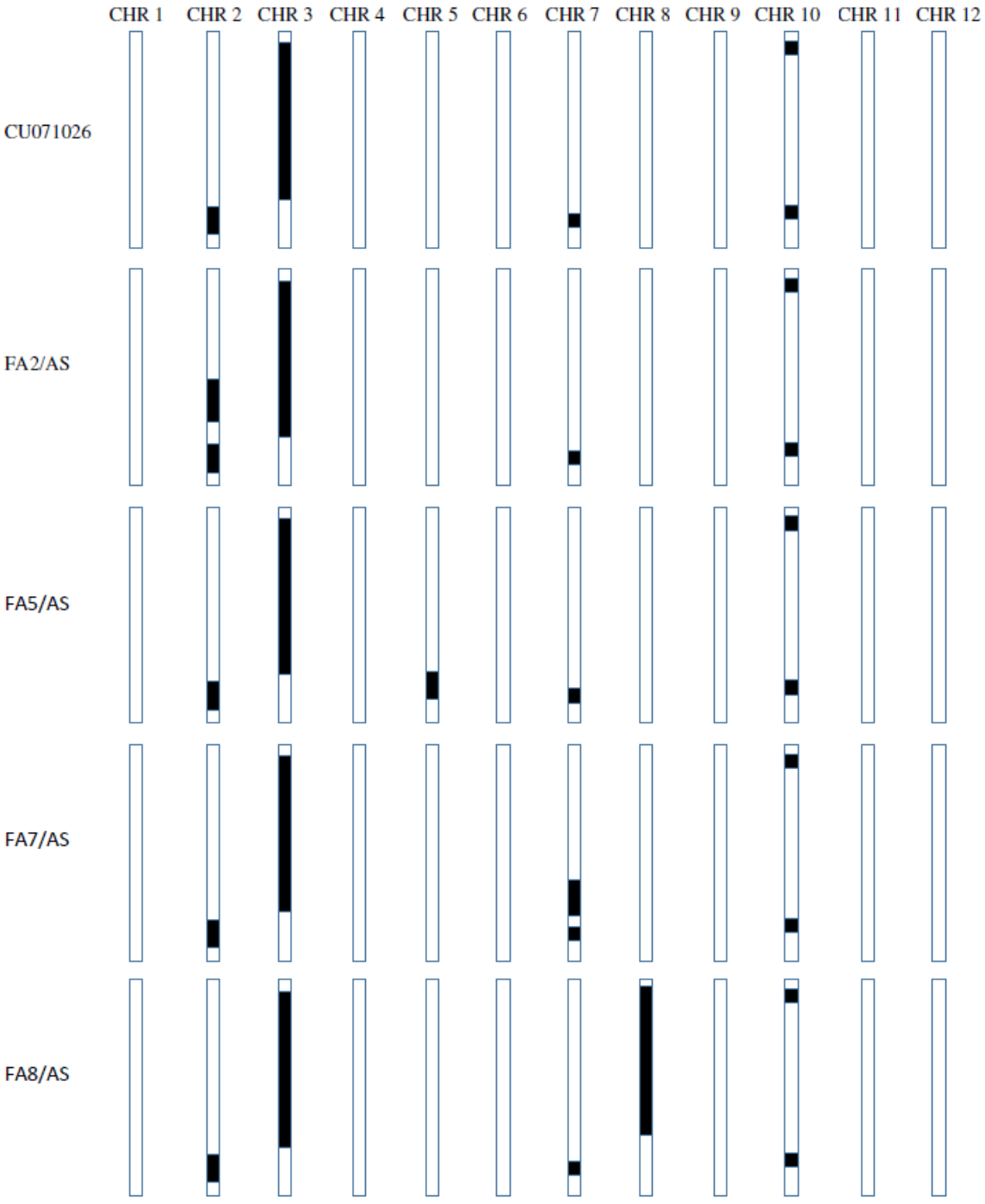
characterize the lines for acylsugar level, density of glandular trichomes, and their acylsugar profile, both for fatty acid acyl group through GC-MS, and type of acylsugar through LC-MS. A schematic of the introgressions contained in each fatty acid line and CU071026 are displayed in Figure 1.4.

Table 1.4. Broad sense heritability estimates for total acylsugar and relative percent of the most predominant fatty acids

| Trait | Broad Sense Heritability[a] |
|---|---|
| Total Acylsugar Level | 0.76 |
| % i-C4 fatty acids | 0.34 |
| % ai-C5 fatty acids | 0.48 |
| % i-C5 fatty acids | 0.67 |
| % n-C10 fatty acids | 0.98 |
| % i-C11 fatty acids | 0.63 |
| % n-C12 fatty acids | 0.84 |
| % i-C13 fatty acids | 0.51 |

[a] Total acylsugar level heritability calculated from 21 entries replicated over five locations and two years. Heritability for the relative percent of each fatty acid calculated from 18 entries replicated over four locations and two years.

Figure 1.4. Depiction of the size and location of the *S. pennellii* LA716 introgressions on each chromosome, based on physical distance, in CU071026 and the fatty acid lines. White is tomato and black is LA716


CHR 1
CHR 2
CHR 3
CHR 4
CHR 5
CHR 6
CHR 7
CHR 8
CHR 9
CHR 10
CHR 11
CHR 12
CU071026
FA2/AS
FA5/AS
FA7/AS
FA8/AS

**Acylsugar level and trichome density**

The fatty acid QTL modified the fatty acids of the acylsugars produced as expected, but also impacted the acylsugar levels of several of the resulting modified fatty acid acylsugar lines. The FA2/AS and FA7/AS lines accumulated higher (119.2% and 121.9%, respectively) acylsugar levels than CU071026 (Table 1.5). It is possible that the introgressions containing FA2QTL and FA7QTL introgressions also contain some minor QTL from *S. pennellii* LA716 affecting acylsugar level, or that the QTL responsible in the introgressions for alteration of fatty acid profile also impact acylsugar level. If there are QTL for increased acylsugar level in the FA2/AS and FA7/AS lines, the effect of these QTL might be influenced by the environment, since past characterization of these lines in field and greenhouse settings (data not shown) indicated that their acylsugar levels are not always significantly different from those of CU071026. The FA8/AS line appeared to have a slightly lower acylsugar level (81.1%) than the CU071026 control, although the difference was not statistically significant. The acylsugar level of the FA5/AS line was strongly reduced, averaging only 16.2% the level of CU071026 (Table 1.5), indicating that the regions from *S. pennellii* LA716 ameliorating this impact of FA5QTL on acylsugar level must be identified and introgressed to obtain a FA5/AS tomato line that produces high acylsugar levels.

Table 1.5. Acylsugar level, trichome density and fatty acid profile characterization of acylsugar lines in greenhouse summer 2015

| Acylsugar Lines | Acylsugar as percent of CU071026 (%)[a] | Type IV trichome density[abc] | Type VI trichome density[abc] | Selected fatty acid profile %[ac] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *i*-C4 | *ai*-C5 | *i*-C5 | *n*-C10 | *i*-C11 | *n*-C12 | *i*-C13 |
| CU071026 | 100.0 b | 67.8 a | 1.6 bc | 3.4 c | 8.5 b | 48.1 b | 1.5 bc | 0.0 bc | 37.7 c | 0.0 b |
| FA2/AS | 119.2 a | 80.3 a | 2.8 ab | 4.4 c | 14.4 a | 58.6 a | 0.5 d | 8.7 a | 9.4 d | 3.2 a |
| FA5/AS | 16.2 c | 48.6 b | 6.3 a | 40.5 a | 3.5 c | 9.4 e | 1.0 c | 0.0 c | 44.4 b | 0.0 b |
| FA7/AS | 121.9 a | 70.5 a | 4.4 a | 2.7 c | 9.1 b | 37.9 c | 11.3 a | 0.1 b | 38.6 bc | 0.0 b |
| FA8/AS | 81.1 b | 54.1 b | 1.1 c | 12.5 b | 9.9 b | 16.1 d | 1.9 b | 0.0 c | 59.3 a | 0.0 b |

[a] Averages from four plants, means followed by different letters within a column are significantly different at $P < 0.05$

[b] Average number of trichomes counted per $mm^2$

[c] i-C4, n-C10 and i-C11 cube root transformed and ai-C5, i-C13, and Type IV and VI trichome density log10(x +0.1) transformed to improve normality

Because prior work showed that acylsugar level could be increased with or without an increase in trichome density (Leckie et al. 2012), we evaluated the density of glandular trichomes among the modified fatty acid lines and their controls. This characterization allows differentiation between increased trichome density and increased acylsugar production per trichome as mechanisms for overall increased acylsugar levels. The density of type IV trichomes varied among the lines tested, with increased trichome density positively correlated with increased acylsugar level ($r = 0.66$). The type IV trichomes density of CU071026 and the FA2/AS and FA7/AS lines were all equivalent, however, the type IV trichome density of the FA5/AS line was lower and the density of type IV trichomes in the FA8/AS line was marginally lower than the other three entries (Table 1.5).

Type IV glandular trichome types have repeatedly been implicated as the predominant trichome type for acylsugar production (Fobes et al. 1985; Goffreda et al. 1990; Slocombe et al. 2008). In particular, the SpASAT1-4 genes, which are integral in attachment of acyl groups to the sugar backbone in acylsugar biosynthesis have been shown to be expressed in type IV trichomes (Schilmiller et al. 2012, 2015; Fan et al. 2016). While density of type IV trichomes was positively correlated with acylsugar level when comparing the fatty acid lines, the density of type IV trichomes alone does not account for differences in acylsugar levels among lines. Type VI glandular trichome density also varied between entries, but was not positively correlated with acylsugar accumulation ($r = -0.16$) as expected based on the role of type VI trichomes in production of other specialized metabolites, such as terpenes (Coates et al. 1988; Frelichowski and Juvik 2001; Li et al. 2004). These results imply that differences in acylsugar levels among lines could be influenced by type IV trichome density, but are more likely due to variation in acylsugar biosynthesis rather than trichome density.

Acylsugar level can also be influenced by QTL that increase or decrease the density of the trichomes that produce and exude these compounds. Comparison of acylsugar producing sister lines with and without the chromosome 6 *S. pennellii* LA716 QTL, named TA6, showed

that its presence increased both the total acylsugar level and the density of type IV trichomes that exude acylsugar droplets (Leckie et al. 2012). While the modified fatty acid lines have variation for acylsugar level and trichome density, none of the fatty acid line possess the TA 6 acylsugar level QTL, or the TA 10.1 acylsugar level QTL that increases acylsugar level without affecting trichome density (Leckie et al. 2012). The acylsugar level and trichome data from the fatty acid lines suggests type IV trichome density is an important morphological feature for acylsugar level in the some of the modified fatty acid lines, but that modulation of acylsugar level through biosynthesis could also be a major component contributing to the differential acylsugar levels observed. It is possible that adding one or both of the acylsugar level QTL (TA 6 or TA 10.1) to the fatty acid lines could further improve the acylsugar level/type characteristics of the resulting lines.

**Fatty acid characterization from GC-MS**

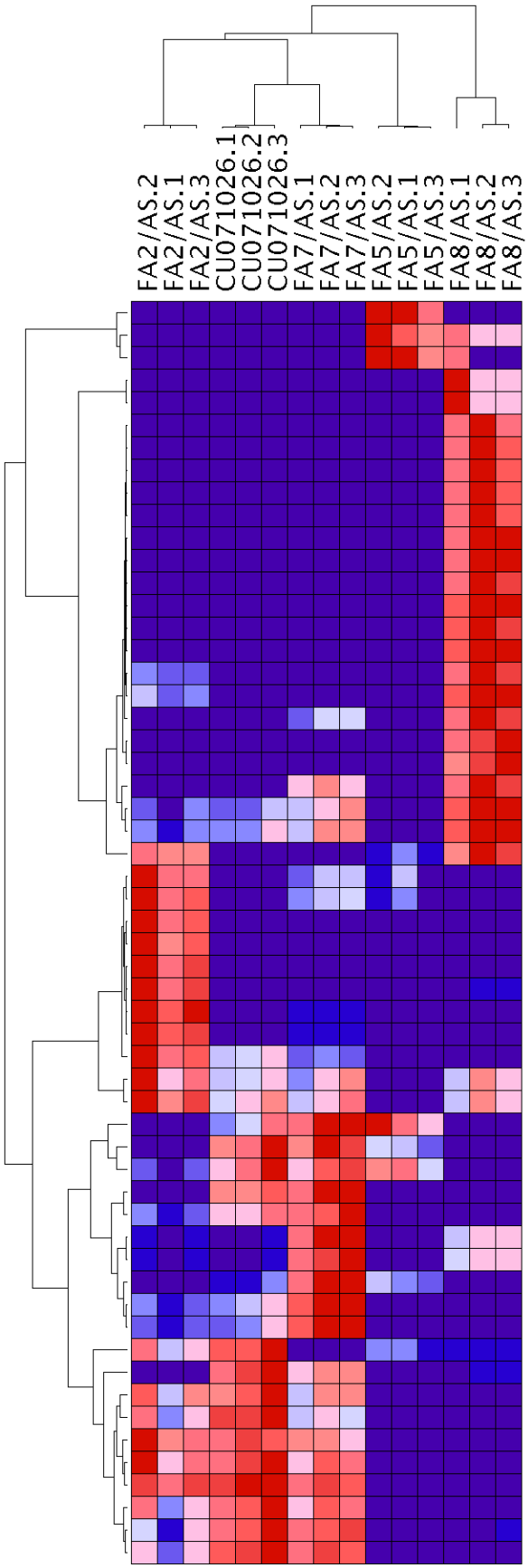
The results from the GC-MS analysis of CU071026 *vs*. the new modified fatty acid lines closely matched predictions based on prior QTL analyses (Leckie et al. 2014). Consistent with previous characterizations, the acylsugars of the FA2/AS line included the extended branch chain fatty acids i-C11 and i-C13 at 8.7% and 3.2% respectably, whereas in the acylsugars of CU071026 these fatty acids were either found at trace levels or not detected (Table 1.5). The acylsugars of the FA2/AS line also possessed reduced levels of n-C12 and slightly increased levels of i-C5 compared to the acylsugars of the CU071026 control. The fatty acid profile of the acylsugars of the FA7/AS line was also consistent with previous characterization of lines during their development; the acylsugars of this line have an increase in n-C10 fatty acids (11.3%) over acylsugars of CU071026 (1.5%), and a reduction in the level of i-C5 (37.9%) compared to that of CU071026 (48.1%) (Table 1.5). The FA8/AS line data were also generally consistent in profile with previous characterization of lines during their development; the acylsugars of the FA8/AS line show increased i-C4 fatty acids (12.5%) over the acylsugars of CU071026 (3.4%)

as well as decreased i-C5 fatty acids (16.1%) versus that in CU071026 (48.1%) (Table 1.5). Additionally, FA8/AS appears to accumulate increased n-C12 fatty acids, which was not previously observed. The FA5/AS line GC-MS data deviated from previous characterization in that the FA5/AS line in the replicated trial accumulated higher levels of i-C4 (40.5%) and very lower levels of ai-C5 (3.5) and i-C5 (9.4%), than was seen in initial characterization of BC1F2 selections homozygous for FA5QTL subregion. This difference this could be due in part to the difficulties inherent in characterizing fatty acids present at low levels by GC-MS. To better understand the effect of FA5QTL on acylsugar chemistry it is necessary to identify and combine any necessary epistatic QTL from *S. pennellii* LA716 that will allow recovery of higher levels of acylsugar in the presence of FA5QTL. With the exception of the FA5/AS line, all modified acylsugar lines displayed remarkable consistency in fatty acid profile at different times and in different environments, suggesting fatty acid profile is minimally impacted by the environment. Broad sense heritability estimates were high for all the major fatty acids accumulated by the acylsugars lines from 2014 and 2015, and are displayed in Table 1.4.

**Acylsugar characterization from LC-MS**

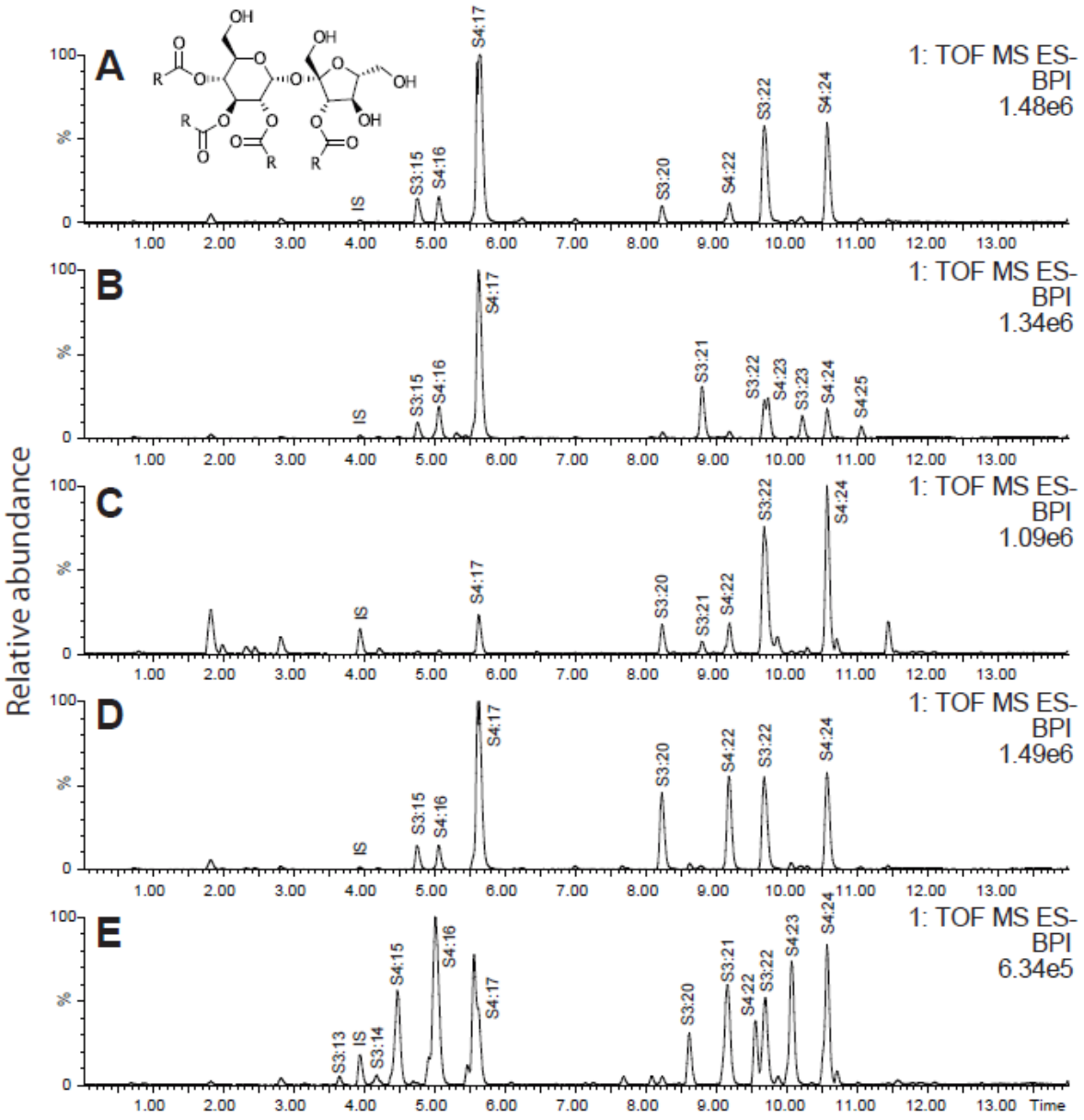
To further characterize the fatty acid lines, LC-MS data was collected to provide information about not only the fatty acids accumulated by each line, but also to provide greater detail on the major acylsugars produced, including the number and length of fatty acids and to which sugar they are esterified (Figure 1.5). Representative LC-MS chromatograms for each fatty acid line are displayed in Figure 1.6. LC-MS is a sensitive technique that can readily detect the low levels of acylsugars produced by cultivated tomato. Although the LC-MS procedure cannot differentiate between orientations in fatty acids of the same length, such as ai-C5 and i-C5, GC-MS can differentiate between these, and therefore the joint analysis using both GC-MS and LC-MS data provides greater detail regarding the acylsugar profiles of each fatty acid line.

Figure 1.5. Hierarchical cluster analysis, with Pearson correlation using a pairwise average-linkage clustering method, indicating the predominant acylsugars accumulated by each fatty acid line. Three samples for each genotype were analyzed. Color across a row indicates relative levels of the respective acylsugar, with red indicating samples with the highest levels detected and blue/purple indicating low or no detection relative to the highest sample. [a] The mass to charge ratio for each acylsugar followed by retention time in min. [b]Acylsugar nomenclature indicates S for sucrose backbone of the molecule, as well as the number of fatty acid acyl chains (2 to 4) with their cumulative length in carbons that are esterified to the sugar followed by the lengths in number of carbons of each acyl group in the respective acylsugar. [c] Proposed acylgroup number and length for acylsugar; identification hampered by low abundance and peak overlap.




| ID | M/z and RT[a] | Acylsugar and acyl groups[b] |
|---|---|---|
| 1 | 653.32 (6.61) | S2:17 (5,12)[c] |
| 2 | 709.34 (8.39) | S3:20 (5,5,10) |
| 3 | 737.37 (9.87) | S3:22 (5,5,12) |
| 4 | 575.35 (11.42) | G3:22 (5,5,12) |
| 5 | 575.35 (11.57) | G3:22 (5,5,12) |
| 6 | 597.25 (3.16) | S3:12 (4,4,4) |
| 7 | 639.33 (6.73) | S2:16 (4,12) |
| 8 | 681.34 (6.99) | S3:18 (4,4,10) |
| 9 | 695.35 (7.53) | S3:19 (4,5,10) |
| 10 | 639.26 (3.94) | S4:14 (2,4,4,4)[c] |
| 11 | 653.35 (7.24) | S2:17 (5,12) |
| 12 | 751.39 (9.38) | S4:22 (2,4,4,12) |
| 13 | 653.35 (7.24) | S4:20 (2,4,4,10) |
| 14 | 681.27 (5.55) | S4:17 (2,5,5,5) |
| 15 | 709.37 (8.61) | S3:20 (4,4,12) |
| 16 | 751.38 (9.55) | S4:22 (2,4,4,12) |
| 17 | 611.26 (3.64) | S3:13 (4,4,5) |
| 18 | 653.27 (4.50) | S4:15 (2,4,4,5) |
| 19 | 723.35 (8.07) | S4:20 (2,4,4,10) |
| 20 | 681.31 (5.46) | S4:17 (2,5,5,5) |
| 21 | 695.32 (6.07) | S4:18 (2,5,5,6) |
| 22 | 681.34 (7.14) | S3:18 (4,4,10) |
| 23 | 765.4 (10.07) | S4:23 (2,4,5,12) |
| 24 | 723.38 (9.14) | S3:21 (4,5,12) |
| 25 | 737.37 (8.46) | S4:21 (2,4,5,10) |
| 26 | 695.35 (7.38) | S3:19 (5,5,9) |
| 27 | 737.34 (8.31) | S4:21 (2,5,5,9) |
| 28 | 709.37 (8.07) | S3:20 (5,5,10) |
| 29 | 737.4 (9.33) | S3:22 (5,6,11) |
| 30 | 793.43 (11.04) | S4:25 (2,5,5,13) |
| 31 | 751.38 (9.02) | S4:22 (2,5,5,10) |
| 32 | 765.39 (9.74) | S4:23 (2,5,5,11) |
| 33 | 723.38 (8.80) | S3:21 (5,5,11) |
| 34 | 751.41 (10.21) | S3:23 (5,6,12) |
| 35 | 625.27 (4.20) | S3:14 (4,5,5) |
| 36 | 667.29 (5.06) | S4:16 (2,4,5,5) |
| 37 | 653.34 (6.45) | S2:17 (5,12) |
| 38 | 723.39 (8.96) | S3:21 (5,5,11) |
| 39 | 821.46 (11.43) | S4:27 (5,5,5,12) |
| 40 | 681.34 (6.79) | S3:18 (4,5,9)/(5,5,8) |
| 41 | 723.35 (7.75) | S4:20 (2,5,5,8)[c] |
| 42 | 695.35 (7.67) | S3:19 (4,5,10) |
| 43 | 737.36 (8.63) | S4:21 (2,4,5,10) |
| 44 | 793.43 (10.29) | S4:25 (5,5,5,10) |
| 45 | 709.37 (8.22) | S3:20 (5,5,10) |
| 46 | 751.38 (9.18) | S4:22 (2,5,5,10)[c] |
| 47 | 751.42 (10.38) | S3:23 (5,6,12) |
| 48 | 793.43 (11.00) | S4:25 (2,5,6,12) |
| 49 | 597.22 (3.00) | S3:12 (2,5,5)[c] |
| 50 | 695.32 (6.23) | S4:18 (2,5,5,6) |
| 51 | 667.29 (5.43) | S4:16 (2,4,5,5) |
| 52 | 639.29 (4.75) | S3:15 (5,5,5) |
| 53 | 681.3 (5.63) | S4:17 (2,5,5,5) |
| 54 | 723.35 (7.00) | S4:20 (5,5,5,5) |
| 55 | 779.41 (10.59) | S4:24 (2,5,5,12) |
| 56 | 737.4 (9.68) | S3:22 (5,5,12) |

Figure 1.6. Representative LC-MS base peak intensity chromatograms for CU071026 (A), FA2/AS (B), FA5/AS (C), FA7/AS (D), and FA8/AS (E). Each compound peak is scaled according to the most abundant peak in the chromatogram to show differences in the acylsugar profiles among the different lines rather than to show differences in abundance. The CU071026, FA2/AS, and FA7/AS samples were diluted 1:10 prior to LC-MS analysis due to high concentrations of acylsugars in the sample. Therefore, the internal standard (IS) peak in these samples is ten-fold lower compared to the FA5/AS and FA8/AS samples (All data in the HCA figure was normalized to the IS peak area which corrected for the dilution). A general acylsucrose structure is shown in panel A.


A
1: TOF MS ES-
BPI
1.48e6
IS
S3:15
S4:16
S4:17
S3:20
S4:22
S3:22
S4:24
B
1: TOF MS ES-
BPI
1.34e6
IS
S3:15
S4:16
S4:17
S3:21
S3:22
S4:23
S3:23
S4:24
S4:25
C
1: TOF MS ES-
BPI
1.09e6
IS
S4:17
S3:20
S3:21
S4:22
S3:22
S4:24
D
1: TOF MS ES-
BPI
1.49e6
IS
S3:15
S4:16
S4:17
S3:20
S4:22
S3:22
S4:24
E
1: TOF MS ES-
BPI
6.34e5
S3:13
IS
S3:14
S4:15
S4:16
S4:17
S3:20
S3:21
S4:22
S3:22
S4:23
S4:24
Relative abundance
%
100
0
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
10.00
11.00
12.00
13.00
Time

CU071026 predominantly accumulates three major acylsugars: S4:17 (ID 53), S3:22 (ID 56) and S4:24 (ID 55) (Figure 1.5). These acylsugars support the GC-MS fatty acid data, as all three acylsugars contain C5 and C12 fatty acids, which are the principal fatty acid chain lengths in CU071026. The FA2/AS line accumulates the three major acylsugars found in CU071026, but additionally possesses significant amounts of two acylsugars that are much lower in CU071026: S3:21 (ID 33) and S4:25 (ID 30). These two acylsugars contain C11 and C13 fatty acids, respectively, which accumulate in the FA2/AS line but are at trace or undetectable amounts in CU071026 (Figure 1.7). The FA7/AS line also accumulates the three major acylsugar peaks found in CU071026, and also has greatly increased relative abundance of two acylsugars accumulated at very low levels in CU071026, S3:20 (ID 45) and S4:22 (ID 46) (Figure 1.8). Additionally, the FA7/AS line accumulates low levels of several other acylsugars that show very low abundance in CU071026, including S3:19 (ID 42) and S4:21 (ID 43). The increase in abundance of C10 containing acylsugars for the FA7/AS line shown by LC-MS is consistent with GC-MS data for which indicates n-C10 fatty acids are more prevalent in this line. The FA8/AS line accumulates the three major acylsugars in CU071026 but also produces significant quantities of a number of acylsugars not significantly accumulated by CU071026, including S4:15 (ID 18), S3:21 (ID 24) and S4:23 (ID 23). These acylsugars all contain C4 fatty acids, which are increased in FA8/AS (Figure 1.9), and some of these acylsugars also contain C12 fatty acids which are increased in the FA8/AS line according to GC-MS data. There were also two acylglucose isomers detected at low abundance in the FA8/AS line (IDs 4 and 5), that were not detected in the other fatty acid lines. As all the fatty acid lines are lacking several important QTL necessary for production of acylglucoses (Smeda et al. accepted), the detection of acylglucoses in the FA8/AS line was unexpected and suggests FA8QTL or a linked QTL within the IL8-1-1 introgression could be involved in acylglucose biosynthesis. The FA5/AS line was found to accumulate similar types of acylsugars to CU071026, however, in contrast to data from the GC-MS-based fatty acid characterization of the FA5/AS with all lines together, LC-MS data

showed the FA5/AS line accumulated an increased proportion of acylsugars containing a long chain fatty acid, and reduced quantities of acylsugars with all short chain acyl groups, consistent with the results in Schilmiller et al. (2016). It is possible that fatty acid levels near the detection limit in GC-MS analysis will result in higher variability between repeated samples and suggests the profile of tomatoes that accumulate low levels of acylsugar, such as the FA5/AS line should be characterized through LC-MS for consistent results.

Figure 1.7. Extracted ion chromatograms (XIC) from CU071026 (A) and FA2/AS (B) for formic acid adducts of acylsugars with m/z 723.4, 751.4, 765.4, and 793.4, as well as the internal standard (m/z 179.08). At higher collision energy where fatty acids fragment from the acylsugar, the C11 (m/z 185.17) and C13 (m/z 213.19) fatty acid anions are seen in FA2/AS (D) but not significantly in CU071026 (C). The lack of C11 or C13 fragments co-chromatographing with peaks in the CU071026 XIC indicate that these peaks are acylsugar isomers different from those in FA2/AS

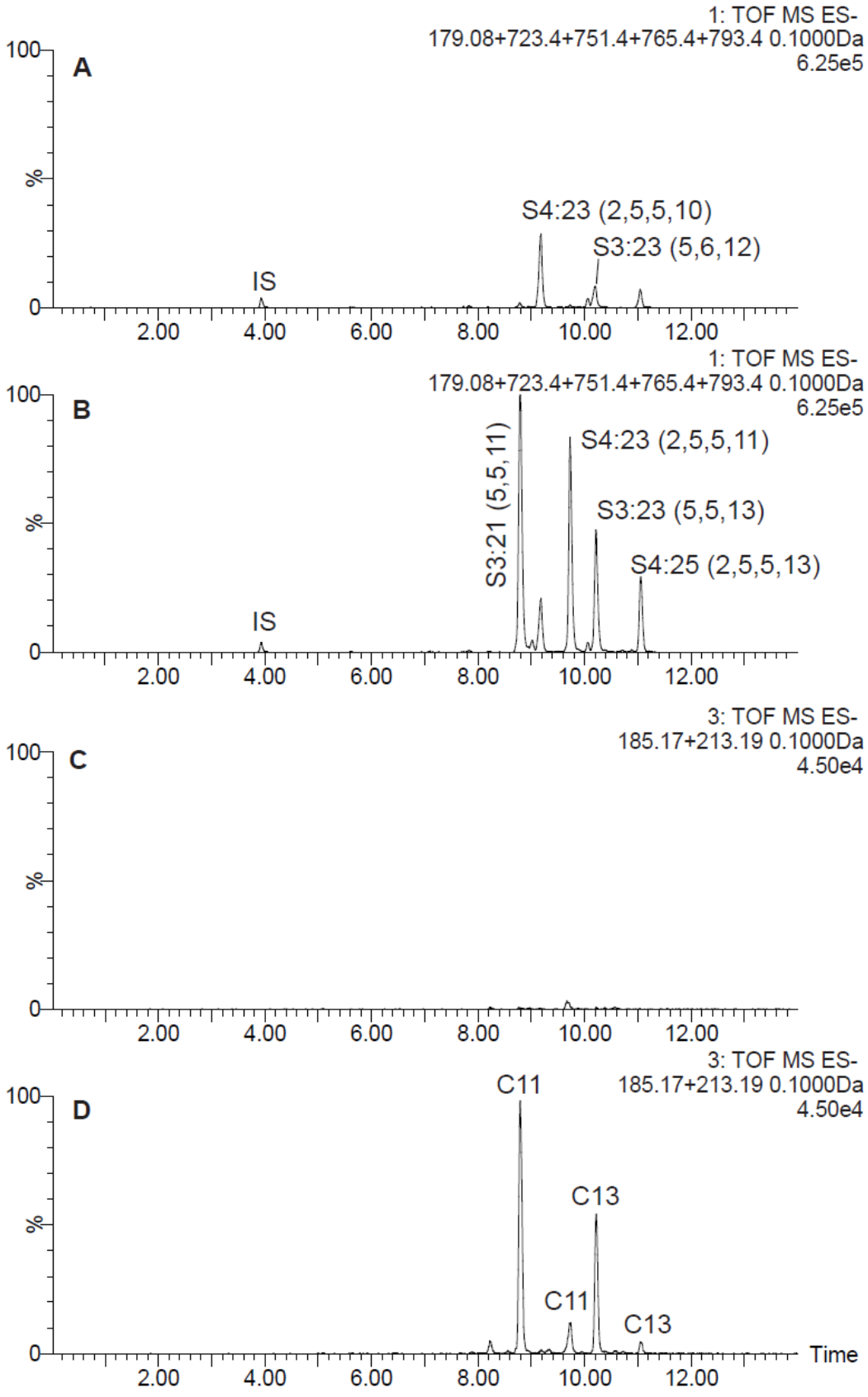
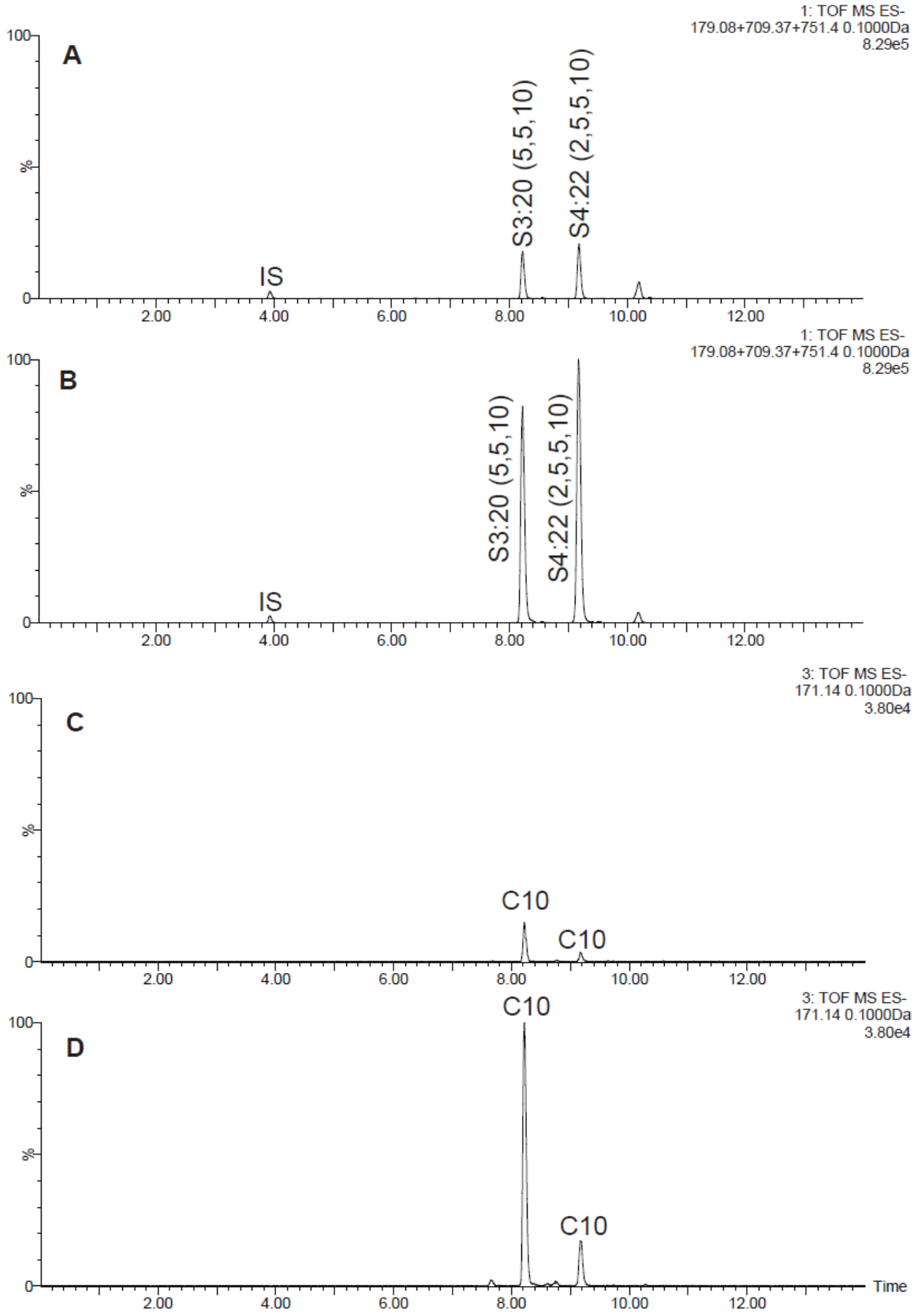

1: TOF MS ES-
179.08+723.4+751.4+765.4+793.4 0.1000Da
6.25e5
A
100
%
0
S4:23 (2,5,5,10)
S3:23 (5,6,12)
IS
2.00
4.00
6.00
8.00
10.00
12.00
1: TOF MS ES-
179.08+723.4+751.4+765.4+793.4 0.1000Da
6.25e5
B
S3:21 (5,5,11)
S4:23 (2,5,5,11)
S3:23 (5,5,13)
S4:25 (2,5,5,13)
IS
3: TOF MS ES-
185.17+213.19 0.1000Da
4.50e4
C
3: TOF MS ES-
185.17+213.19 0.1000Da
4.50e4
D
C11
C13
C11
C13
Time

Figure 1.8. Extracted ion chromatogram from CU071026 (A) and FA7/AS (B) for m/z 179.08 (internal standard, IS), 709.37 (S3:20) and 751.4 (S4:22). At higher collision energy where fatty acids fragment from the acylsugar, the C10 fatty acid anion (m/z 171.14) is seen in CU071026 (C), but found at higher abundance in FA7/AS (D).


A
1: TOF MS ES-
179.08+709.37+751.4 0.1000Da
8.29e5
100
%
0
IS
S3:20 (5,5,10)
S4:22 (2,5,5,10)
2.00
4.00
6.00
8.00
10.00
12.00
B
1: TOF MS ES-
179.08+709.37+751.4 0.1000Da
8.29e5
100
%
0
IS
S3:20 (5,5,10)
S4:22 (2,5,5,10)
2.00
4.00
6.00
8.00
10.00
12.00
C
3: TOF MS ES-
171.14 0.1000Da
3.80e4
100
%
0
C10
C10
2.00
4.00
6.00
8.00
10.00
12.00
D
3: TOF MS ES-
171.14 0.1000Da
3.80e4
100
%
0
C10
C10
Time
2.00
4.00
6.00
8.00
10.00
12.00

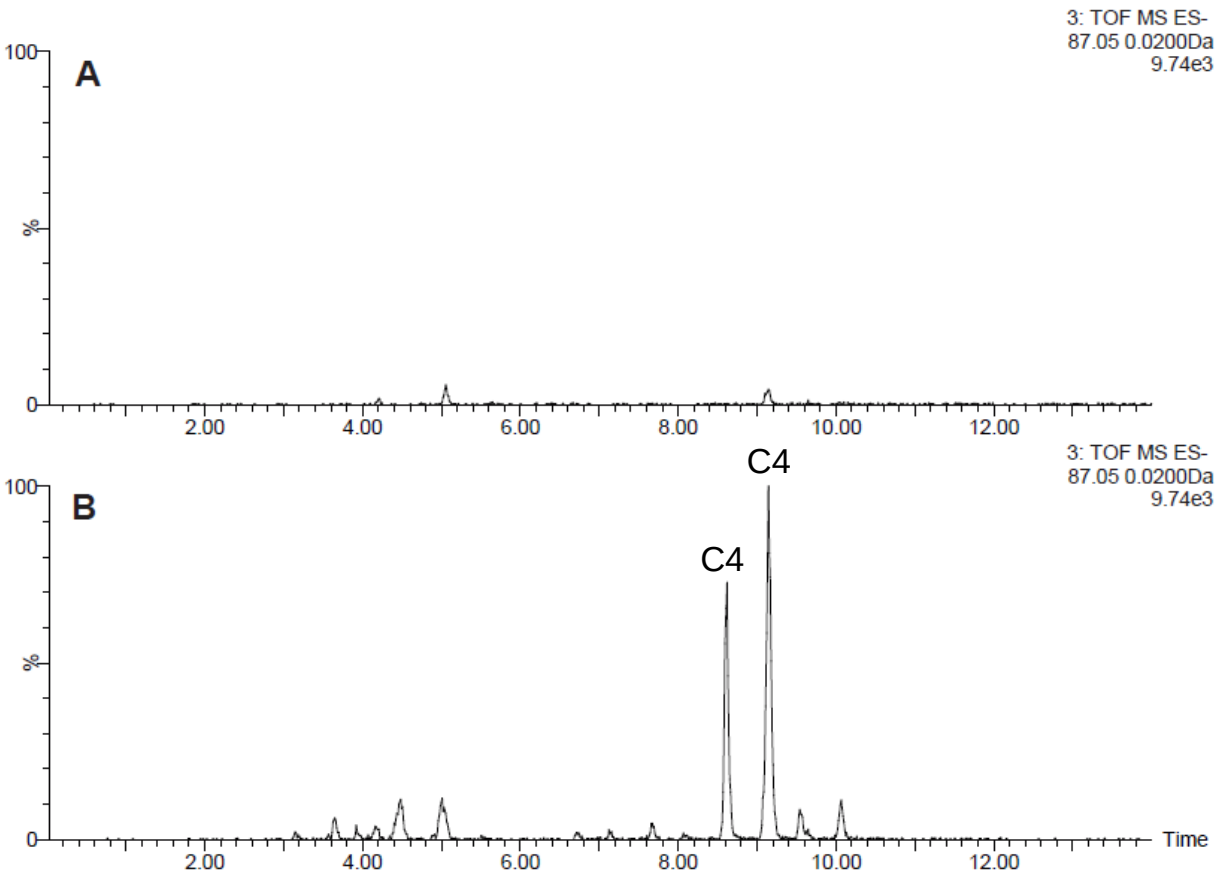



Figure 1.9. Extracted ion chromatogram from CU071026 (A) and FA8/AS (B) for m/z 87.05 at higher collision energy showing the C4 fatty acid anion present in FA8/AS and at trace levels in CU071026.

For several acylsugars identified in the LC-MS analysis, there was clear chromatographic separation of compounds having identical mass and indistinguishable mass spectra, for example S3:20 (5,5,10) (ID 45) with a retention time of 8.22 min and S3:20 (5,5,10) (ID 28) with a retention time of 8.07 min (Figure 1.5). These acylsugar isomers likely differ in either the position of acyl chain attachment or in the branching of the acyl chains. There are several instances of fatty acid QTL influencing the relative abundance of different acylsugar isomers. For example, each line accumulates some level of S3:20 (5,5,10) (ID 45) (see Supplementary Table S4 of Smeda et al. 2016), however, the FA2/AS line also accumulates a significant amount of S3:20 (5,5,10) (ID 28) that is not detected in CU071026 or the FA7/AS line and is seen at only very low levels in the FA5/AS and FA8/AS lines. The difference between these two isomers would require purification and nuclear magnetic resonance (NMR) analysis for clarification, and could be pursued in future work.

The hierarchical clustering analysis (HCA) in Figure 1.5 separates the fatty acids lines into several clades. As expected, the presence of FA8QTL largely separates the fatty acid lines into two main clusters. Acylsugars with IDs 6 to 25 commonly contain C4 fatty acids and are significantly accumulated in the FA8/AS line and largely absent in the other acylsugar lines, which is due to the effect of FA8QTL. Acylsugars with IDs 26 to 56, conversely, are generally accumulated in CU071026, and the FA2/AS and FA7/AS lines, and largely absent in the FA8/AS line. Further elucidation of the impact of fatty acid QTL on the fatty acid combinations within acylsugars and the locations of fatty acid attachment could allow greater understanding of acylsugar biosynthesis, how acylsugars interact with insects, and selection of optimal acylsugar lines for insect resistance.

**The effect of the fatty acid QTL on acylsugar diversity**

A broad question that can be addressed by looking at the combination of GC-MS and LC-MS data on the acylsugar lines is whether addition of the fatty acid QTL into CU071026 increases

the diversity of fatty acid acyl groups, and or the diversity of acylsugars accumulated. It is possible that when a novel acylsugar is accumulated in a line possessing an additional QTL, the acylsugar replaces acylsugar(s) accumulated in lines lacking the QTL, so that the addition of fatty acid QTL might lead to accumulation of acylsugars not seen in CU071026, but that this gain could be coupled with the loss of acylsugars produced in CU071026. Alternately, addition of a novel acylsugar not accumulated in CU071026 could be additive rather than a replacement of a previously accumulated acylsugar. GC-MS data indicates there are five fatty acids acyl groups (i-C4, ai-C5, i-C5, n-C10 and n-C12) that are significantly accumulated by all the fatty acid lines and CU071026 (Table 1.5). The FA5/AS, FA7/AS and FA8/AS lines vary in the relative proportions of these five fatty acids, but do not accumulate detectable levels of novel fatty acids not found in CU071026. Conversely, the FA2/AS line accumulates the five fatty acids in CU071026 as well as detectable levels of two fatty acids (i-C11 and i-C13) not abundant in CU071026. These data indicate that addition of FA2QTL increases acyl chain diversity compared to CU071026, but the QTL in lines FA5QTL, FA7QTL, and FA8QTL do not increase acyl chain diversity.

Concerning LC-MS data, 56 acylsugars are identifiable among the fatty acid lines and CU071026 (Figure 1.5). We can evaluate the effect of introgression of the fatty acid QTL on acylsugar diversity by comparing the lines for acylsugars that are accumulated at moderate to high levels in respective lines. The acylsugars that are the darkest blue color in Figure 1.5 are accumulated at low levels or are not detectable in the respective line. If at least two of the three samples per genotype are above the background dark blue, that acylsugar is considered present in that line. Using this metric, CU071026 accumulates 23 of the 56 identifiable acylsugars. Not surprising, the FA2/AS line accumulated an increased number of acylsugars (31), some of which contained C11 and C13 acyl groups, likely the novel i-C11 and i-C13 fatty acid acyl groups detected in GC-MS analysis. LC-MS analysis revealed the FA7/AS and FA8/AS lines also accumulated an increased diversity of acylsugars over CU071026, (30 each),

comparable to the FA2/AS line. Unlike FA2QTL, which partly seems to increase acylsugar diversity by incorporating novel acyl groups, FA7QTL and FA8QTL seem to increase diversity by modulating increased or decreased incorporation of existing upregulated or downregulated fatty acid acyl groups. The FA5/AS line displayed a lower diversity of acylsugars due to the low levels of acylsugar in this line, likely resulting from the presence of the acylhydrolases discussed in Schilmiller et al. (2016). Taken together the LC-MS data indicate that addition of FA2QTL, FA7QTL and FA8QTL each leads to an increased diversity of acylsugars accumulated.

The additional acylsugars in the FA7/AS line did not result in a substantial tradeoff since all but one of the acylsugars moderately accumulated by CU071026 were still accumulated by the FA7/AS line (Figure 1.5). In the FA2/AS line, the addition of eight acylsugars resulted in a slight tradeoff; four acylsugars accumulated by CU071026 were no longer moderately accumulated in the FA2/AS line. The FA8/AS line, accumulated seven additional acylsugars, but this resulted in a strong tradeoff, (Figure 1.5), with only six acylsugars in common between CU071026 and the FA8/AS line.

Perspective to the diversity metrics discussed in this study can be gained by comparison with recent published work concerning acylsugar biosynthesis and chemistry (Schilmiller et al. 2010, 2015; Fan et al. 2016). It is evident that there is, at most, one long (C9 to C13) chain acyl group found in each of the 56 identifiable acylsugars accumulated by the fatty acid lines and CU071026 (Figure 1.5); the long chain acyl group is almost exclusively found in one location in tomato acylsucroses, whereas there are up to four positions at which short chain (C4, C5, C6) acyl groups are commonly found in tomatoes (Schilmiller et al. 2015; Fan et al. 2016). With this in mind, the added diversity in acylsugars provided by the addition of FA2QTL and FA7QTL have limited impact in diversifying the acylsugars produced since these two QTL mostly alter the long chain fatty acids. Despite the addition of novel fatty acids, the FA2/AS line only accumulates moderate levels of one additional acylsugar compared with the FA7/AS line because there are limited long chain acyl group attachment sites open to diversification. There

is not much tradeoff in acylsugar diversity produced in the FA2/AS and FA7/AS lines compared to CU071026, since these three lines have similar levels of ai-C5 and i-C5 fatty acids, and these short chain acyl groups are ubiquitous in almost all acylsugars accumulated by these lines. On the other hand, FA8QTL increases the diversity of short chain fatty acids, which leads to increased diversity of acylsugar chemotypes accumulated in the FA8/AS line, but the loss of some of the acylsugar chemotypes found in CU071026. The FA8/AS line has increased levels of i-C4, which is only found at low levels in CU071026, and greatly reduced levels of i-C5, which is the most common short chain acyl group in CU071026. Furthermore, since short chain acyl groups are part of every acylsugar identified in the fatty acid lines and CU071026 (Figure 1.5), there are few acylsugars in the FA8/AS line that do not incorporate at least one i-C4 acyl group. In fact, many of the acylsugars no longer accumulated at substantial levels in the FA8/AS line contain at least two C5 acyl groups. This replacement of at least one i-C5 group with one i-C4 acyl group is most likely responsible for the limited overlap of acylsugars between CU071026 and the FA8/AS line. This idea is supported by LC-MS data from Schilmiller et al. (2010) showing i-C5 replacement by i-C4 acyl groups in the line IL8-1-1 *vs.* M82. Additionally, Ning et al. (2015) showed that a feedback insensitive isopropylmalate synthase on chromosome 8 governs an increase in C4 and a decrease in C5 acyl groups in cultivated and wild tomato.

## CONCLUSIONS

An important question when transferring the FA5, FA7 and FA8QTL, was whether the QTL have the impact on acylsugar accumulation in the resulting line that was predicted based on prior QTL analysis. As noted by Bernardo (2008) regarding work in maize, the majority of QTL identified never find their way into released varieties. The common explanation for this observation across crops is that the QTL do not have the expected impact after transfer from the background in which they were discovered, possibly due to missing epistatic interactions.

When dealing with qualitative traits governed by one or two QTL, the likelihood of successful transfer and expected function is greater than when dealing with quantitative traits. Since there are many QTL that affect both acylsugar level and/or chemistry, it is difficult to predict how a QTL will behave in a different genetic background. For example, pertinent to FA5QTL, the recently discovered acylhydrolases (Schilmiller et al. 2016) that cleave particular acyl groups from specific locations on acylsucroses, depend on the ASAT genes, which is likely why the acylsucroses of *S. pennellii* LA716 are less subject to acyl group cleavage than the acylsucroses of tomato, such as in the FA5/AS line. Therefore, it is imperative to understand acylsugar biochemistry to successfully utilize QTL that impact acylsugar chemistry because of the potential for epistatic interactions.

Analysis of the new tomato lines bred by transfer of FA2QTL, FA5QTL, and FA7QTL showed alteration in acylsugar profiles that largely matched the expectations for these QTL based on QTL analysis. The existence of epistatic interactions among acylsugar QTL affecting acylsugar level, sugar component of acylsugars, and fatty acid components of acylsugars was already demonstrated in QTL mapping populations (Leckie et al. 2012, 2013, 2014), and will be a factor in development of lines with desired acylsugar levels and chemotypes. Production of lines combining two or more of the fatty acid QTL would be needed to test whether similar epistatic relationships are observed in the resulting lines.

The incorporation of FA2QTL, FA7QTL and FA8QTL into CU071026, creating the lines FA2/AS, FA7/AS, and FA8/AS, respectively, led to an increase in the diversity of either fatty acid acyl groups or acylsugars accumulated in these lines. A logical question to ask is whether the functionality of the acylsugars is altered by the increased diversity? From the results of Leckie et al. (2016) it is evident that acylsugar chemistry can have an impact on the efficacy of insect deterrence. It is possible that production of particular acyl groups, or modulation of the proportions of particular acyl groups, played a role in the differential insect control observed in that study. The results of Leckie et al. (2016) also demonstrate that synergy is an important

element in the functionality of acylsugars as a defense, which suggests that an increased diversity of acyl groups and acylsugars could lead to greater opportunity for synergy and therefore improved insect resistance. Whether the fatty acid lines differ for control of various insects, and the role of acyl group and acylsugar diversity in mediating the efficacy of insect resistance will be evaluated in future studies.

The tomato lines completed to date, and additional lines nearing completion, are being developed to serve as a research platform with broad utility. These lines could be used: (1) for research directed at further elucidating acylsugar biosynthesis and its regulation, such as the genes controlling FA2QTL and FA7QTL, which have not yet been elucidated (2) for a range of entomological research including efficacy of acylsugar-mediated control of diverse tomato insect and arthropod pest species by the different lines, identification of acylsugar levels and chemotypes with the optimal impact on each insect species, and study of the mechanism by which acylsugar mediated insect resistance operates against insect and arthropod pest species (3) to be utilized as breeding material for the transfer of acylsugar QTL to tomato lines with optimal acylsugar profiles for control of targeted insect and arthropod pest species with reduction/elimination of pesticides. As each line is completed, characterized, and its seed is sufficiently increased, the line will be provided, under MTA, upon request.

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## CHAPTER 2

## Combination of QTL Affecting Acylsugar Chemistry Reveals Additive and Epistatic Genetic Interactions to Increase Acylsugar Profile Diversity


### ABSTRACT

The tomato breeding line, CU071026, was bred to accumulate high levels of the insect control compounds called acylsugars, which are exuded from glandular trichomes. The acylsugars of CU071026 exhibit a characteristic profile of acylsugar composition and constituent fatty acid acyl groups that is distinct from that of the progenitor wild tomato, *Solanum pennellii* LA716. A prior study reported transfer of three QTL (FA2, FA7, and FA8), from *S. pennellii* LA716, that are associated with changes in acylsugar chemistry into CU071026 and demonstrated that the resulting lines, each of which possesses one of these QTL displayed a unique acylsugar and fatty acid profile distinct from that characteristic of the acylsugars of CU071026 and each other. The current study utilized marker-assisted backcrossing to combine pairs of two of these QTL or all three of these QTL. This created a new set of lines, which allowed evaluation of the combinatory effects of FA2QTL, FA7QTL, and FA8QTL, on acylsugar level and acylsugar and fatty acid profile and diversity. The resulting high-acylsugar accumulating tomato lines possessing combinations of two or all three QTL revealed that the QTL interacted additively and epistatically to alter acylsugar level and chemistry, increasing the diversity of fatty acid constituents and/or or acylsugar chemotypes present in the exudates of some of the lines. Extensive characterization of the lines for acylsugar level through a spectrophotometric invertase assay and acylsugar chemistry, through gas and liquid chromatography mass spectrometry allowed association of the QTL interactions with aspects of acylsugar chemotype. The evaluated fatty acids and acylsugars accumulated by the set of lines generally displayed high heritability and minimal environmental effect, which is discussed. The QTL interactions that

govern a more diverse acylsugar and fatty acid profile provide valuable information for the generation of tomato lines with improved acylsugar efficacy against pests of tomato.

## INTRODUCTION

Plants produce a large diversity of specialized metabolites, of which some mediate resistance to pathogens and herbivores and have been suggested as viable targets in breeding for sustainable pest control. One promising class of specialized metabolites receiving increased attention for their potential to provide resistance to a wide array of insect herbivores is a family of sugar polyesters known as acylsugars. Acylsugars are accumulated by various species in the nightshade family (Solanaceae), including several wild relatives of tomato, such as *Solanum pennellii*, *Solanum galapagense,* and *Solanum habrochaites* (Fobes et al. 1985; Burke et al. 1987; King et al. 1986, 1988; Shapiro et al. 1994; Kim et al. 2012; Schilmiller et al. 2015). Acylsugars are implicated in mediating a variety of plant-insect interactions, including feeding deterrence and oviposition preference by a number of insect species (Severson et al. 1985, Goffreda and Mutschler 1989; Hawthorne et al. 1992; Rodriguez et al. 1993; Juvik et al. 1994, Liedl et al. 1995, Fancelli et al. 2005; Leckie et al. 2016).

*Solanum pennellii* (Correll) D'Arcy accession LA716 accumulates substantial amounts of acylsugars which have been shown to effectively control many insects and provides a promising source of direct insect resistance (Goffreda et al. 1990; Hawthorne et al. 1992; Rodriguez et al. 1993; Juvik et al. 1994; Shapiro et al. 1994; Liedl et al. 1995) that can be transferred to cultivated tomato (Mutschler and Wintermantel 2006; Leckie et al. 2012). The Cornell benchmark acylsugar tomato line, CU071026, was bred from *S. pennellii* LA716, and contains five introgressions from *S. pennellii* LA716 on chromosomes 2, 3, 7 and 10, which are called AS2, AS3, AS7, AS10.1, and AS10.2, respectively (Leckie et al. 2012). The acylsugars secreted by CU071026 and related lines significantly reduced *Bemisia tabaci* oviposition on lines grown

in field cages (Leckie et al. 2012) and acylsugar producing hybrids reduced the incidence of tomato infectious chlorosis virus in fields with heavy pressure from the whitefly *Trialeurodes vaporariorum* (Mutschler and Wintermantel 2006).

In wild and cultivated tomato species, the acylsugars produced and secreted from glandular trichomes are composed of a sugar backbone (sucrose or glucose) to which several short to medium chain aliphatic acids are esterified. These fatty acids can be either straight-chained or branched (Fobes et al. 1985; Burke et al. 1987; Shapiro et al. 1994; Schilmiller et al. 2010, 2012, 2016; Fan et al. 2016). *S. pennellii* accessions have various acylsugar chemotypes (number, length and branching orientation of acyl groups and to which sugar they are esterified) that vary with geographical location (Shapiro et al. 1994 and Ning et al. 2015), suggesting the possibility of adaptation and selection of specific metabolic profiles in response to local herbivore pressures. The acylsugars of *S. pennellii* LA716 are predominantly acylglucoses with a characteristic array of fatty acids including 2-Methylpropanoate (i-C4), 2-Methylbutanoate (ai-C5), 3-Methylbutanoate (i-C5), 8-Methylnonanoate (i-C10), n-Decanoate (n-C10), and n-Dodecanoate (n-C12) (Burke et al. 1987; Shapiro et al. 1994; Blauth et al. 1999). In contrast, the profile of CU071026, bred from *S. pennellii* LA716, is almost exclusively acylsucroses with predominantly ai-C5, i-C5 and n-C12 fatty acids, and only trace or undetectable levels of i-C4, i-C10 and n-C10 (Leckie et al. 2014). The fatty acid profile of CU071026 is similar to that of cultivated tomato, which also predominantly accumulates i-C5, ai-C5 and n-C12 fatty acids (Schilmiller et al. 2010 and Ghosh et al. 2014).

Leckie et al. (2016) evaluated the deterrence effect of purified acylsugars from CU071026 and several *S. pennellii* accessions, including *S. pennellii* LA716 on thrips and whitefly feeding and oviposition. Results indicated that the purified acylsugars of CU071026 were less effective at equimolar levels than purified acylsugars of the *S. pennellii* accessions (especially *S. pennellii* LA1376) at controlling whitefly (*B. tabaci*) and western flower thrips (*Frankliniella occidentalis*) feeding and oviposition in laboratory assays. Furthermore, the results

of Leckie et al. (2016) revealed synergistic interaction between acylsugar fractions, which led to improved insect resistance. These results suggest that greater diversity of acylsugars could lead to synergistic interactions and greater efficacy against insects. An increased diversity of acylsugars and fatty acid acyl groups could also provide a sophisticated plant defense that impedes the selection of insects that can overcome acylsugar mediated resistance. These hypotheses are in agreement with a number of other studies evaluating plant-herbivore interactions with a focus on specialized metabolites that suggested chemical diversity could decrease insect feeding, adaptation and survival (Duffey and Stout 1996; Castellanos and Espinosa-Garcia 1997; Akhtar and Isman 2003). The mechanism of resistance mediated by acylsugars has not been elucidated, but as acylsugars are broadly deterrent to insects, understanding of the mode of action could have a significant impact on breeding for resistance to many insects.

QTL from *S. pennellii* LA716 that impact acylsugar chemistry have been identified (Blauth et al. 1998, 1999; Schilmiller et al. 2010, 2012, 2015; Leckie et al. 2013, 2014; Fan et al. 2016). Some QTL were shown to alter the chemotype of acylsugar accumulating tomato lines (Schilmiller et al. 2010) such as the mono-introgression lines (ILs) created by Eshed and Zamir (1994, 1995). Addition of several fatty acid altering QTL (FA2QTL, FA7QTL, FA8QTL) into CU071026 led to the creation of three fatty acid acylsugar lines (FA2/AS, FA7/AS, and FA8/AS respectively) that produced high levels of acylsugars with fatty acid profiles distinct from that of CU071026 (Leckie et al. 2014; Smeda et al. 2016). Characterization of the acylsugars produced by this new set of tomato lines revealed that FA2QTL mediates production of novel fatty acids not significantly accumulated in CU071026 (i-C11 and i-C13), and FA7QTL and FA8QTL moderate variation in the proportions of several fatty acids, such as i-C4, i-C5 and n-C10. Additionally, LC-MS analysis demonstrated that all three QTL function in the resulting lines and lead to accumulation of a greater diversity of acylsugars over CU071026 (Smeda et al. 2016). While these three lines have increased acylsugar and or fatty acid diversity compared to

CU071026, the acylsugar compound diversity of FA2/AS, FA7/AS and FA8/AS is still far less than that of the *S. pennellii* accessions tested by Leckie et al (2016).

The objectives of this study were to combine the previously introgressed FAQTL (FA2QTL, FA7QTL and FA8QTL) in all possible pairs and all together in pursuit of lines with increased acylsugar and fatty acid diversity over the single QTL lines. After generating these lines, we wanted to characterize them for acylsugar level, acylsugar profile, and fatty acid profile to enhance the current platform of knowledge and germplasm. Several of the QTL were combined with FA5QTL, which was previously linked to low acylsugar level (Leckie et al. 2014; Schilmiller et al. 2010, 2016; Smeda et al. 2016) to determine if the low acylsugar phenotype could be rescued through combination with some of the fatty acid QTL (FAQTL). Additionally, we attempted to identify combinations of QTL that would result in a greater quantity of acylsugars accumulated and or increased diversity of acylsugars and fatty acids to increase the likelihood of synergistic interactions and improved efficacy of insect deterrence. Specifically, we wanted to determine what degree of fatty acid and acylsugar profile diversity could be obtained through combination of the FAQTL, and if compound tradeoffs, such as the simultaneous gain/loss of accumulation of particular acylsugars similar to that observed in Smeda et al. (2016), would restrict our ability to increase the number of acylsugars and fatty acids and profile diversity in the combination FAQTL germplasm above that observed in the single QTL lines. We anticipated that combination of the FAQTL would lead to a greater number of compounds and a more diverse fatty acid and acylsugar profile than the single FAQTL lines. Finally, we sought to evaluate the heritability of selected fatty acids and acylsugars and the impact of the environment on these traits to inform breeding objectives, such as increasing the diversity of the acylsugar and fatty acid profiles. We characterized the acylsugars accumulated by the combination QTL lines through a spectrophotometric acylsugar assay to measure total acylsugar level, GC-MS analysis to evaluate the type and percentage of fatty acid acyl groups present, and LC-MS to determine the relative proportions of acylsugars accumulated. Discussion of the implications of

these data includes evaluation of the interactive effect of the FAQTL on acylsugar level, fatty acid acyl group diversity and acylsugar diversity.

## MATERIALS AND METHODS

### Plant materials

CU071026 is an acylsugar-producing tomato line bred using *S. pennellii* LA716 as the donor of the QTL necessary for substantial acylsugar accumulation. See Supplementary Table 1 of Leckie et al. 2012 for markers and map positions of the *S. pennellii* LA716 introgressions in CU071026. The acylsugar lines that each possess one of the fatty acids QTL (FA2QTL, FA7QTL, FA5QTL or FA8QTL) are named FA2/AS, FA5/AS, FA7/AS and FA8/AS, respectively. These acylsugar-producing tomato lines with modified fatty acid profiles were developed by the Cornell University tomato-breeding program, bred as described in Smeda et al (2016) using CU071026 and specific *S. pennellii* LA716 mono-introgression lines developed by Eshed and Zamir (1994, 1995). Visual representation of the introgressions contained in CU071026 and the single fatty acid QTL acylsugar lines is depicted in Figure 1.4.

### Plant growth conditions

Seed were germinated in 32 cell flat cups with LM1 (Lambert, Rivière-Ouelle, Quebec, Canada) mix until *ca.* 5 weeks of age, during which time any necessary marker based genetic analysis could be completed. Selected plants were transplanted to 8 inch clay pots of LM111 (Lambert, Rivière-Ouelle, Quebec, Canada) mixed with turface (Turface Athletics, Buffalo Grove, IL) in a 1:1.8 ratio, with 0.3% unimix (10-5-10) and calcium sulfate additive. Plants for all populations and experiments were grown in a greenhouse in the Guterman Bioclimatic Laboratory and

Greenhouse Complex at Cornell University in Ithaca NY, and were typically maintained at 29°C: 20C day night temperatures with a 16:8 hr light:dark photoperiod.

**Genotypic screening**

Identity and location of all molecular markers utilized to select for CU071026 regions can be found in Table 2.1. Identity and location of markers used to pyramid FA2QTL, FA7QTL, and FA8QTL can be found in Table 2.2.

Table 2.1. Markers and map locations delineating CU071026 introgressions based on Tomato-EXPEN SL2.50 ITAG2.4

| Marker[a] | Chromosome | Start Position (Bp) | End Position (Bp) |
|---|---|---|---|
| C2_At4g37300 | 2 | 53,834,679 | 53,835,655 |
| C2_At3g26900 | 2 | 54,947,728 | 54,950,298 |
| solcap_snp_sl_63290[b] | 3 | 1,390,271 | 1,390,304 |
| TG130 | 3 | 1,755,716 | 1,756,224 |
| C2_At5g24120 | 3 | 1,914,316 | 1,920,336 |
| C2_At3g02420 | 3 | 11,509,743 | 11,514,318 |
| C2_At5g23060 | 3 | 64,448,262 | 64,451,474 |
| C2_At3g15430 | 7 | 65,800,017 | 65,803,497 |
| TG303 | 10 | 1,773,625 | 1,774,114 |
| C2_At5g60990 | 10 | 1,853,562 | 1,864,123 |
| SSR85 | 10 | 61,580,912 | 61,581,577 |
| C2_At3g12290 | 10 | 62,141,901 | 62,147,562 |

[a] Full marker information provided by The Sol Genomics Network (SGN; http://solgenomics.net/)
[b] A cleaved amplified polymorphic sequence (CAPS) marker was designed from this SNP

Table 2.2. Markers and map locations used to introgress FAQTL based on Tomato-EXPEN SL2.50 ITAG2.4

| Marker[a] | Chromosome | Start Position (Bp) | End Position (Bp) |
|---|---|---|---|
| solcap_snp_sl_51242[b] | 5 | 16,840,699 | 16,840,733 |
| T1601 | 5 | 41,511,439 | 41,512,805 |
| solcap_snp_sl_69382[b] | 5 | 54,980,534 | 54,980,568 |
| solcap_snp_sl_29[b] | 5 | 60,842,571 | 60,842,605 |
| C2_At1g10500 | 5 | 62,107,578 | 62,107,796 |
| solcap_snp_sl_25859[b] | 5 | 62,738,979 | 62,739,013 |
| TG23 | 5 | 63,347,293 | 63,348,190 |
| C2_At2g26590 | 7 | 6,878,227 | 6,899,018 |
| C2_At4g29490 | 7 | 32,758,106 | 32,807,954 |
| solcap_snp_sl_52215[b] | 7 | 38,290,705 | 38,290,739 |
| solcap_snp_sl_70150[b] | 7 | 51,197,900 | 51,197,934 |
| solcap_snp_sl_100893[b] | 7 | 54,044,220 | 54,044,254 |
| C2_At2g30520 | 7 | 56,723,902 | 56,725,342 |
| U221657 | 8 | 3,279,725 | 3,283,085 |
| solcap_snp_sl_51919[b] | 8 | 3,781,348 | 3,781,382 |
| solcap_snp_sl_51931[b] | 8 | 4,103,545 | 4,103,579 |
| solcap_snp_sl_51969[b] | 8 | 4,744,567 | 4,744,601 |
| solcap_snp_sl_69286[b] | 8 | 5,597,495 | 5,597,529 |
| solcap_snp_sl_69336[b] | 8 | 9,125,184 | 9,125,218 |
| C2_At5g27390 | 8 | 22,850,567 | 22,857,802 |
| C2_At1g30360 | 8 | 27,897,600 | 27,905,822 |
| C2_At4g33030 | 8 | 52,633,704 | 52,637,369 |

[a] Full marker information provided by The Sol Genomics Network (SGN; http://solgenomics.net/).
[b] A cleaved amplified polymorphic sequence (CAPS) marker was designed from this SNP

## Phenotypic screening

*Acylsugar level* Levels of acylsugar for control plants and populations in the development of the combination FA/QTL lines were measured on 9-10-week-old plants using the method of Leckie et al. (2012), which was modified from a prior method described by Goffreda et al. (1990). For the segregating populations, four samples of two primary lateral leaflets were taken per plant. For the replicated screen, four plants of each entry were sampled, collecting four samples of two primary lateral leaflets from leaves that were two to three nodes from the apex of stems. Each two-leaflet sample was placed in a wide mouth plastic scintillation vial and completely dried in a seed dryer at $29^0$ C. Fully dried leaflets were rinsed with 3 ml of methanol

containing methyl heptanoate (30 mg $L^{-1}$), an internal standard for fatty acid analysis. The assay uses 100 ul of each rinsate. Leaflets were re-dried after rinsing and weighed, so that acylsugar level could be expressed per weight dried leaf. Acylsugar level data were analyzed using ANOVA in JMP Pro 12 (SAS Institute Inc. 2015), and means were separated by Tukey-Kramer HSD ($p < 0.05$). Prior to analysis, acylsugar level data were $Ln_{(x)}$ transformed to improve normality.

*Fatty acid characterization* Percentages of each type of fatty acids from each sample were ascertained by collecting pairs of young, fully expanded primary lateral leaflets, rinsing leaflets with 3 ml of methanol containing methyl heptanoate (30 mg $L^{-1}$) as an internal standard, and then utilizing transmethylation/GC-MS analysis, as described in Leckie et al. (2014). Peak areas of the resulting chromatograms were calculated using Varian MS Workstation Version 6.9.1 (Agilent Technologies, Santa Clara, CA) and levels of respective fatty acids were determined through comparison with levels of the internal standard to generate relative proportions of each fatty acid. Percent fatty acid GC-MS data was analyzed using ANOVA in JMP Pro 12 (SAS Institute Inc. 2015), and means separated by Tukey-Kramer HSD ($p < 0.05$). Prior to analysis, data for i-C4, n-C10 and n-C12 aliphatic acids were cube root $_{(x + 0.1)}$ transformed and the data for ai-C5, i-C9, i-C10, i-C12, i-C13 (11-methyldodecanoate) and i-C14(likely 12-methyltridecanoate) were $\log_{10(x+1)}$ transformed to improve normality.

*Acylsugar composition characterization* LC-MS was utilized to analyze the composition of acylsugars accumulated in each line. Three samples of a single primary lateral leaflet per genotype were taken and extracted with a buffer consisting of isopropanol:acetonitrile:water (3:3:2 v/v/v) containing 0.1% formic acid and 10 µM of propyl-4- hydroxybenzoate, an internal standard, and processed as described in Schilmiller et al. (2015). Acylsugar results from the LC-MS analysis are described using the nomenclature of Schilmiller et al. (2010), in which the

acylsugar name S4:17 indicates a sucrose backbone sugar, with four fatty acid acyl chains that have a total of 17 carbons. LC-MS data were analyzed by hierarchical clustering with a Pearson correlation using pairwise average-linkage clustering for both genotypes and acylsugars using the hierarchical clustering tools provided by GenePattern (Reich et al. 2006).

**Heritability estimation and environmental impact on traits**

Broad sense heritability estimates and environmental impact for fatty acids were calculated using the dataset from Smeda et al. (2016), but with an additional year of data from 2016. Acylsugar heritability and environmental impact was calculated from data spanning 2015 and 2016 from two greenhouse experiments. Heritability for the major fatty acids and acylsugars (only those whose levels were at least 1% of the internal standard) was calculated according to Holland et al. (2003) using variances obtained from the lmer function in the lme4 package in R (Bates et al. 2014) where genotype, location, year, genotype by location, and genotype by year were treated as random effects. The lmer function was used to fit a second model with genotype, location and genotype by location interaction effects, where each was treated as a random effect to calculate the relative variance contribution from environment and genotype by environment interaction.

**Non metric multidimensional scaling (NMDS)**

NMDS analysis for Figure 2.1, 2.2 and 2.3 was performed in R using the meta MDS function in the Vegan Package (Oksanen et al. 2013). Ten-twelve samples from each genotype were used for calculation, and prior to the analysis data were square-root transformed and then standardized using a Wisconsin double standardization, which is the default data treatment in the meta MDS function to diminish the range of values. A data matrix of pairwise comparisons among samples was calculated using the Bray-Curtis distance index, for NMDS analysis, which ranges between 0 and 1. NMDS was used to find the best low-dimensional representation of the

distance matrix that minimized stress. The NMDS analysis was performed using two dimensions, which resulted in a stress of 0.1, with profiles of plants of the same genotype clustering well. The solution was rotated according to PCA (Vegan default) so that the largest variance of samples was on the first axis.

**Shannon's diversity index (H')**

Assessments of profile diversity for fatty acids and acylsugars was conducted in R using the diversity function within the Vegan package. Shannon's Diversity Index has been adapted from ecological species diversity analyses to evaluate plant chemical profiles (Becerra et al. 2009; Cacho et al. 2015). H' values were calculated for fatty acids as proportions and as presence/absence, whereby fatty acid data were recoded 0 for absence and 1 for presence of a compound within each acylsugar line. Similarly, H' values were calculated for acylsugar profile, both as relativized peak areas, and as presence/absence, whereby acylsugar data were recoded 0 for absence and 1 for presence of an acylsugar within each acylsugar line. The specific diversity index values are not informative, but increased index values correspond to an increased profile diversity.

## RESULTS

### *Combination of FA2QTL/FA7QTL and initial assessment of impacts on acylsugar level and fatty acid profile*

An F1 plant (131540-2) heterozygous for FA2QTL was crossed with a selection (131285-176) from the BC1F1 FA7QTL population (Smeda et al. 2016) that was heterozygous for the *ca.* 3.2 Mbp FA7QTL sub introgression from IL7-4-1 and also heterozygous for the AS2 and AS7 *S. pennellii* introgressions from CU071026, resulting in a 32 plant F1 population. Three plants were identified that lacked both FA2QTL and FA7QTL, and two plants were identified that were

heterozygous for both FA2QTL and FA7QTL. One plant each was also identified that were heterozygous for just FA2QTL or FA7QTL.

Evaluation of the acylsugar level and fatty acid profile of the selections from the F1 population were as conducted. Those plants that were homozygous for the AS2, AS3, AS10.1 and AS10.2 *S. pennellii* introgressions of CU071026 and heterozygous for AS7 and both FA2QTL and FA7QTL accumulated high levels of acylsugars (Table 2.3). Specifically, selections that contained both FA2QTL and FA7QTL accumulated acylsugars at 125.1% the level of CU071026, comparable to internal control selections lacking either QTL, which accumulated acylsugars at 117.8% the level of CU071026. The fatty acid profile of the selections revealed that plants containing the FA2QTL and FA7QTL individually in the heterozygous condition accumulated increased proportions of i-C11/i-C13 (9.3% and 1.0%, respectively) and n-C10 (7.6%) fatty acids, respectively (Table 2.3). The selections lacking both FA2QTL and FA7QTL accumulated low to trace levels of i-C11/i-C13 (0.0% and 0.0%, respectively) and n-C10 (2.2%), and the selections heterozygous for AS7 and both FA2QTL and FA7QTL accumulated increased proportions of both i-C11 (8.7%) and n-C10 (5.5%), but low levels of i-C13 (0.1%). In addition, the selections heterozygous for AS7/FA2QTL/FA7QTL accumulated detectable levels of i-C9 (0.5%) which was not detectable in the other haplotypes.

Table 2.3. Initial characterization of the heterozygous effect of FA2QTL and FA7QTL on acylsugar level and fatty acid profile

| QTL[ab] | | n[c] | Acylsugar as percent of CU071026 (%)[de] | Selected fatty acid profile (%)[de] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| FA2 | FA7 | | | i-C4 | ai-C5 | i-C5 | i-C9 | n-C10 | i-C11 | n-C12 | i-C13 |
| 1 | 1 | 3 | 117.8 a | 2.7 a | 15.0 a | 54.0 a | 0.0 b | 2.2 c | 0.0 b | 25.8 a | 0.0 c |
| 1 | 2 | 1 | 104.2 a | 2.6 a | 12.1 a | 49.0 a | 0.0 b | 7.6 a | 0.1 b | 28.1 a | 0.0 c |
| 2 | 1 | 1 | 137.6 a | 4.3 a | 15.6 a | 57.5 a | 0.0 b | 0.9 d | 9.3 a | 11.2 b | 1.0 a |
| 2 | 2 | 2 | 125.1 a | 2.7 a | 13.8 a | 49.3 a | 0.5 a | 5.5 b | 8.7 a | 19.0 ab | 0.1 b |

[a] 1 = locus homozygous for *S. lycopersicum* alleles; 2 = locus heterozygous for *S. lycopersicum* / *S. pennellii* LA716
[b] Haplotype 1 1 are selections derived in this F1 population and are equivalent to CU071026
[c] Number of plants per haplotype that were identified and averaged for acylsugar level and fatty acid profile
[d] Means followed by different letters within a column are significantly different at $P < 0.05$
[e] i-C4, n-C10, n-C12 Cuberoot(x+.1) transformed, % CU071026 ln(x) transformed and ai-C5, i-C9, i-C10, i-C12, i-C13 Log10(x+.1) transformed prior to analysis to improve normality

A 187 plant F2 population was generated from one of the selections in the F1 that was segregating for FA2QTL, FA7QTL, and the AS7 introgression from CU071026. Eight of the 187 plants had the desired recombination so that the AS7 and FA7QTL regions were both homozygous and one of the eight recombinant plants was also homozygous for FA2QTL. Six selections were identified that were homozygous for both FA2QTL and FA7QTL, but that had lost the AS7 CU071026 introgression. Additionally, one plant was identified that was homozygous for just FA2QTL, and two plants were identified that were homozygous for just the FA7QTL. Characterization of the acylsugar level and fatty acid profile of the selections from the F2 population was conducted and suggested interaction between the QTL on acylsugar level and fatty acid profile. In the F2 population selections, plants homozygous for just the FA2QTL or FA7QTL displayed higher acylsugars levels, 149.6% and 126.6% the level, respectively, of CU071026. Additionally, we observed that plants homozygous for both FA2QTL and FA7QTL accumulated increased levels of acylsugars as well, compared to CU071026 (Population I Table 2.4). Specifically, the six selections homozygous for FA2QTL/FA7QTL, but lacking the AS7 introgression, accumulated 170% the level of acylsugars of CU071026, and the selection

homozygous for AS7/FA2QTL/FA7QTL accumulated 150.3% the level of acylsugars of CU071026.

Table 2.4. Initial characterization of the homozygous combination of FAQTL in four populations: I (FA2/FA7), II (FA2/FA8), III (FA7/FA8), IV (FA2/FA7/FA8) in the presence and absence of the AS7 CU071026 *S. pennellii* introgression

| Population | QTL[ab] | | | | n[c] | Acylsugar as percent of CU071026 (%)[de] | Selected fatty acid profile (%)[de] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | FA2 | FA7 | AS7 | FA8 | | | i-C4 | ai-C5 | i-C5 | i-C9 | i-C10 | n-C10 | i-C11 | i-C12 | n-C12 | i-C13 |
| I | 1 | 1 | 3 | 1 | 3 | 100.0 b | 0.3 a | 11.4 ab | 55.6 a | 0.0 b | 0.0 b | 1.0 b | 0.0 b | 0.0 a | 31.2 a | 0.0 c |
| | 3 | 1 | 1 | 1 | 1 | 149.6 ab | 0.7 a | 11.3 ab | 63.5 a | 0.0 b | 0.1 a | 0.6 b | 5.0 a | 0.0 a | 16.8 b | 1.6 a |
| | 1 | 3 | 1 | 1 | 2 | 126.6 b | 0.5 a | 10.5 b | 51.5 a | 0.0 b | 0.0 b | 7.1 a | 0.0 b | 0.0 a | 29.9 ab | 0.0 c |
| | 1 | 3 | 3 | 1 | 1 | 125.0 ab | 0.2 a | 16.7 a | 55.9 a | 0.0 b | 0.0 b | 4.6 a | 0.0 b | 0.0 a | 22.5 ab | 0.0 c |
| | 3 | 3 | 1 | 1 | 6 | 170.0 a | 0.4 a | 12.4 ab | 53.5 a | 0.2 a | 0.1 a | 5.5 a | 3.8 a | 0.0 a | 23.5 ab | 0.1 b |
| | 3 | 3 | 3 | 1 | 1 | 150.3 ab | 0.7 a | 12.0 ab | 53.6 a | 0.3 a | 0.1 a | 6.0 a | 3.5 a | 0.0 a | 23.2 ab | 0.1 b |
| II | 1 | 1 | 3 | 1 | 3 | 100.0 a | 0.3 c | 11.4 b | 55.6 a | 0.0 b | 0.0 b | 1.0 a | 0.0 b | 0.0 b | 31.2 b | 0.0 b |
| | 1 | 1 | 3 | 3 | 2 | 86.6 a | 9.4 b | 9.6 b | 20.9 c | 0.0 b | 0.0 b | 1.3 a | 0.1 b | 0.0 b | 58.3 a | 0.0 b |
| | 3 | 1 | 3 | 3 | 4 | 91.5 a | 15.0 a | 16.1 a | 41.7 b | 0.1 a | 3.1 a | 0.7 b | 7.1 a | 0.9 a | 11.8 c | 2.8 a |
| III/IV | 1 | 1 | 3 | 1 | 3 | 100.0 b | 0.8 d | 13.5 b | 57.7 a | 0.0 b | 0.0 c | 0.4 c | 0.0 c | 0.0 b | 27.1 c | 0.0 b |
| | 3 | 1 | 3 | 1 | 3 | 87.0 bc | 3.6 c | 17.5 a | 60.2 a | 0.0 b | 0.1 b | 0.1 c | 9.9 a | 0.0 b | 6.6 d | 1.9 a |
| | 1 | 3 | 3 | 1 | 3 | 139.8 a | 0.6 d | 11.9 bc | 52.1 b | 0.0 b | 0.0 c | 4.9 b | 0.0 c | 0.0 b | 30.1 bc | 0.0 b |
| | 1 | 1 | 3 | 3 | 3 | 53.1 e | 18.3 a | 14.1 ab | 21.5 c | 0.0 b | 0.0 c | 0.5 c | 0.0 c | 0.0 b | 45.4 a | 0.0 b |
| | 1 | 3 | 1 | 3 | 2 | 65.6 de | 14.0 ab | 10.3 c | 17.6 cd | 0.0 b | 0.0 c | 9.5 a | 0.1 c | 0.0 b | 48.0 a | 0.0 b |
| | 1 | 3 | 3 | 3 | 1 | 73.9 cd | 12.0 b | 11.6 bc | 13.2 d | 0.0 b | 0.0 c | 9.3 a | 0.0 c | 0.0 b | 53.5 a | 0.0 b |
| | 3 | 3 | 3 | 3 | 1 | 108.3 ab | 17.3 a | 13.8 ab | 13.6 d | 0.1 a | 9.3 a | 6.0 ab | 2.6 b | 0.7 a | 36.0 b | 0.0 b |

[a] 1 = locus homozygous for *S. lycopersicum* alleles; 3 = locus homozygous for *S. pennellii* LA716 alleles

[b] Plants with haplotype 1 1 3 1 for FA2, FA7, AS7, and FA8 are CU071026

[c] Number of plants with respective haplotype that were identified and averaged for acylsugar level and fatty acid profile

[d] Means followed by different letters within a column are significantly different at $P < 0.05$

[e] i-C4, n-C10, n-C12 Cuberoot(x+.1) transformed, % CU071026 ln(x) transformed and ai-C5, i-C9, i-C10, i-C12, i-C13, i-C14 Log10(x+.1) transformed prior to analysis

The fatty acid profile of the selections homozygous for just FA2QTL or FA7QTL showed the characteristic increase in i-C11/i-C13 (5.0/1.6%) and n-C10 (6.3%), respectively (Population I Table 2.4). Consistent with the FA2QTL/FA7QTL F1 selections, plants homozygous for FA2QTL/FA7QTL exhibited an increase in both n-C10 (ca. 5.6%) and i-C11 (ca 3.8%), but low levels of i-C13 (ca. 0.1%). The FA2QTL/FA7QTL selections also accumulated increased levels of i-C9 (ca. 0.2%), which was undetectable in the other haplotypes. We also observed that the selection homozygous for FA2QTL/FA7QTL and homozygous for the AS7 introgression had a profile consistent with the six selections homozygous for FA2QTL/FA7QTL, but lacking the AS7 introgression. The selection homozygous for FA2QTL, AS7 and FA7QTL was selected as the initial plant for the new line, FA2/FA7/AS. However, since this selection had reduced seed set, we also selected a higher seed set plant homozygous for FA2QTL and FA7QTL, but that lost the AS7 introgression, as the initial plant for the new line, FA2/FA7/-AS7/AS.

***Combining FA2QTL/FA8QTL and initial assessment of impacts on acylsugar level and fatty acid profile***

Parallel to combining FA2QTL/FA7QTL, pyramiding FA2QTL/FA8QTL was also begun to characterize the interaction of these QTL in the homozygous condition on the accumulated acylsugars. An F1 plant (131540-4), heterozygous for FA2QTL, and equivalent to the 131540-2 plant used to pyramid FA2QTL/FA7QTL, was crossed to the FA8/AS line (Smeda et al. 2016). The resulting 32 plants of the F1 population segregated for the introgression containing FA2QTL, with all plants heterozygous for the full length introgression carrying FA8QTL. Four plants were identified that were heterozygous for just FA8QTL, and 4 plants were identified that were heterozygous for both FA2QTL and FA8QTL.

The acylsugar levels and fatty acid profile of the selections and control CU071026 plants were evaluated (Table 2.5). The levels of acylsugars accumulated in plants heterozygous for just FA8QTL were comparable to the levels of acylsugars accumulated in CU071026 plants.

Conversely, the plants heterozygous for FA2QTL/FA8QTL were slightly higher at 124.4% the levels of acylsugars accumulated in CU071026. The fatty acid profiles of the CU071026 plants and selections heterozygous for just FA8QTL were quite similar, with the plants containing FA8QTL slightly higher for i-C4 fatty acids. The selections containing the FA2QTL/FA8QTL together in the heterozygous condition exhibited an increase in i-C11/i-C13 fatty acids at 8.1% and 1.0%, respectively, typical of plants containing FA2QTL, as well as a slight increase in i-C4 fatty acids.

Table 2.5. Initial characterization of heterozygous effect of FA2QTL and FA8QTL on acylsugar level and fatty acid profile

| QTL[ab] | | n[c] | Acylsugar as percent of CU071026 (%)[d] | Selected fatty acid profile (%)[de] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| FA2 | FA8 | | | i-C4 | ai-C5 | i-C5 | n-C10 | i-C11 | n-C12 | i-C13 |
| 1 | 1 | 4 | 100.0 b | 1.9 b | 12.6 a | 52.4 b | 2.3 a | 0.1 b | 30.2 a | 0.0 b |
| 1 | 2 | 4 | 107.0 b | 3.4 a | 12.8 a | 50.4 b | 2.5 a | 0.1 b | 30.3 a | 0.0 b |
| 2 | 2 | 4 | 124.4 a | 3.1 a | 13.2 a | 55.7 a | 1.6 b | 8.1 a | 16.6 b | 1.0 a |

[a] 1 = locus homozygous for S. lycopersicum alleles; 2 = locus heterozygous for S. lycopersicum / *S. pennellii* LA716
[b] Haplotype 1 1 is CU071026
[c] Number of plants per haplotype that were identified and averaged for acylsugar level and fatty acid profile
[d] Means followed by different letters within a column are significantly different at $P < 0.05$
[e] i-C4, n-C10, n-C12 Cuberoot(x+.1) transformed, and ai-C5, i-C13 Log10(x+.1) transformed prior to analysis to improve normality

An F2 population of 160 plants was generated from an F1 selection heterozygous for both FA2QTL and FA8QTL to evaluate the interactive effect of these QTL in the homozygous condition. Two of the 160 F2 plants screened were identified to be homozygous for just FA8QTL, and four plants were identified that were homozygous for both FA2QTL and FA8QTL. The acylsugar levels and fatty acid profiles of the selections and CU071026 control were evaluated (Population II Table 2.4). Selections homozygous for the FA8QTL alone accumulated lower levels of acylsugars (86.6% of CU071026) similar to selections homozygous for

FA2QTL/FA8QTL which accumulated levels of acylsugars at 91.5% the level of CU071026. Selections homozygous for just the recessive FA8QTL accumulated a characteristic increase of i-C4 fatty acid proportion (9.4%) compared to CU071026 (0.3% i-C4). Similarly, the selections homozygous for FA2QTL/FA8QTL also accumulated an increase in i-C4 (15.0%), but also accumulated an increase in i-C11/i-C13 (7.1% and 2.8%, respectively), characteristic of the presence of FA2QTL. In addition, the FA2QTL/FA8QTL selections displayed a significant accumulation of i-C10 (3.1%) and i-C12 (0.9%) fatty acids, which were not detectable in selections containing just FA8QTL, or in the CU071026 control. It was also observed that the selections homozygous for both FA2QTL/FA8QTL accumulated higher levels of i-C5 fatty acids and much lower levels of n-C12 fatty acids than selections homozygous for just FA8QTL. One of the four selections homozygous for FA2QTL/FA8QTL with the best seed set was chosen to be the initial plant to establish the new line, FA2/FA8/AS.

***Combining FA7QTL/FA8QTL and FA2/FA7QTL/FA8QTL and initial assessment on acylsugar level and fatty acid profile***

Simultaneously with the combination of FA2QTL/FA7QTL and FA2QTL/FA8QTL, breeding was initiated to combine FA7QTL and FA8QTL, and FA2QTL, FA7QTL, and FA8QTL to observe the interaction of FA7QTL and FA8QTL, and all three QTL when homozygous, on the accumulated acylsugars. An F1 between the FA8/AS line and a selection heterozygous for FA2QTL, FA7QTL, and the AS7 region was made, and the resulting 186 plant F1 population segregated for the FA2QTL, FA7QTL, and AS7 introgressions, with all plants heterozygous for the full length introgression (ca. 50 Mbp) (Smeda et al. 2016) carrying FA8QTL. Two selections heterozygous for FA2QTL, FA7QTL, the AS7 introgression, and FA8QTL, (151142-016 and 151142-088) were used to generate F2 populations of 192 plants each, to obtain plants homozygous for both FA7QTL and FA8QTL, and all three FAQTL.

Out of the two 192 plant populations, 13 plants were identified as being homozygous for both FA7QTL and AS7, which required recombination to bring into coupling. Of these 13 selections, only one was also homozygous for the FA8QTL introgression and had lost the FA2QTL introgression. In addition, two other selections out of the population were homozygous for FA7QTL, and FA8QTL, but had lost the AS7 introgression. Out of the 13 selections with the necessary recombination between FA7QTL and AS7, one plant was also identified that was homozygous for FA2QTL, FA7QTL and FA8QTL.

The acylsugar level and fatty acid profile of the selections was evaluated (Population III/IV Table 2.4). The level of acylsugars in the FA8/AS controls was low, at 53.1% of CU071026, while the level of acylsugars accumulated in FA7/AS controls were higher, at 139.8% the level of CU071026. For the selection homozygous for FA7QTL/FA8QTL, and homozygous for AS7 from CU071026, the level of acylsugars accumulated was also lower, at 73.9% the level of acylsugars in CU071026. Similarly, the level of acylsugars accumulated in the selections homozygous for FA7QTL/FA8QTL, but lacking the AS7 introgression were low, at 65.6% the level of CU071026. The plant identified that was homozygous for FA2QTL/FA7QTL/FA8QTL and homozygous for the AS7 introgression accumulated high levels of acylsugars at 108.3% the level of CU071026. Evaluation of the fatty acid profile revealed that the selections homozygous for FA7QTL/FA8QTL accumulated increased i-C4 and n-C10 fatty acids. Specifically, the selections homozygous for FA7QTL/FA8QTL and lacking the AS7 introgression accumulated 14.0% i-C4 and 9.5% n-C10 fatty acids, while the selection homozygous for FA7QTL/FA8QTL/AS7 accumulated a similar profile with 12.0% i-C4 and 9.3% n-C10. The selection homozygous for FA2QTL/FA7QTL/FA8QTL and homozygous for AS7 accumulated increased levels of i-C4, i-C9, i-C10, n-C10, and i-C11, but did not accumulate detectable levels of i-C13. The selection homozygous for the FA7QTL/FA8QTL and AS7 introgressions was chosen as the initial plant for the new line, FA7/FA8/AS. Additionally, since the AS7 introgression did not appear to have a significant effect on acylsugar level or chemistry

in this population, we also chose among the selections homozygous for the FA7QTL and FA8QTL introgressions but missing AS7 for the plant that set the most seed as the initial plant for the new line, FA7/FA8/-AS7/AS. The selection homozygous for the FA2QTL, FA7QTL, FA8QTL and AS7 introgressions was chosen as the initial plant for the new line, FA2/FA7/FA8/AS.

***Characterization of combination QTL lines***

To appropriately compare the single and multiple FAQTL lines and to evaluate whether interactions between the FAQTL could increase acylsugar level and acylsugar and fatty acid diversity compared to the single FAQTL lines, all lines were simultaneously grown and sampled for acylsugar level, fatty acid profile and acylsugar profile. To build upon the heritability results mentioned in Smeda et al. (2016), heritability analysis was conducted for the predominant fatty acids accumulated by the combination QTL lines, as well as for a selected number of the predominant acylsugars accumulated by the lines. In addition, to evaluate the impact of the environment on these traits to inform breeding objectives, the environment (E) and genotype by environment (G X E) values for these traits were also calculated.

*Acylsugar level* The levels of acylsugars in the single QTL fatty acid lines were largely consistent with previous characterization (Smeda et al. 2016). FA8/AS accumulated lower levels of acylsugars (58.6%) compared to CU071026, while the FA2/AS and FA7/AS lines accumulated levels of acylsugars comparable to CU071026 (Table 2.6). The FA2/FA8/AS line accumulated less acylsugars compared to CU071026 (76.0%), whereas the FA7/FA8/AS line accumulated comparable levels of acylsugars (99.9%) similar to CU071026. Conversely, the FA2/FA7/AS and FA2/FA7/FA8/AS lines were observed to accumulate higher levels of acylsugars (141.0% and 169.8% respectively) than CU071026.

Table 2.6. Acylsugar level and fatty acid profile of acylsugars from single and multiple FAQTL lines in the greenhouse trial, summer 2016

| Acylsugar Lines | Acylsugar as percent of CU071026 (%)[ab] | Selected fatty acid profile %[bc] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *i-C4* | *ai-C5* | *i-C5* | *i-C9* | *i-C10* | *n-C10* | *i-C11* | *i-C12* | *n-C12* | *i-C13* | *i-C14* |
| CU071026 | 100.0 c | 0.7 d | 8.3 c | 52.6 ab | 0.0 d | 0.0 f | 1.2 c | 0.1 d | 0.0 e | 36.7 b | 0.0 c | 0.0 d |
| FA2/AS | 117.8 bc | 2.4 c | 11.2 ab | 58.3 a | 0.05 c | 0.08 d | 0.6 d | 8.5 a | 0.09 c | 14.5 e | 3.7 a | 0.09 b |
| FA7/AS | 102.0 c | 1.0 d | 8.6 c | 52.0 b | 0.0 d | 0.0 f | 8.6 a | 0.1 d | 0.0 e | 29.3 c | 0.0 c | 0.0 d |
| FA8/AS | 58.6 e | 21.7 a | 9.9 bc | 24.6 d | 0.0 d | 0.03 e | 1.3 c | 0.0 d | 0.05 d | 42.0 ab | 0.0 c | 0.04 c |
| FA2/FA7/AS | 141.0 ab | 1.0 d | 9.0 bc | 54.4 ab | 0.29 a | 0.12 c | 6.5 b | 5.8 b | 0.01 e | 22.3 d | 0.2 b | 0.0 d |
| FA2/FA8/AS | 76.0 d | 25.4 a | 13.0 a | 35.1 c | 0.1 b | 2.97 b | 0.5 d | 8.1 a | 1.88 a | 8.7 f | 3.2 a | 0.78 a |
| FA7/FA8/AS | 99.9 c | 15.4 b | 9.5 bc | 18.9 d | 0.0 d | 0.0 f | 8.3 a | 0.1 d | 0.0 e | 47.6 a | 0.0 c | 0.0 d |
| FA2/FA7/FA8/AS | 169.8 a | 16.8 b | 10.2 bc | 21.5 d | 0.27 a | 4.2 a | 6.5 b | 3.5 c | 0.37 b | 35.8 b | 0.1 b | 0.07 b |

[a] Acylsugar level data ln(x) transformed prior to analysis to improve normality

[b] Means followed by different letters within a column are significantly different at $P < 0.05$

[c] i-C4, n-C10, n-C12 Cuberoot (x+0.1) transformed and ai-C5, i-C9, i-C10, i-C12, i-C13, i-C14 Log10(x+0.1) transformed prior to analysis

*Fatty acid profile* Consistent with preliminary characterization during development, the FA2/FA7/AS line accumulated significant levels of n-C10 and i-C11 fatty acids, which are found at low and trace levels, respectively in CU071026 (Table 2.6). The proportion of n-C10 (6.5%) in FA2/FA7/AS was slightly lower than that in FA7/AS (8.6%), and the proportion of i-C11 (5.8%) in FA2/FA7/AS was slightly lower than that in FA2/AS (8.5%). The FA2/FA7/AS line was found to accumulate a decreased proportion of i-C13 (0.2%) compared to FA2/AS (3.7%) and an increased proportion of i-C9 fatty acids (0.29%), compared to FA2/AS (0.05%) and which is not detectable in FA7/AS or CU071026. FA2/FA8/AS accumulated an increased proportion of i-C4 (25.4%) fatty acids, similar to FA8/AS (21.7% i-C4) and significant levels of i-C11 (8.1%) and i-C13 (3.2%), similar to FA2/AS. FA2/FA8/AS, however, also accumulated significant levels of i-C10 (2.97%), i-C12 (1.88%), and i-C14 (0.78%) fatty acids, not detectable in CU071026, and virtually undetectable in the FA2/AS and FA8/AS lines. FA7/FA8/AS accumulated an increased proportion of i-C4 (15.4%), slightly less than FA8/AS, and an increased proportion of n-C10 (8.3%), similar to FA7/AS. The FA7/FA8/AS line was also observed to possess an extremely low proportion of i-C5 fatty acids (18.9%), lower than FA7/AS (52.0%) and slightly less than FA8/AS (24.6%), although not significant. The interaction of all three FAQTL in the FA2/FA7/FA8/AS line displayed an increased proportion of i-C4 (16.8%), slightly less than FA8/AS, and increased proportions of n-C10 (6.5%) and i-C11 (3.5%), slightly less than FA7/AS and FA2/AS, respectively. The FA2/FA7/FA8/AS line also accumulated increased i-C9 fatty acids (0.27%) and decreased i-C13 fatty acids (0.1%), similar to the FA2/FA7/AS line and increased i-C10 and i-C12 fatty acids (4.2% and 0.37% respectively), similar to the FA2/FA8/AS line.

Non-metric multidimensional scaling (NMDS) analysis was conducted to further evaluate whether the fatty acid profile of each FAQTL line was distinct, and to visualize the results (Figure 2.1). The NMDS analysis clearly showed clustering of the profiles of plants of the same genotype, but also revealed that a pair of genotypes clustered closely together, CU071026 with

FA7/AS, while the rest of the genotypes were cleanly separated. Additionally, FA2/AS, FA8/AS and FA2/FA8/AS showed greater profile variance between plant samples than the other genotypes (Figure 2.1). Two further NMDS analyses of the FAQTL lines helped illustrate the effects of the single and combination FAQTL to separate genotypes based on their fatty acid profiles. For example, NMDS analysis was performed, but the single and multiple FAQTL line and CU071026 samples were colored according to whether they possessed the single FAQTL (FA2, FA7, or FA8); even more clearly than in Figure 2.1 presence of the FA7QTL did not differentiate samples (Figure 2.2.b). Conversely, presence of the FA2QTL and FA8QTL cleanly separated samples of the different genotypes (Figure 2.2.a and 2.2.c, respectively). Additionally, the NMDS analysis was recoded so that the samples were distinguished according to the FAQTL combination classes, which furthered clarified the ability of the FAQTL to differentiate fatty acid profiles. For example, in Figure 2.3.a, the combination of FA2QTL/FA7QTL demonstrated that the samples with just FA7QTL did not separate well from samples that had neither FAQTL, but the FA2QTL/FA7QTL class did separate well from the FA2QTL alone class. In contrast to the FA7QTL, the samples with just FA2QTL separated well from the other classes, especially the class with neither QTL. In Figure 2.3.b, the four classes with both FA2QTL/FA8QTL, just FA2QTL, just FA8QTL, or neither, all separated cleanly. Lastly, the combination of FA7QTL/FA8QTL, depicted in Figure 2.3.c. showed that the FA7QTL class did not separate well from the neither QTL class, and that the FA7QTL/FA8QTL class did not separate well from the FA8QTL class. The FA8QTL class, however, did separate well from the neither QTL class and the FA7QTL class.

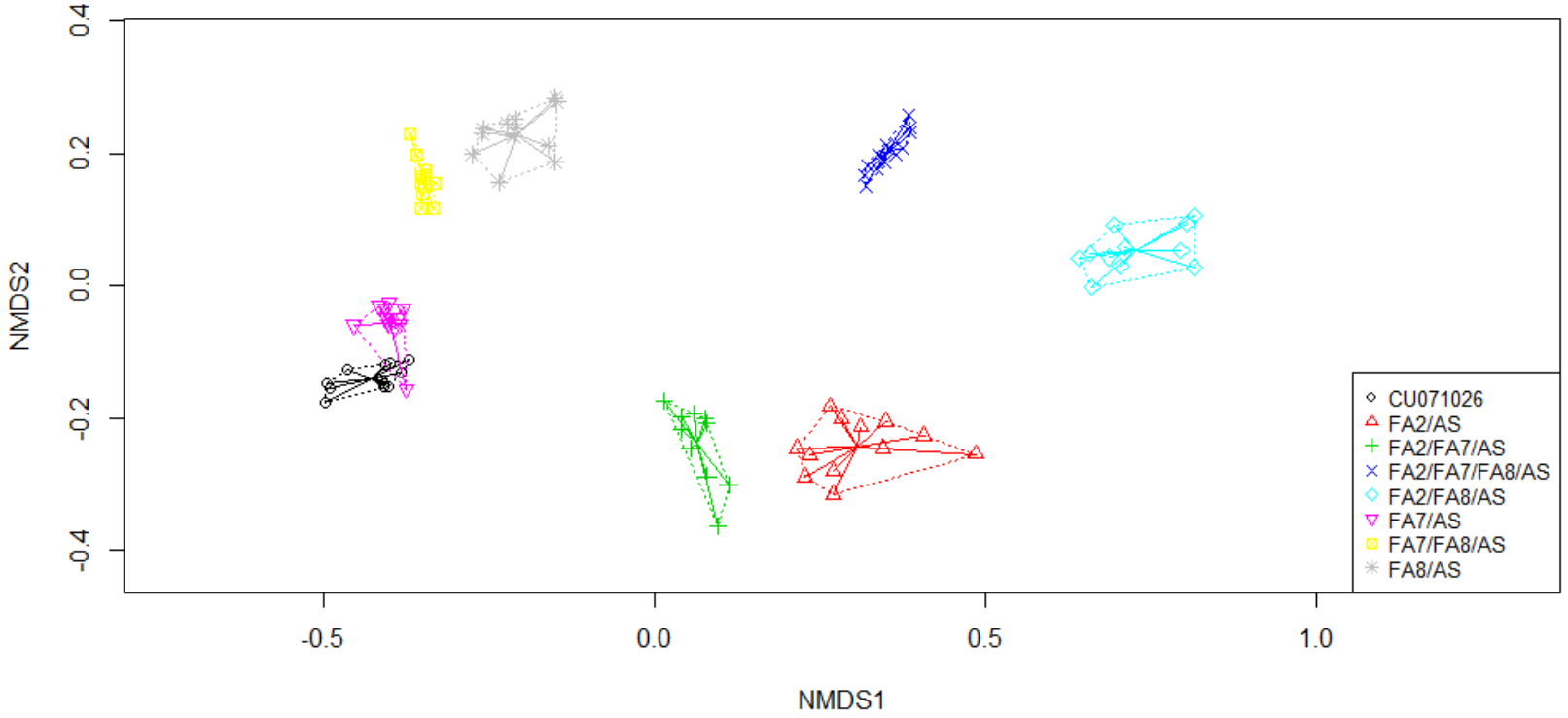



**Figure 2.1.** Non-metric multidimensional scaling (NMDS) ordination in two dimensions of the fatty acid profile composition of the FAQTL acylsugar lines and CU071026, based on Bray-Curtis distance, rotated by principal component analysis (Different shapes and colors correspond to samples from each acylsugar line).

Figure 2.2. NMDS ordination of the fatty acid profile of the FAQTL lines and CU071026, based on Bray-Curtis distance, rotated by principal component analysis. Samples are separated by FA2QTL (a), FA7QTL (b) and FA8QTL (c), illustrating the effect of each QTL to alter and distinguish fatty acid profile

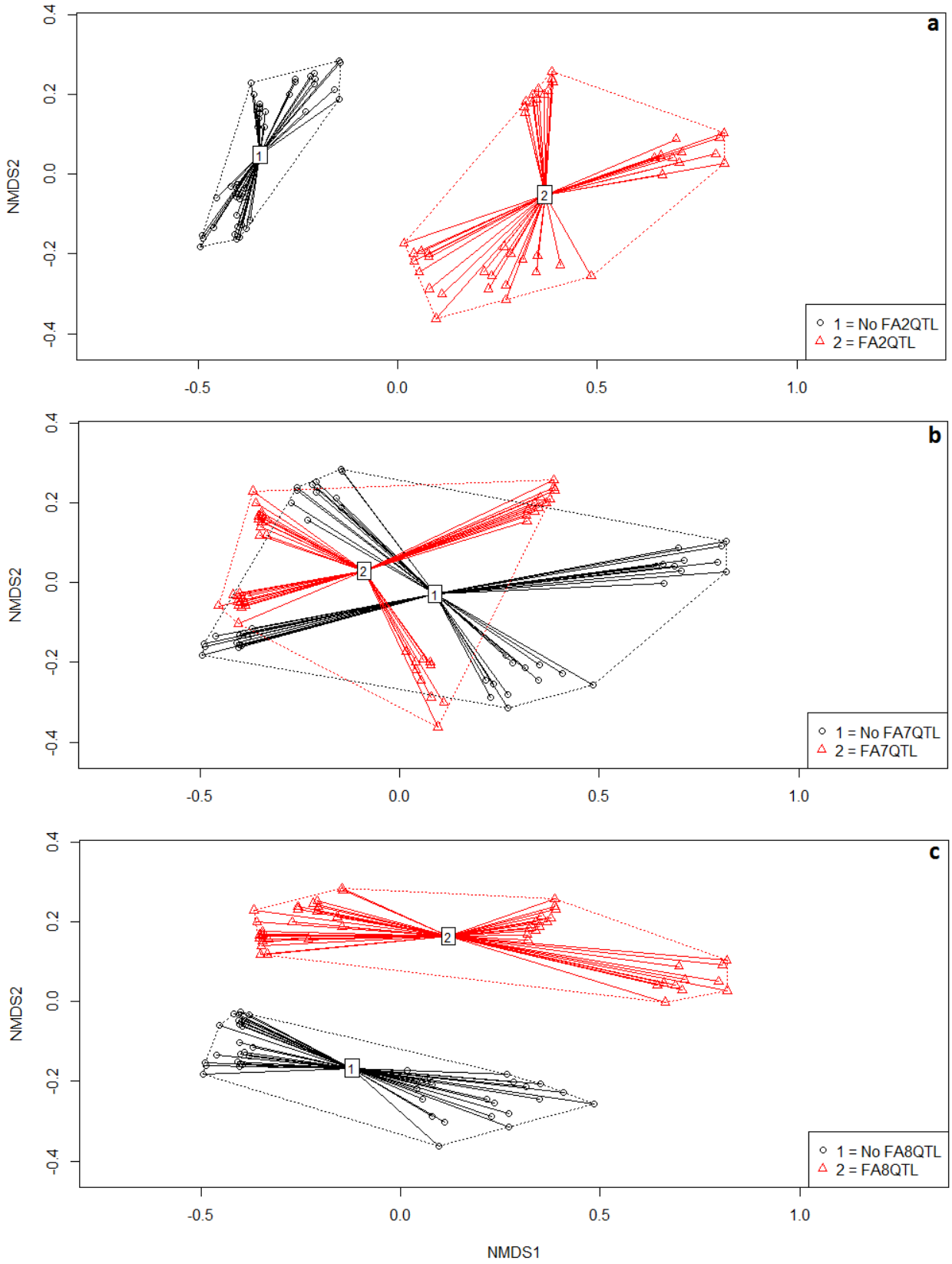
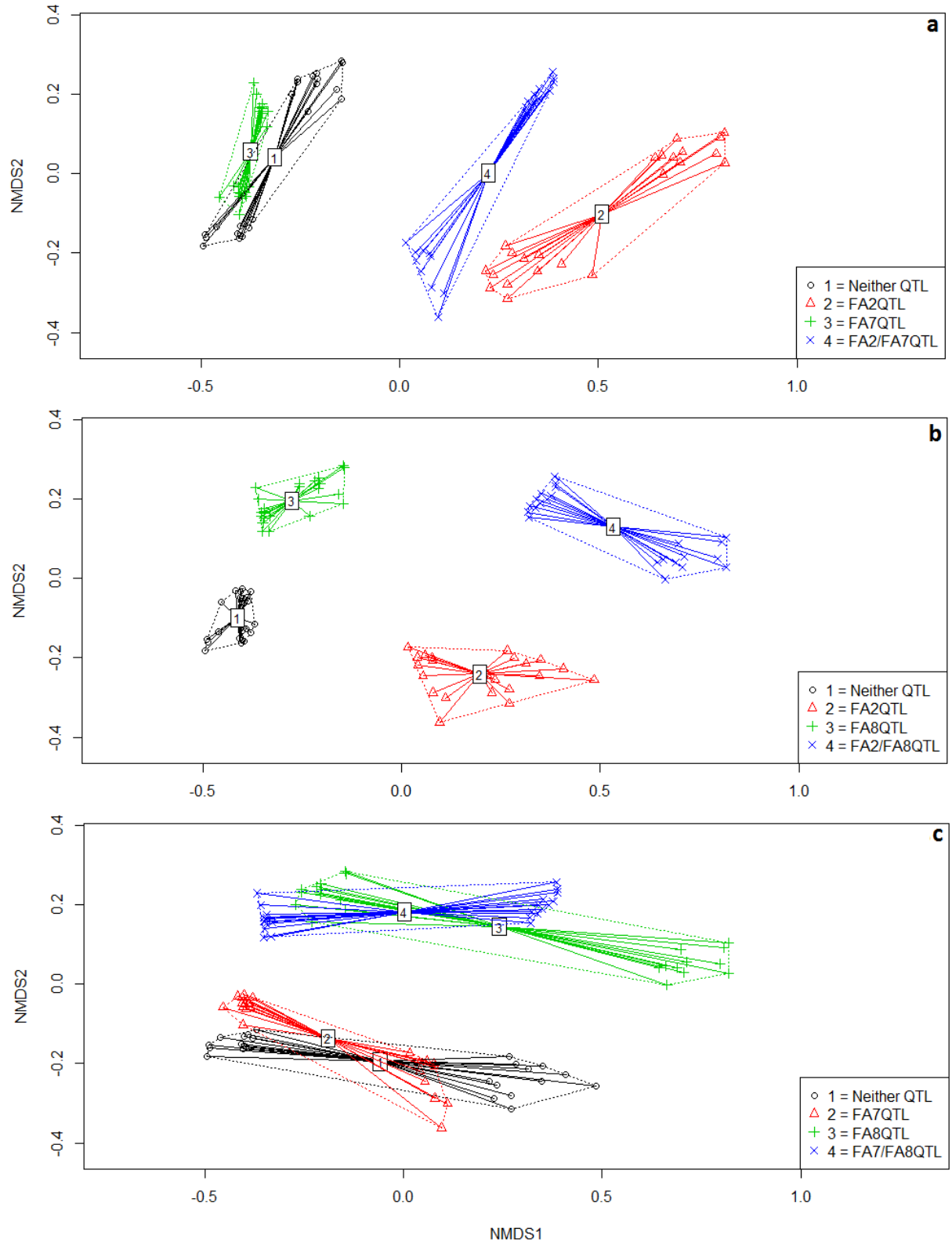

a
NMDS2
0.4
0.2
0.0
-0.2
-0.4
-0.5
0.0
0.5
1.0
1
2
1 = No FA2QTL
2 = FA2QTL
b
NMDS2
1 = No FA7QTL
2 = FA7QTL
c
NMDS2
NMDS1
1 = No FA8QTL
2 = FA8QTL

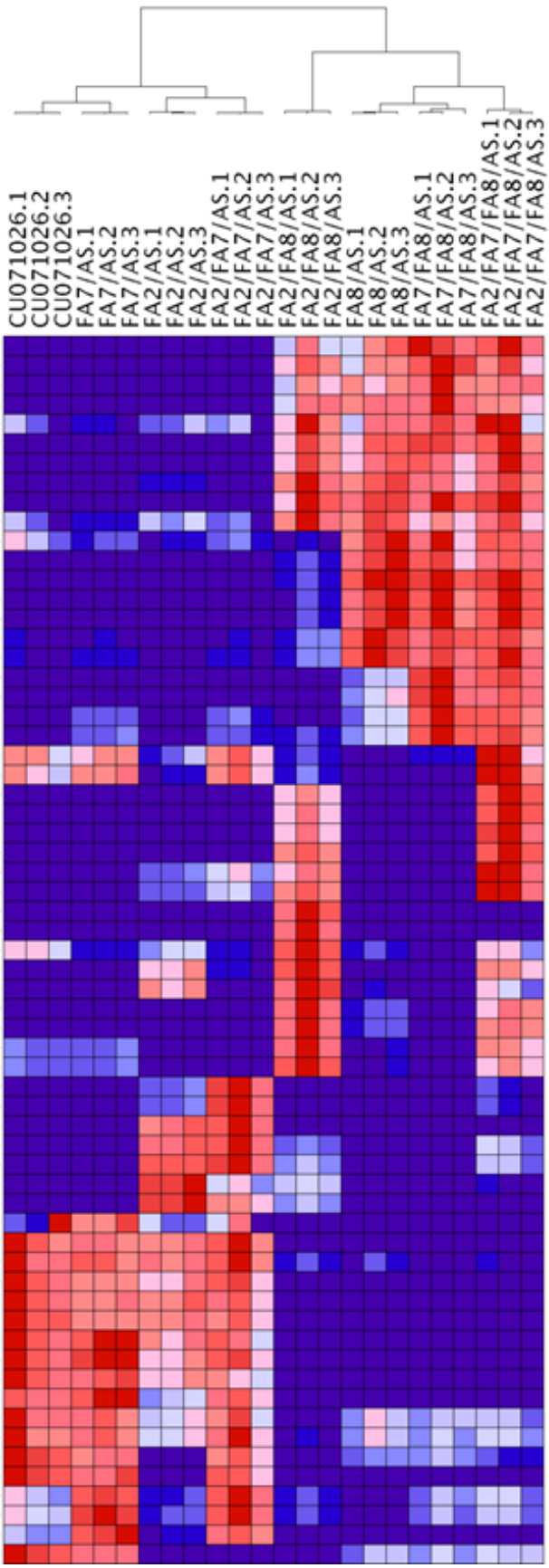
Figure 2.3. NMDS ordination of the fatty acid profile of the FAQTL lines and CU071026, based on Bray-Curtis distance, rotated by principal component analysis. Samples are separated by FA2QTL/FA7QTL (a), FA2QTL/FA8QTL (b) and FA7QTL/FA8QTL (c), illustrating the effect of FAQTL pairs to alter and distinguish fatty acid profile


a
NMDS2
0.4
0.2
0.0
-0.2
-0.4
-0.5
0.0
0.5
1.0
1
2
3
4
1 = Neither QTL
2 = FA2QTL
3 = FA7QTL
4 = FA2/FA7QTL
b
NMDS2
1 = Neither QTL
2 = FA2QTL
3 = FA8QTL
4 = FA2/FA8QTL
c
NMDS2
NMDS1
1 = Neither QTL
2 = FA7QTL
3 = FA8QTL
4 = FA7/FA8QTL

*Acylsugar profile* To evaluate the diversity of acylsugars accumulated by the combination fatty acid lines, LC-MS data was collected to complement the GC-MS data and provide a holistic picture of the acylsugar composition of each line (Figure 2.4). LC-MS revealed that the three predominant acylsugars accumulated by CU071026 are S4:17 (ID 48), S3:22 (ID 56) and S4:24 (ID 57), which matches previous characterization of this line in Smeda et al. (2016). The predominant acylsugars accumulated by the single FAQTL lines and discussed in Smeda et al. (2016) also closely matched previous characterization. The combination FAQTL lines displayed both additive and epistatic impacts on acylsugar profile. Specifically, the FA2/FA7/AS line was observed to accumulate the predominant acylsugars identified in CU071026, FA2/AS and FA7/AS, but also accumulated significant amounts of S3:19 (5,5,9) ID 39 and S4:21 (2,5,5,9) ID 40, which are not detectable in CU071026, and detectable in FA2/AS and FA7/AS, but in much lower levels. For the ID 39/40 acylsugars, FA2/FA7/AS accumulated *ca*. 15x the levels of these acylsugars compared to FA2/AS and *ca*. 100x the levels of these acylsugars compared to FA7/AS. The FA2/FA8/AS line was observed to accumulate, but at lower levels, the three major acylsugars that dominated the profile of CU071026 and additionally accumulated the predominant acylsugars in the FA2/AS and FA8/AS lines. The FA2/FA8/AS line also accumulated significant amounts of S4:22 (2,5,5,10) ID 29, S4:21 (2,4,5,10) ID 27, S4:20 (2,4,4,10) ID 25, and S3:19 (4,5,10) ID 26, all of which are undetectable or at trace levels in CU071026, and undetectable or much lower in FA2/AS and FA8/AS. The levels of S4:21 (2,4,5,10) ID 27, for example, were 85x higher in FA2/FA8/AS compared to FA8/AS and 165x higher than in FA2/AS. Additionally FA2/FA8/AS significantly accumulated S3:22 (5,5,12) ID 31, which was not detected in CU071026, FA2/AS or FA8/AS. FA2/FA8/AS also significantly accumulated an acylsugar with a C14 group, S3:24 (5,5,14) ID 34, which was detected at moderate levels in FA2/FA7/FA8/AS and FA2/AS. The FA7/FA8/AS line was observed to accumulate the three major acylsugars of CU071026, as well as the major acylsugars of FA7/AS and FA8/AS. Additionally, the FA7/FA8/AS line accumulated significant amounts of S3:18

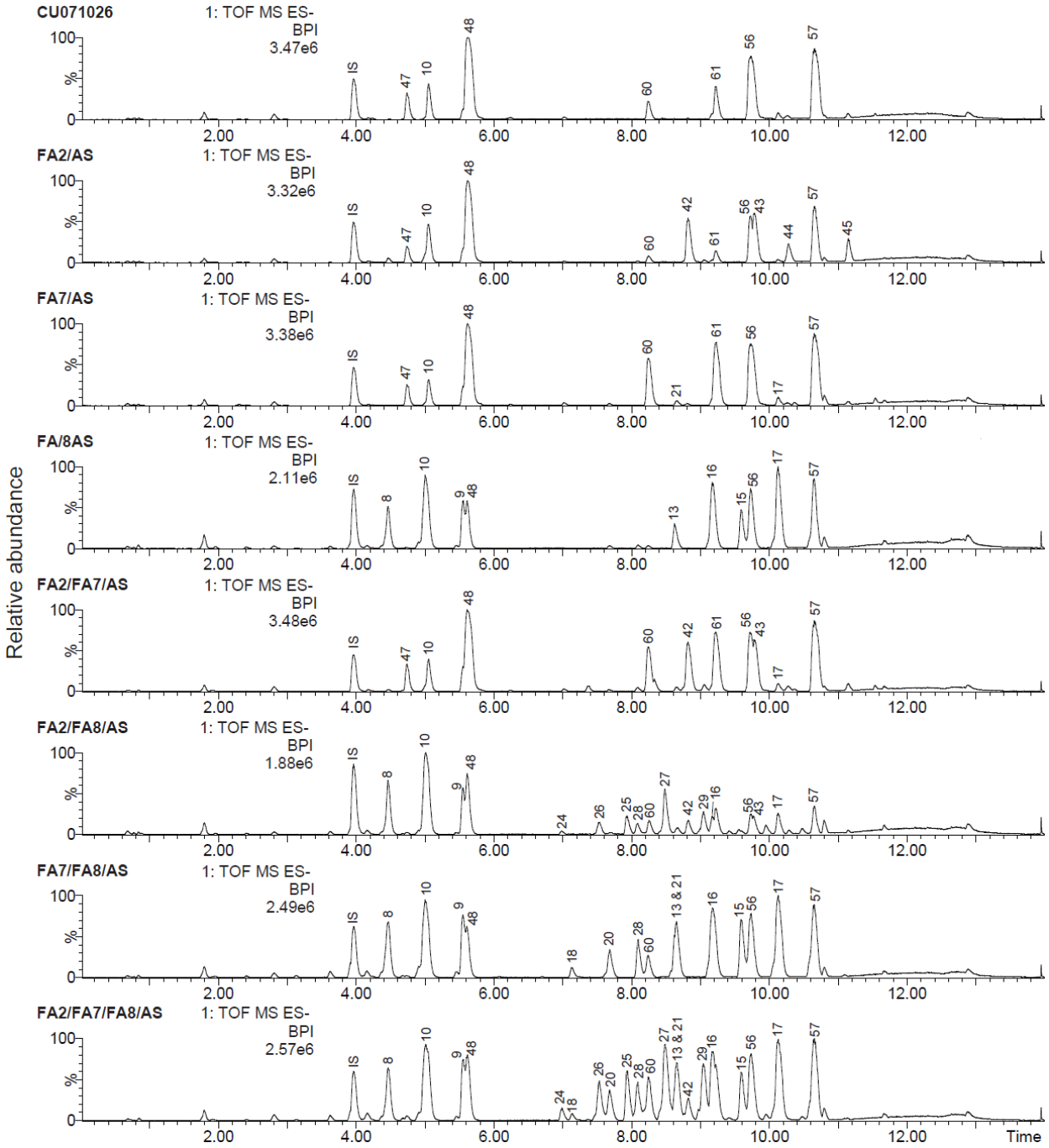
(4,4,10) ID 18 and S4:20 (2,4,4,10) ID 19, which are accumulated at low or undetectable levels in FA7/AS and FA8/AS. Specifically, for S4:20 (2,4,4,10) ID 19, FA7/FA8/AS accumulated 5x higher levels than FA8/AS and 79x higher levels than FA7/AS. The FA2/FA7/FA8/AS line was observed to significantly accumulate a large number of acylsugars including the major acylsugars of CU071026, FA2/AS, FA7/AS, and FA8/AS. In addition to accumulating the major acylsugars of the single FAQTL lines, the FA2/FA7/FA8/AS line also accumulated significant amounts of the major acylsugars found in the binary combination lines (FA2/FA7/AS, FA2/FA8/AS, FA7/FA8/AS). Chromatograms of each combination line are displayed in Figure 2.5 and illustrate the dominant acylsugars accumulated in each line.

Figure 2.4. Hierarchical cluster analysis with Pearson correlation using a pairwise average-linkage clustering method, indicating the predominant acylsugars accumulated by each tomato line. Three samples were analyzed per line. Color across a row indicates relative levels (peak area/g leaf weight) of the respective acylsugar, with red indicating samples with the highest levels detected and blue/purple indicating low or no detection relative to the highest sample for the particular type of acylsugar. [a]The mass to charge ratio for each acylsugar followed by retention time in minutes. [b]Acylsugar nomenclature indicates S for sucrose backbone of the molecule, as well as the number of fatty acid acyl chains (2 to 4) with their cumulative length in carbons that are esterified to the sugar followed by the lengths in carbons of each acyl group in the respective acylsugar. A question mark indicates the proposed acylgroup number and length for an acylsugar whose identification was hampered by low abundance and peak overlap.




| ID | M/z and RT[a] | Acylsugar and acyl groups[b] |
|---|---|---|
| 1 | 597.25 (3.16) | S3:12 (4,4,4) |
| 2 | 639.29 (4.67) | S3:15 (5,5,5) |
| 3 | 681.30 (5.46) | S4:17 (2,5,5,5) |
| 4 | 695.32 (6.07) | S4:18 (2,5,5,6) |
| 5 | 625.27 (4.20) | S3:14 (4,5,5) |
| 6 | 611.26 (3.64) | S3:13 (4,4,5) |
| 7 | 639.26 (3.94) | S4:14 (2,4,4,4) |
| 8 | 653.27 (4.50) | S4:15 (2,4,4,5) |
| 9 | 681.30 (5.55) | S4:17 (2,5,5,5) |
| 10 | 667.29 (5.06) | S4:16 (2,4,5,5) |
| 11 | 653.35 (7.24) | S2:17 (5,12) |
| 12 | 639.33 (6.73) | S2:16 (4,12) |
| 13 | 709.37 (8.58) | S3:20 (4,4,12) |
| 14 | 751.38 (9.50) | S4:22 (2,4,4,12) |
| 15 | 751.39 (9.55) | S4:22 (2,4,4,12) |
| 16 | 723.38 (9.14) | S3:21 (4,5,12) |
| 17 | 765.40 (10.07) | S4:23 (2,4,5,12) |
| 18 | 681.34 (7.14) | S3:18 (4,4,10) |
| 19 | 723.35 (8.07) | S4:20 (2,4,4,10) |
| 20 | 695.35 (7.67) | S3:19 (4,5,10) |
| 21 | 737.36 (8.63) | S4:21 (2,4,5,10) |
| 22 | 681.34 (6.79) | S3:18 (4,5,9)/(5,5,8) |
| 23 | 723.35 (7.75) | S4:20 (2,5,5,8) |
| 24 | 681.34 (6.99) | S3:18 (4,4,10) |
| 25 | 723.36 (7.91) | S4:20 (2,4,4,10) |
| 26 | 695.35 (7.53) | S3:19 (4,5,10) |
| 27 | 737.37 (8.46) | S4:21 (2,4,5,10) |
| 28 | 709.37 (8.07) | S3:20 (5,5,10) |
| 29 | 751.38 (9.02) | S4:22 (2,5,5,10) |
| 30 | 695.36 (7.78) | S3:19 (4,5,10) |
| 31 | 737.37 (9.87) | S3:22 (5,5,12) |
| 32 | 751.42 (10.38) | S3:23 (5,6,12) |
| 33 | 737.41 (9.52) | S3:22 (5,5,12) |
| 34 | 765.44 (10.90) | S3:24 (5,5,14) |
| 35 | 709.34 (8.39) | S3:20 (5,5,10) |
| 36 | 751.39 (9.38) | S4:22 (2,4,4,12) |
| 37 | 723.39 (8.96) | S3:21 (5,5,11) |
| 38 | 765.40 (9.89) | S4:23 (2,5,5,11) |
| 39 | 695.35 (7.38) | S3:19 (5,5,9) |
| 40 | 737.34 (8.31) | S4:21 (2,5,5,9) |
| 41 | 737.40 (9.33) | S3:22 (5,6,11) |
| 42 | 723.38 (8.80) | S3:21 (5,5,11) |
| 43 | 765.39 (9.74) | S4:23 (2,5,5,11) |
| 44 | 751.41 (10.21) | S3:23 (5,5,13) |
| 45 | 793.42 (11.04) | S4:25 (2,5,5,13) |
| 46 | 681.30 (5.16) | S4:17 (2,5,5,5) |
| 47 | 639.29 (4.75) | S3:15 (5,5,5) |
| 48 | 681.30 (5.63) | S4:17 (2,5,5,5) |
| 49 | 597.22 (3.00) | S3:12 (2,5,5)? |
| 50 | 667.29 (5.43) | S4:16 (2,4,5,5) |
| 51 | 695.32 (6.23) | S4:18 (2,5,5,6) |
| 52 | 723.35 (7.47) | S3:21 (5,5,11) |
| 53 | 723.35 (7.00) | S4:20 (5,5,5,5) |
| 54 | 723.35 (7.10) | S4:20 (5,5,5,5) |
| 55 | 821.46 (11.43) | S4:27 (5,5,5,12) |
| 56 | 737.40 (9.68) | S3:22 (5,5,12) |
| 57 | 779.41 (10.59) | S4:24 (2,5,5,12) |
| 58 | 751.41 (10.21) | S3:23 (5,6,12) |
| 59 | 765.40 (10.34) | S4:23 (2,4,5,12) |
| 60 | 709.37 (8.23) | S3:20 (5,5,10) |
| 61 | 751.38 (9.18) | S4:22 (2,5,5,10) |
| 62 | 793.43 (10.29) | S4:25 (5,5,5,10) |
| 63 | 793.43 (11.04) | S4:25 (2,5,6,12) |

Figure 2.5. Representative LC-MS base peak intensity chromatograms for the FAQTL lines and CU071026. Each chromatogram is scaled according to the most abundant peak in the chromatogram to show differences in acylsugar profiles. Peaks are labeled with the ID number system used in the HCA analysis (Figure 2.4)


CU071026
1: TOF MS ES-
BPI
3.47e6
FA2/AS
1: TOF MS ES-
BPI
3.32e6
FA7/AS
1: TOF MS ES-
BPI
3.38e6
FA/8AS
1: TOF MS ES-
BPI
2.11e6
FA2/FA7/AS
1: TOF MS ES-
BPI
3.48e6
FA2/FA8/AS
1: TOF MS ES-
BPI
1.88e6
FA7/FA8/AS
1: TOF MS ES-
BPI
2.49e6
FA2/FA7/FA8/AS
1: TOF MS ES-
BPI
2.57e6
Relative abundance
Time
100
%
0
2.00
4.00
6.00
8.00
10.00
12.00

Clear chromatographic separation was observed for several acylsugars identified in LC-MS analysis with identical mass and indistinguishable mass spectra, such as S4:16 (2,4,5,5) ID 10 with a retention time of 5.06 min and S4:16 (2,4,5,5) ID 50 with a retention time of 5.43 min. We also detected influence of the FAQTL alone and in combination on presence/absence and relative abundance of acylsugar isomers. We observed that acylsugar S3:20 ID 60 with a retention time of 8.23 min was highly accumulated in FA7/AS, and accumulated at lower levels in CU071026 and FA2/AS, and very low levels in FA8/AS. Acylsugar S3:20 ID 28 with a retention time of 8.07 min, however, was significantly accumulated by FA2/AS but was present only at very low levels in CU071026, FA7/AS and FA8/AS. A possible example of differential isomer abundance due to acyl group branching orientation involved four isomers: S4:17 (2,5,5,5) ID 3 with a retention time of 5.46 min, S4:17 (2,5,5,5) ID 9 with a retention time of 5.55 min, S4:17 (2,5,5,5) ID 48 with a retention time of 5.63 min and S4:17 (2,5,5,5) ID 46 with a retention time of 5.16 min. CU071026, FA2/AS, FA7/AS, and FA2/FA7/AS only accumulated detectable levels of the isomers with IDs 46 and 48, whereas FA8/AS, FA2/FA8/AS, FA7/FA8/AS, and FA2/FA7/FA8/AS accumulated detectable levels of the isomers with IDs 3, 9, and 48. The isomer with ID 46 was not detectable in lines containing FA8QTL.

To further evaluate the acylsugar profiles of the FAQTL lines, the hierarchical clustering analysis (HCA) in Figure 2.4 was utilized to compare how the acylsugars clustered between genotypes. The clustering analysis revealed separation of the fatty acid lines into four main clusters. Acylsugars with IDs 1-23 were more abundant in lines with FA8QTL (FA8/AS, FA2/FA8/AS, FA7/FA8/AS, and FA2/FA7/FA8/AS), whereas acylsugars with IDs 24-38 (particularly 24-27) were most abundant in lines with both FA2QTL and FA8QTL. Acylsugars with IDs 39-45 were most abundant in lines containing FA2QTL, and acylsugars with IDs 46-61 were most abundant in lines lacking the FA8QTL. Only one of the QTL combinations (FA2QTL/FA8QTL) was observed to generate a novel cluster in the HCA. The

FA2QTL/FA7QTL, FA7QTL/FA8QTL, and FA2QTL/FA7QTL/FA8QTL did not lead to novel clusters of acylsugars unique to these QTL combinations.

***Heritability estimates for acylsugar and fatty acid traits and environmental impact***

*Fatty acid heritability and environmental impact* Heritability estimates (Figure 2.6.a) for the fatty acids were high, with all fatty acids displaying broad sense heritability of at least 0.5. Estimates were particularly high for the heritability of the three novel fatty acids, increased in the combination FAQTL lines (i-C9, i-C10, and i-C12), all greater than 0.9, and together the estimates revealed a trend for the longer chain fatty acids (C > 8), except i-C13, to exhibit higher heritability than the shorter fatty acids. Looking beyond heritability, we also generated estimates for the effect of the environment and genotype by environment interaction on the evaluated fatty acids (Figure 2.6.a). The shorter chain fatty acids were more impacted by the environment, which was not unexpected based on the lower heritability; in particular, the i-C4 fatty acids experienced considerable genotype by environmental interaction, whereas the i-C5 fatty acids experienced greater interaction with the environment directly. Consistent with the lower heritability, the i-C13 fatty acids also experienced greater interaction with the environment, in particular through genotype by environmental interaction.

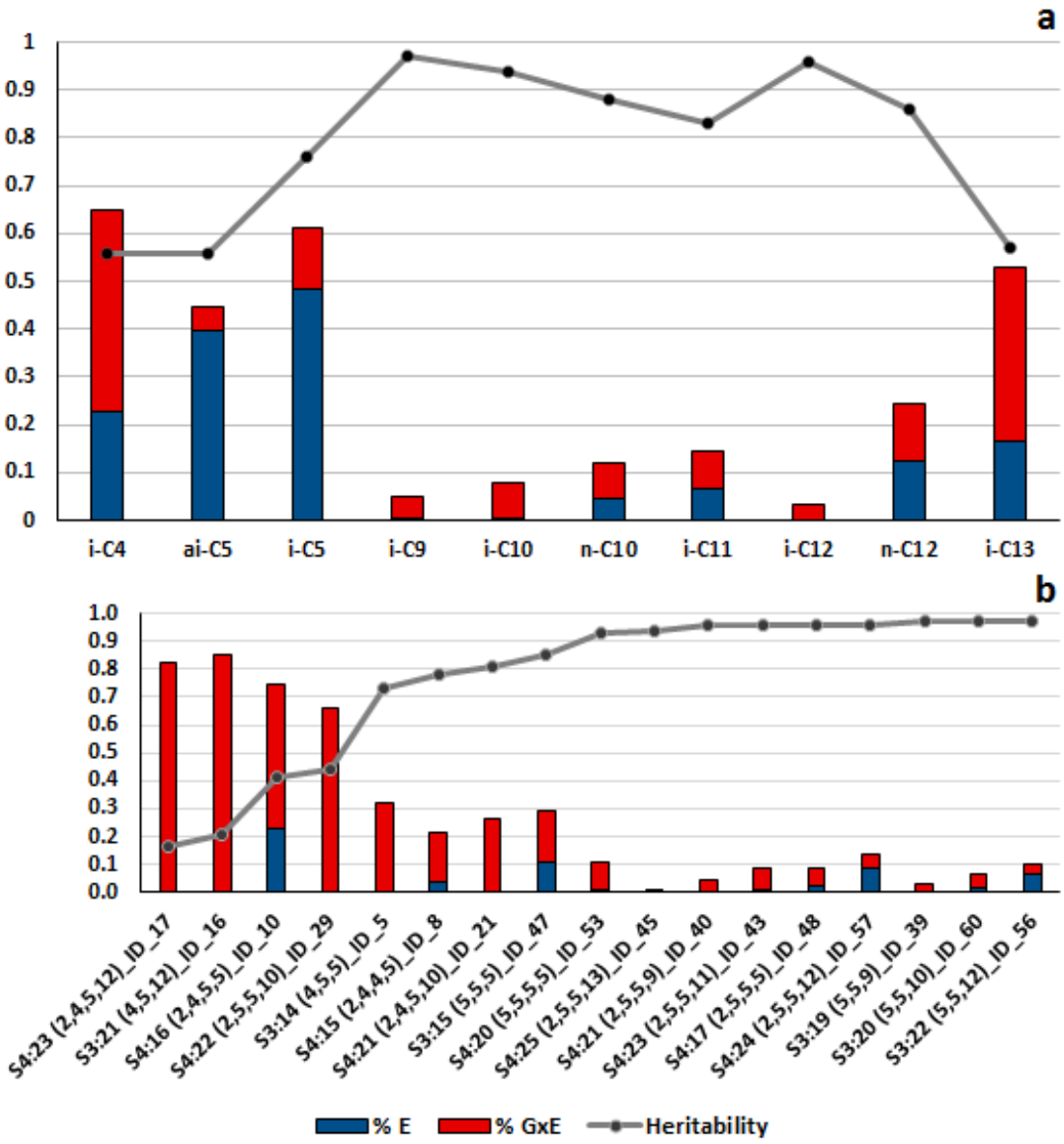



Figure 2.6. Heritabilities, environmental impact (E) and genotype by environmental impact (GxE) for proportions of specific fatty acid components of acylsugars (**a**) and specific acylsugars (**b**). Calculations for fatty acids were performed using data from 2014-2016 and calculations for acylsugars were performed using data from 2015-2016. Full acylsugar ID information is displayed in Figure 2.4.

*Acylsugar heritability and environmental impact* Estimates were generally high for the heritability of the acylsugars looked at in this study as well, and a selection of them are displayed in Figure 2.6.b. Consistent with the lower heritability for the shorter chain acyl groups, there was a trend for the acylsugars containing all short chain acyl groups to display lower heritability, although most evaluated acylsugars exhibited a broad sense heritability of at least 0.4. In particular, the acylsugars with C4 and C5 chains together displayed relatively lower heritability, ranging from ca. 0.16 to 0.8, versus many of the acylsugars which displayed heritability values of at least 0.9. Unlike the fatty acid data, where the i-C13 acyl groups displayed lower heritability, the heritability for the acylsugar containing a C13 group (ID 45) was high (> 0.9). Heritability estimates for many of the acylsugars displayed in Figure 2.4 that are not included in Figure 2.6 are displayed in Table 2.7. Like we did for the fatty acids, we also generated estimates for the effect of the environment and genotype by environment interaction on selected acylsugars (Figure 2.6.b). The acylsugars with lower heritability estimates were generally impacted to a greater degree by the environment; in particular, the acylsugars with combinations of C4 and C5 acyl groups displayed considerable genotype by environmental interaction.

Table 2.7. Broad sense heritability estimates and environmental (Percent E) and genotype by environmental (Percent GxE) impact on acylsugars

| Acylsugar ID[a] | Mass to charge ratio and retention times | Acylsugar and acyl groups | Heritability[b] | Percent E[b] | Percent GxE[b] |
|---|---|---|---|---|---|
| 5 | 625.27 (4.20) | S3:14 (4,5,5) | 0.73 | 0.00 | 0.32 |
| 7 | 639.26 (3.94) | S4:14 (2,4,4,4)? | 0.86 | 0.04 | 0.16 |
| 8 | 653.27 (4.50) | S4:15 (2,4,4,5) | 0.78 | 0.04 | 0.17 |
| 10 | 667.29 (5.06) | S4:16 (2,4,5,5) | 0.41 | 0.23 | 0.52 |
| 16 | 723.38 (9.14) | S3:21 (4,5,12) | 0.21 | 0.00 | 0.85 |
| 17 | 765.4 (10.07) | S4:23 (2,4,5,12) | 0.16 | 0.00 | 0.82 |
| 20 | 695.35 (7.67) | S3:19 (4,5,10) | 0.87 | 0.00 | 0.18 |
| 21 | 737.36 (8.63) | S4:21 (2,4,5,10) | 0.81 | 0.00 | 0.26 |
| 25 | 723.36 (7.91) | S4:20 (2,4,4,10) | 0.73 | 0.01 | 0.41 |
| 26 | 695.35 (7.53) | S3:19 (4,5,10) | 0.69 | 0.01 | 0.44 |
| 27 | 737.37 (8.46) | S4:21 (2,4,5,10) | 0.42 | 0.00 | 0.63 |
| 28 | 709.37 (8.07) | S3:20 (5,5,10) | 0.79 | 0.00 | 0.31 |
| 29 | 751.38 (9.02) | S4:22 (2,5,5,10) | 0.44 | 0.00 | 0.66 |
| 39 | 695.35 (7.38) | S3:19 (5,5,9) | 0.97 | 0.00 | 0.03 |
| 40 | 737.34 (8.31) | S4:21 (2,5,5,9) | 0.96 | 0.00 | 0.04 |
| 42 | 723.38 (8.80) | S3:21 (5,5,11) | 0.96 | 0.02 | 0.07 |
| 43 | 765.39 (9.74) | S4:23 (2,5,5,11) | 0.96 | 0.01 | 0.07 |
| 44 | 751.41 (10.21) | S3:23 (5,5,13) | 0.97 | 0.00 | 0.00 |
| 45 | 793.42 (11.04) | S4:25 (2,5,5,13) | 0.94 | 0.00 | 0.00 |
| 47 | 639.29 (4.75) | S3:15 (5,5,5) | 0.85 | 0.11 | 0.19 |
| 48 | 681.3 (5.63) | S4:17 (2,5,5,5) | 0.96 | 0.02 | 0.06 |
| 51 | 695.32 (6.23) | S4:18 (2,5,5,6) | 0.77 | 0.00 | 0.32 |
| 53 | 723.35 (7.00) | S4:20 (5,5,5,5) | 0.93 | 0.01 | 0.10 |
| 55 | 821.46 (11.43) | S4:27 (5,5,5,12) | 0.69 | 0.05 | 0.38 |
| 56 | 737.4 (9.68) | S3:22 (5,5,12) | 0.97 | 0.06 | 0.04 |
| 57 | 779.41 (10.59) | S4:24 (2,5,5,12) | 0.96 | 0.09 | 0.05 |
| 58 | 751.41 (10.21) | S3:23 (5,6,12) | 0.65 | 0.00 | 0.48 |
| 60 | 709.37 (8.22) | S3:20 (5,5,10) | 0.97 | 0.01 | 0.05 |
| 61 | 751.38 (9.18) | S4:22 (2,5,5,10) | 0.96 | 0.03 | 0.06 |
| 62 | 793.43 (10.29) | S4:25 (5,5,5,10) | 0.64 | 0.00 | 0.48 |
| 63 | 793.43 (11.04) | S4:25 (2,5,6,12) | 0.84 | 0.00 | 0.25 |

[a] Acylsugars listed include acylsugars with levels at least 1% of the internal standard

[b] Calculated from eight entries, replicated over two years

***The impact of pyramiding FAQTL on acylsugar and fatty acid richness/diversity***

To more holistically evaluate the fatty acid and acylsugar diversity, we measured the profile richness through ANOVA and the profile diversity and evenness using Shannon's Diversity Index (H') (Shannon and Weaver 1949). Richness focuses on comparing the number of significantly accumulated compounds between genotypes whereas evenness is a measure of the spread of accumulated compounds among the possible compound classes. The diversity metric is a combination of the richness and evenness measures. Specifically, a higher H' index score equates to a generally more even profile with greater compound richness. It is important to clearly define these labels, as much confusion in the literature results from ambiguous terminology concerning diversity, which is articulated in Spellerberg and Fedor (2003).

*Fatty acid richness.* Evaluation of fatty acid profile using the relative percent of fatty acids from the whole fatty acid profile of each FAQTL line and CU071026 revealed that the acylsugar lines separated into two main groups, with acylsugar lines containing the FA2/AS introgression demonstrating increased fatty acid richness (Table 2.8). The FA7/AS and FA7/FA8/AS lines did not have greater fatty acid richness than CU071026, but the FA8/AS line did have a slight increase in richness. It was observed that the QTL in FA7/AS was associated with a decrease in fatty acid richness, as the FA7/FA8/AS line had less richness than FA8/AS and the FA2/FA7/AS line has less richness than FA2/AS. Additionally, it was observed that the FA2/AS line had a comparable level of fatty acid richness as the FA2/FA7/AS, FA2/FA8/AS and FA2/FA7/FA8/AS combination QTL lines.

Table 2.8. Richness of fatty acids and acylsugars accumulated by each FAQTL line and CU071026

| Genotype | Fatty Acid Richness[ab] | Acylsugar Richness[ac] |
|---|---|---|
| FA7/FA8/AS | 6.8 a | 42.3 a |
| CU071026 | 6.7 a | 43.3 ab |
| FA7/AS | 6.9 a | 44.0 bc |
| FA2/AS | 11.6 d | 45.0 c |
| FA8/AS | 8.2 b | 46.7 d |
| FA2/FA7/AS | 10.6 c | 47.0 d |
| FA2/FA8/AS | 11.6 d | 53.0 e |
| FA2/FA7/FA8/AS | 12.0 d | 53.0 e |

[a] Means followed by different letters within a column are significantly different at P < 0.05

[b] Number of distinct fatty acid acyl groups identified from the acylsugars of each genotype

[c] Number of distinct acylsugars identified to be accumulated by each genotype

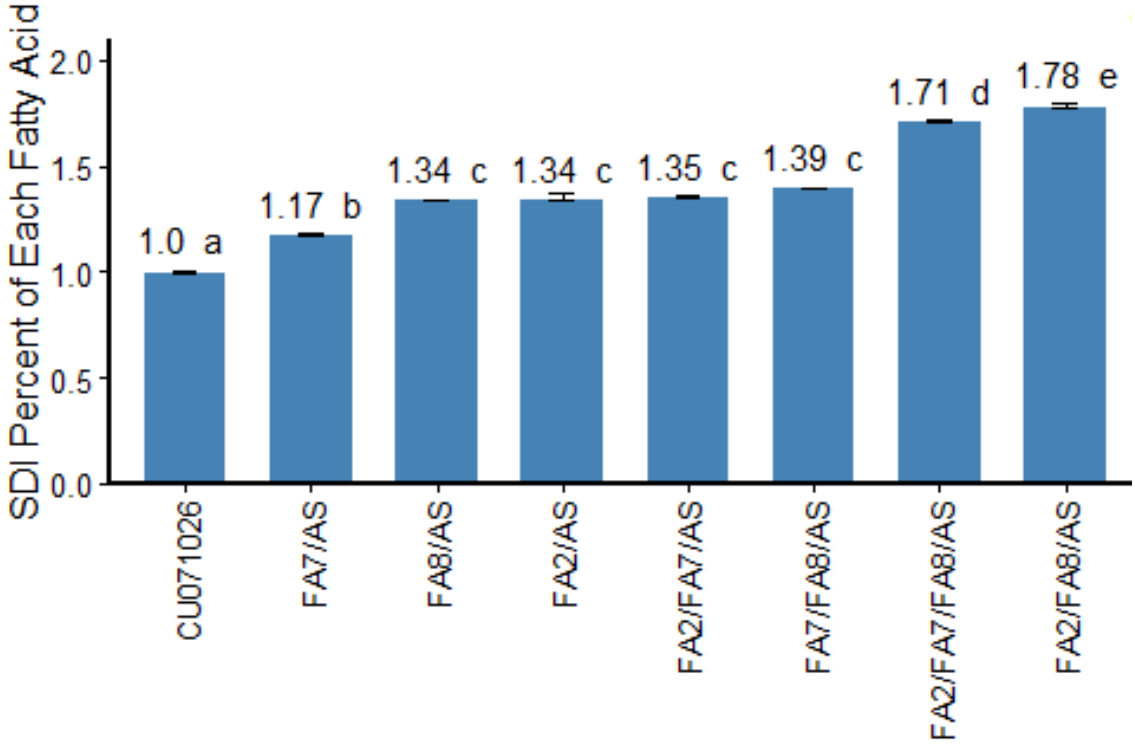



Figure 2.7. Shannon's Diversity Index (SDI) values for fatty acid profile using relative percent of fatty acids for CU071026 and the FAQTL lines. Higher values indicate greater profile diversity through a combination of a greater number of types of fatty acids present and greater evenness of fatty acids. Genotypes not sharing the same letter are significantly different (p < 0.05) Tukey HSD. Error bars represent one standard error of the mean.

*Fatty acid diversity and evenness* Using H' with the relative proportions of the fatty acids for each genotype revealed a more sophisticated pattern than the richness analysis. For example, CU071026 possessed the lowest diversity index score; FA7/AS, on the other hand, had a slightly higher index score, and FA7/FA8/AS had a much higher diversity score (Figure 2.7). FA2/AS and FA8/AS were observed to have a more diverse fatty acid profile than CU071026 or FA7/AS, and a comparable level of profile diversity to FA2/FA7/AS and FA7/FA8/AS. The FA2/FA8 and FA2/FA7/FA8/AS lines, unlike the richness analysis, displayed the highest H' values, and therefore had the most diverse fatty acid profiles. The fatty acid profile evenness analysis revealed a slightly different pattern where CU071026 had the lowest evenness score, similar to FA2/AS, whereas the FA7/AS and FA8/AS lines had increased fatty acid profile evenness (Figure 2.8). The QTL in FA2/AS appeared to slightly lower evenness when combined with the QTL in FA7/AS, but combination of FA7QTL/FA8QTL and FA2QTL/FA8QTL increased fatty acid profile evenness.

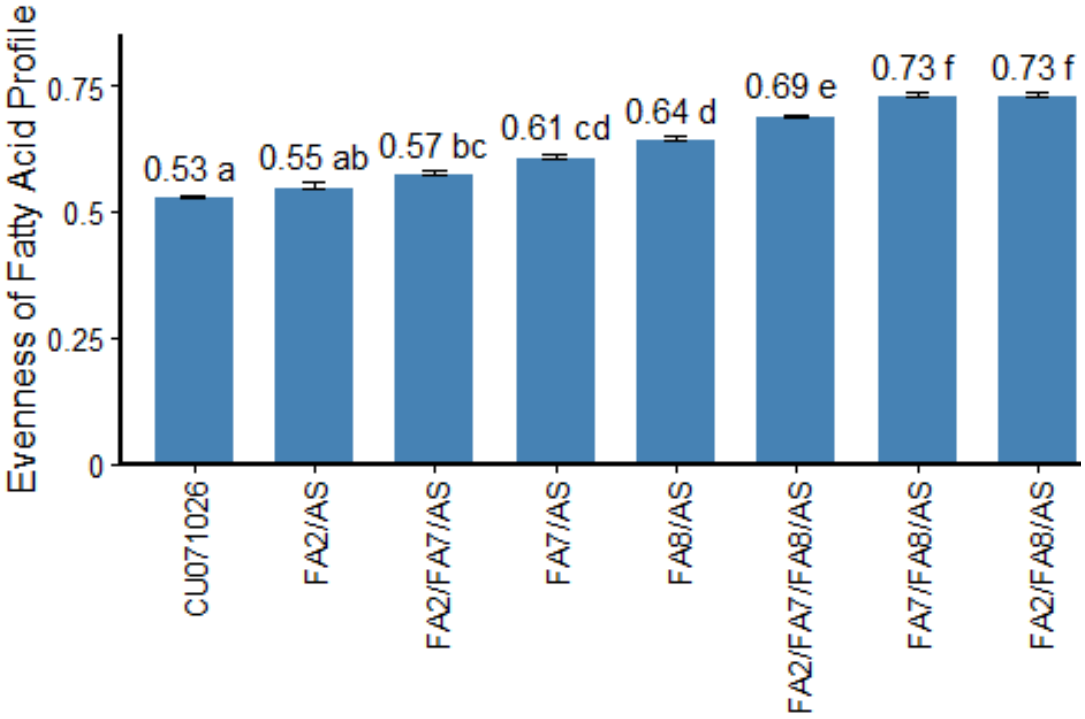



Figure 2.8. Fatty acid profile evenness values for CU071026 and the FAQTL lines, as calculated from the diversity values in Figure 2.7. Values can range from 0 (no evenness) to 1 (complete evenness). Genotypes not sharing the same letter are significantly different ($p < 0.05$) Tukey HSD. Error bars represent one standard error of the mean.

*Acylsugar richness.* Evaluation of acylsugar profile richness using the 63 acylsugars from the whole acylsugar profile of each FAQTL line and CU071026 (Figure 2.4) revealed a more complex pattern of acylsugar richness than fatty acid richness (Table 2.8). Specifically, the FA7/FA8/AS and CU071026 profiles displayed the lowest acylsugar richness, similar to FA7/AS. FA2/AS and FA7/AS had similar richness values, but the FA2/FA7/AS line had increased acylsugar richness. FA8/AS displayed an increase in acylsugar richness and FA2/FA8/AS, and FA2/FA7/FA8/AS had the highest acylsugar richness values.

Utilization of the HCA in Figure 2.4 demonstrated that some FAQTL combinations led to significant tradeoffs in acylsugar accumulation. For example, FA7/FA8/AS lost moderate accumulation of 19 acylsugars that were accumulated by at least one of the FA7/AS or FA8/AS genotypes. Similarly, FA2/FA8/AS also lost moderate accumulation of 19 acylsugars significantly accumulated in FA2/AS or FA8/AS. FA2/FA7/FA8/AS lost moderate accumulation of slightly less acylsugars (15) that were significantly accumulated in at least one of the single or binary FAQTL genotypes. Contrary to the other combinations, the FA2QTL/FA7QTL, in FA2/FA7/AS only resulted in the loss of moderate accumulation of five acylsugars that were accumulated in FA2/AS or FA7/AS, indicating these two FAQTL experience fewer acylsugar tradeoffs when combined.

*Acylsugar diversity and evenness* Using H' with the relative peak areas of each of the 63 acylsugars accumulated by the FAQTL lines and CU071026 (Figure 2.4) revealed that CU071026 possessed the least diverse acylsugar profile (Figure 2.9). Consistent with the fatty acid profile diversity, FA2/AS and FA2/FA7/AS had comparable levels of diversity, with both exhibiting a more diverse acylsugar profile than FA7/AS. FA2/FA8/AS and FA7/FA8/AS exhibited a more diverse acylsugar profile than any of the single FAQTL lines with FA2/FA8/AS slightly more diverse than FA7/FA8/AS. FA2/FA7/FA8/AS displayed the highest H' values, indicating the most diverse acylsugar profile, which was slightly more than the FA2/FA8/AS line. The acylsugar evenness analysis demonstrated the same pattern as the diversity analysis with FA7/FA8/AS demonstrating a higher evenness value than any of the single QTL lines and comparable evenness to the FA2/FA8/AS line; the FA2/FA7/FA8/AS line had the most even acylsugar profile, slightly more even than FA2/FA8/AS (Figure 2.10).

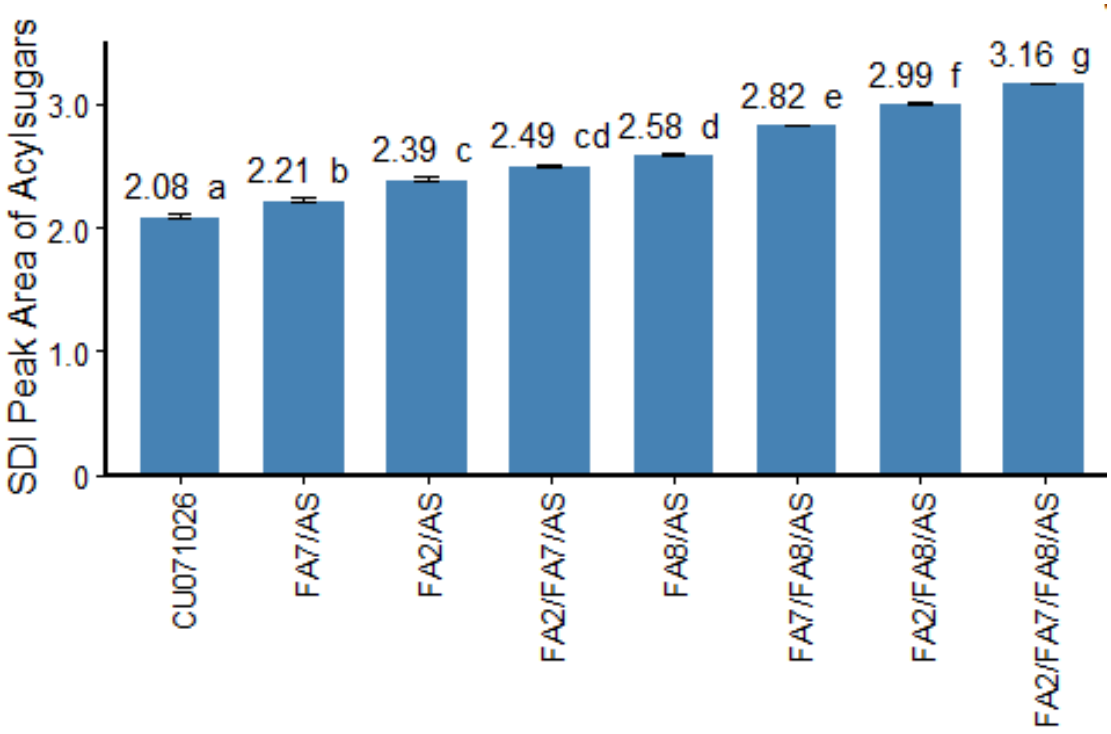



Figure 2.9. Shannon's Diversity Index (SDI) values for acylsugar profile using peak area of acylsugar abundance from CU071026 and the FAQTL lines. Higher values indicate greater profile diversity through a greater number of compounds present and greater acylsugar profile evenness. Genotypes not sharing the same letter are significantly different ($p < 0.05$) Tukey HSD. Error bars represent one standard error of the mean.

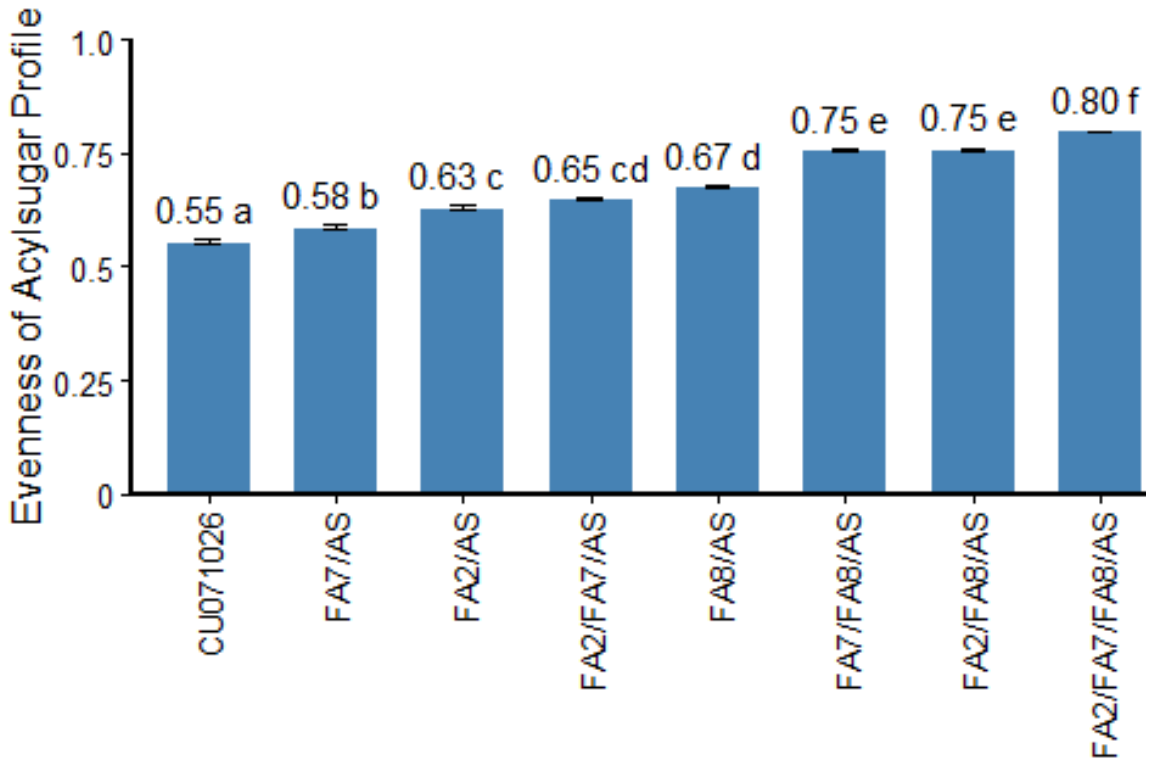



Figure 2.10. Acylsugar profile evenness values for CU071026 and the FAQTL lines as calculated from the diversity values from Figure 2.9. Values can range from 0 (no evenness) to 1 (complete evenness). Genotypes not sharing the same letter are significantly different ($p < 0.05$) Tukey HSD. Error bars represent one standard error of the mean.

## DISCUSSION

There were two major questions we hoped to answer through pyramiding the FAQTL. One question in combining the three FAQTL (FA2, FA7, FA8), was whether we could identify combinations of these QTL that increased acylsugar level over the single QTL lines. Closely related, a second question to answer was whether combinations of the three FAQTL could increase the diversity of the fatty acids and acylsugars accumulated compared to the single QTL lines. In this study, we show that combination of the FAQTL has a profound impact on altering the accumulated levels of acylsugars, the fatty acid and acylsugar profiles, and the fatty acid and acylsugar profile diversity (richness and evenness) in the resulting lines. Additionally, we show that significant interactions between the FAQTL, both additive and epistatic, modulate the acylsugar level and chemotype changes in the FAQTL lines.

Evaluation of the F1 and F2 population selections combining FA2QTL and FA7QTL heterozygously and homozygously suggested these QTL in combination were providing additive and epistatic impacts on acylsugar level and fatty acid profile. In the heterozygous condition and homozygous condition, pairing the FA2QTL and FA7QTL appeared to increase acylsugar levels relative to CU071026. Additionally, pairing the QTL in both the heterozygous and homozygous conditions resulted in a fatty acid profile that exhibited increased levels of i-C9, and decreased levels of i-C13, indicative of positive epistatic interaction between FA2QTL and FA7QTL for i-C9, and negative interaction for i-C13. Together, the data for the FA2QTL/FA7QTL population selections indicate these QTL function together heterozygously and homozygously to generate high levels of acylsugars with modified fatty acid profiles.

Evaluation of the F1 and F2 population selections combining FA2QTL and FA8QTL in the heterozygous and homozygous condition indicated that in combination these QTL were additively impacting acylsugar level and additively and epistatically altering the fatty acid profile. FA8QTL is largely recessive in function (Leckie et al. 2014), and as such FA8QTL minimally

impacted acylsugar level or fatty acid profile when heterozygous. When homozygous, FA8QTL negatively impacted acylsugar levels compared to CU071026, but pairing FA2QTL/FA8QTL in the homozygous condition revealed additive action since the levels of acylsugars accumulated were increased back toward the level of CU071026. Pairing the FA2QTL/FA8QTL in the homozygous condition resulted in a distinct fatty acid profile highlighted by the marked increase in several extended, even, branched fatty acids through epistatic interaction. Together the data from the F2 population selections indicated FA2QTL and FA8QTL function together in the homozygous condition to allow accumulation of moderate levels of acylsugars with a unique fatty acid profile.

Similarly, assessment of the population selections combining FA7QTL/FA8QTL and FA2QTL/FA7QTL/FA8QTL suggested that in combination these QTL were additively and epistatically affecting acylsugar level and fatty acid profile. Pairing of the FA7QTL and FA8QTL in the homozygous condition led to levels of acylsugar higher than when FA8QTL was alone, indicating additive impact on acylsugar level. The selection that combined all three FAQTL in the homozygous condition displayed levels of acylsugars at or above the level observed in CU071026, which suggested all three FAQTL could be interacting epistatically to increase the accumulated levels of acylsugars. Combination of FA7QTL/FA8QTL in the homozygous condition resulted in a fatty acid profile additive of the profiles associated with the FA7/AS lines and FA8/AS lines. Pairing of the FA2QTL/FA7QTL/FA8QTL in the homozygous condition displayed a profile that appeared additive to that observed in the FA2QTL/FA7QTL selections and the FA2QTL/FA8QTL selections. Together the data from the FA7QTL/FA8QTL and FA2QTL/FA7QTL/FA8QTL population selections indicated these QTL functioned together homozygously as a FA7QTL/FA8QTL pair, and all together to affect acylsugar levels and modify the fatty acid profile. Preliminary evaluations of selections among the populations containing two or all three of the FAQTL suggested that some combinations led to increased acylsugar levels and increased fatty acid profile diversity, such as the increased accumulation of novel fatty acids

like i-C9/i-C10/i-C12. Observation of increased acylsugar levels and fatty acid profile richness and evenness in some FAQTL combinations illustrates the intricacy of acylsugar biosynthesis, and suggests acylsugar phenotypes depend on the interaction of a number of QTL.

Replicated evaluation of the FAQTL lines supported the initial characterization from selections in the breeding populations, but also revealed additional additive and epistatic interactions to increase acylsugar level and acylsugar and fatty acid profile complexity. Based on the trend for FA2QTL and FA7QTL, individually, to increase acylsugar levels and FA8QTL, individually, to decrease acylsugar levels (Smeda et al. 2016) we expected to see levels of acylsugars in the FA2/FA8/AS line and FA7/FA8/AS lines that were comparable to CU071026, and levels of acylsugars in the FA2/FA7/AS line that were at or above the level of CU071026. Consistent with the initial FA2QTL/FA8QTL F2 population selections though, the FA2/FA8/AS line accumulated less acylsugars then CU071026, while conversely, the FA7/FA8/AS line accumulated comparable levels of acylsugars (99.9%) to CU071026, somewhat higher than that observed in the FA7QTL/FA8QTL F2 population selections. The FA7/FA8/AS line matched our hypothesis, suggesting FA7QTL can mediate the negative effect of FA8QTL on total acylsugar level, possibly through epistatic interaction. Conversely, while there was a trend for acylsugar levels in the FA2/FA8/AS line to be higher than the FA8/AS line, these data suggest the FA2QTL introgression cannot fully compensate for the lower acylsugar levels in the FA8/AS line. We also observed that the FA2/FA7/AS and FA2/FA7/FA8/AS lines accumulated much higher levels of acylsugars than CU071026. The increase in acylsugar levels in FA2/FA7/AS suggests that the FA2QTL and FA7QTL introgressions are combining in a non-additive manner, since in this study the acylsugar levels observed in the FA2/FA7/AS line are greater than the summed increase in acylsugar levels of the FA2/AS and FA7/AS lines. Furthermore, the FA2/FA7/FA8/AS line accumulated acylsugars at least as high as the FA2/FA7/AS line despite addition of FA8QTL, which suggests epistatic interaction between all three FAQTL when combined to increase acylsugar levels.

NMDS analysis (Figure 2.1) clearly distinguished FAQTL lines based on their accumulated fatty acids, which demonstrated that each FAQTL line had a unique fatty acid profile. FA7QTL had the least impact on altering a fatty acid profile, in particular when alone. Figures 2.2 and 2.3 provided additional perspective on the separation of FAQTL lines based on the single and multiple FAQTL. In particular, Figure 2.1 and 2.3 demonstrated a limited ability to separate plants with just FA7/QTL and those without FA7QTL, and plants with both FA7QTL/FA8QTL from plants with just FA8QTL. This suggested that FA7QTL in combination with FA8QTL had little epistatic impact on generating a unique fatty acid profile. Conversely, Figure 2.3 suggested that FA7QTL interacted with FA2QTL to impact fatty acid profile, causing distinct separation of the FA2/AS and FA2/FA7/AS samples, and the FA2/FA8/AS and FA2/FA7/FA8/AS samples. The separation of these samples and other FAQTL line pairs provides further support for the presence of epistatic genetic interactions that modify fatty acid profile.

The FA7QTL/FA8QTL interaction in the FA7/FA8/AS line was largely additive of the fatty acid profile of the FA7/AS and FA8/AS line profiles, with no novel fatty acids accumulated that were unique to this line. The FA2/FA7/AS line profile was largely additive of the fatty acid profiles of FA2/AS and FA7/AS, but an epistatic effect was apparent in the increased production of a novel fatty acid (i-C9), and decreased production of i-C13. Based on previous knowledge about FA7QTL, it is not surprising that the FA2QTL/FA7QTL combination would lead to increased i-C9 and decreased i-C13. For example, presence of FA7QTL results in accumulation of shorter iso-branched and straight chain fatty acids above C8 in length (Leckie et al. 2014). It is possible that FA7QTL prevents the last two carbon extension (Slocombe et al. 2008; Walters and Steffens 1990) of a portion of iso-branched and straight chain fatty acids such as n-C10 to n-C12 or to i-C11 to i-C13. Due to the presence of FA2QTL, which generates accumulation of both i-C11 and i-C13, the addition of FA7QTL possibly prevents some of the i-C9 fatty acids from being extended to i-C11, and some of the i-C11 fatty acids from being extended to i-C13.

The FA2/FA8/AS and FA2/FA7/FA8/AS line profiles were substantially more complex than the individual FAQTL lines, which was largely due to epistatic interaction between FA2QTL and FA8QTL to increase accumulation of novel fatty acids, such as i-C10, i-C12, and i-C14. Extension of fatty acids to 14 carbons has not been previously reported, and to our knowledge, this is the first observation of a 14 carbon fatty acid that is part of an acylsugar molecule. The orientation of this fatty acid is likely i-C14 because it was mostly detected in FAQTL lines containing FA2QTL, and was greatly upregulated in FA2/FA8/AS, which also has increased accumulation of i-C10, and i-C12. Based on knowledge of how the individual FAQTL function (Leckie et al. 2014; Smeda et al. 2016), it is not surprising that FA2QTL and FA8QTL would lead to a more complex fatty acid profile. For example, the FA8QTL allows substantial accumulation of i-C4, which is largely absent in genotypes lacking the FA8QTL, but still maintains a decent proportion of i-C5 as well. These two branched-chain fatty acids are the starting point for the two-carbon extension method in acylsugar biosynthesis by which extended straight chain and branched-chain fatty acids are generated (Slocombe et al. 2008; Walters and Steffens 1990). FA2QTL extends short, branched-chain chain fatty acids, and leads to a greater proportion of extended branched fatty acids (Leckie et al. 2014). Therefore, when FA2QTL is combined with FA8QTL, this leads to extended odd and even branched-chain fatty acids. These data demonstrate that FA2QTL, FA7QTL and FA8QTL function together in all combinations to distinctly alter the fatty acid profile.

Similar to the analysis of the fatty acid profiles, the analysis of the acylsugar profiles of the combination QTL lines revealed both additive and epistatic interactions. In combination, the FA7QTL/FA8QTL in the FA7/FA8/AS line appear to function mostly in an additive manner, but there was some evidence of non-additive interaction to modify the acylsugar profile. The epistatic impact on acylsugar profile in the FA7/FA8/AS line was limited to increased levels of a few acylsugars over that accumulated in the single FAQTL lines (FA7/AS and FA8/AS). There was also no accumulation of novel acylsugars in FA7/FA8/AS, which is supported by analysis of

the HCA in Figure 2.4 that showed no clustering of acylsugars specific to the FA7QTL/FA8QTL combination. The few acylsugars increased in the FA7/FA8/AS line over FA7/AS and FA8/AS contain C4 and C10 acyl groups, which are likely i-C4, and n-C10, both of which are increased in FA7/FA8/AS due to combination of FA7QTL and FA8QTL (Table 2.6).

Together, the FA2QTL/FA7QTL in FA2/FA7/AS also functioned in a mostly additive manner, but demonstrated some non-additive impact on acylsugar profile as well. The epistatic impact of FA2QTL/FA7QTL was limited to a couple acylsugars, in this case containing C9 acyl groups that were significantly upregulated in the FA2/FA7/AS line over the FA2/AS and FA7/AS lines. This was supported by the HCA in Figure 2.4 that showed there was no cluster unique to the FA2QTL/FA7QTL combination. The C9 acyl group in the upregulated acylsugars in this line is likely i-C9 based on GC characterization of FA2QTL/FA7QTL combination plants, showing increased levels of i-C9 compared to the single QTL parent lines (Table 2.6). An explanation for why the epistatic increase in i-C9 fatty acids in FA2/FA7/AS, did not lead to a unique cluster of acylsugars in the HCA is that i-C9 comprises less than 1% of the fatty acid profile, therefore limiting the incorporation of this fatty acid into a large number of acylsugars. Additionally, acylsugars in the FAQTL lines only incorporated one longer chain fatty acid (C > 8) (Figure 2.4), which is consistent with prior studies (Schilmiller et al. 2015; Fan et al. 2016). Since FA2QTL and FA7QTL increase longer chain fatty acids such as n-C10 and i-C11, the effect of combining these QTL to generate novel acylsugars is limited because it does not appear possible in this germplasm to combine two longer chain acyl groups on the same sugar residue.

Together, the FA2QTL/FA8QTL in FA2/FA8/AS revealed additive impact, but also substantial epistatic impact on modifying the acylsugar profile. For example, analysis of the HCA in Figure 2.4 identified a cluster of about 15 acylsugars greatly upregulated in the FA2/FA8/AS line, a few of which were only detectable in the FA2QTL/FA8QTL combination lines (FA2/FA8/AS and FA2/FA7/FA8/AS). Almost all the upregulated acylsugars in the FA2/FA8/AS line contain an extended even acyl group of C10, C12 or C14 length. It is likely

most of these extended acyl groups are iso orientation because the combination of FA2QTL/FA8QTL uniquely allows increased accumulation of extended even branched chain fatty acids (Table 2.6).

When all three FAQTL in the FA2/FA7/FA8/AS line were combined, they demonstrated both additive and epistatic impacts on acylsugar profile. Moreover, the combination of all three FAQTL revealed that the epistatic interactions identified in the FA2/FA7/AS, FA2/FA8/AS and FA7/FA8/AS lines were largely combined in the FA2/FA7/FA8/AS line. For example, FA2/FA7/FA8/AS significantly accumulates acylsugars with IDs 24-27, similar to FA2/FA8/AS, suggesting the FA2QTL/FA8QTL epistatic interaction remains with or without addition of FA7QTL. FA2/FA7/FA8/AS also accumulates detectable levels of the epistatically upregulated acylsugars in FA2/FA7/AS (IDs 39 and 40), although at lower levels, suggesting presence of FA8QTL diminishes the epistatic effect of FA2QTL/FA7QTL on these acylsugars.

Chromatographic separation of acylsugars through LC-MS with identical mass and indistinguishable mass spectra was observed. These differences could represent positional isomers where acyl chains are esterified to different positions, allowing for chromatographic separation (likely explanation for acylsugar IDs 10 and 50) or to differential acyl group branching orientation. Moreover, there were differences between genotypes for a number of these acylsugars with identical mass and indistinguishable mass spectra. The differential accumulation of four S4:17 (2,5,5,5) acylsugars with varying retention times (IDs 3, 9, 46, and 48) is likely an example of the FAQTL interacting to control isomer abundance through acyl group branching orientation. Because FA8QTL decreases the amount of i-C5 fatty acids and increases the relative amount of ai-C5 fatty acids, some or all of the C5 acyl groups in the acylsugars with IDs 3, 9 and 48 could be ai-C5 fatty acids in place of i-C5 fatty acids. Conversely, the isomer with ID 46 is not detectable in lines containing FA8QTL, possibly because all C5 acyl groups in this acylsugar could be in the iso orientation; i-C5 is greatly reduced in lines containing FA8QTL according to GC-MS analysis (Table 2.6). For the observed

isomers that have differential abundance due to presence or absence of FAQTL, it would require nuclear magnetic resonance analysis (NMR) spectroscopy with purified compounds to clarify if separation was due to location changes in attachment site for acyl groups or acyl group branching orientation.

Evaluation of the heritability of the fatty acids revealed a trend for the shorter chain acyl groups to display a lower heritability than the longer chain fatty acids. This trend was evident in evaluation of the heritability of the acylsugars as well, with acylsugars containing all short chain acyl groups often displaying lower heritability. A possible explanation for this pattern is that the shorter chain acyl groups are the starting point for extension, and once a shorter chain fatty acid has been extended, there is no evidence that those extended acyl groups are shortened or deconstructed, although there is evidence that acylsugar acylhydrolase genes (Schilmiller et al. 2016) can remove acyl chains from acylsugars. In particular, it was also observed that acylsugars with combinations of C4 and C5 acyl groups displayed some of the lowest heritabilities and experienced considerable genotype by environmental interaction. It is unclear if there is a biological basis for this trend, but it is possible that these C4 and C5 acyl groups are i-C4 and i-C5 branching orientations. Ning et al. (2015) demonstrated that a non-functional isopropylmalate synthase gene from *S. pennellii* LA716, contained in the FA8QTL introgression, governs a shift from i-C4 to i-C5 fatty acid production. As seen in the fatty acid characterization in Smeda et al. (2016), and in Table 2.6, FAQTL lines containing FA8QTL accumulate a mixture of i-C4/i-C5. Therefore, it is possible that the isopropylmalate synthase gene governing the shift could be influenced to a greater degree by environmental conditions, resulting in greater flux in the resulting proportions of i-C4 and i-C5. Further evaluation of this germplasm over additional years and environments could help provide an explanation for this observation. Together the data showed that heritabilities for the novel fatty acids due to the FAQTL interactions were high, as were most of the evaluated acylsugars. The high heritabilities for these traits are promising for selection of lines with increased fatty acid and acylsugar complexity, and suggest selection

can be made in a variety of environments. Additionally, the disproportionate impact of the environment and genotype by environment interaction on particular fatty acids and acylsugars can inform target environments for deployment of particular acylsugar chemotypes.

An important question to answer in this paper was what the combinatory effect of the FAQTL is on acylsugar and fatty acid diversity. The richness/evenness diversity analysis of the fatty acids and acylsugars demonstrated positive and negative FAQTL interactions, mediated both additively and epistatically. Generally, addition of FAQTL led to a greater diversity of fatty acids and acylsugars, however, contribution of the richness and evenness of the profiles to increase diversity depended on genotype. Interestingly, increased fatty acid richness did not always correspond with acylsugar richness. For example, the FA2/AS line displayed greater fatty acid richness, yet lower acylsugar richness compared to FA2/FA7/AS. One explanation for this disparity is that some FAQTL lines like FA2/AS accumulated trace levels of a larger number of fatty acids, but it is possible that some of the trace fatty acids in FA2/AS were not abundant enough to be incorporated into acylsugars to the extent that they could be detected in LC-MS analysis. Alternatively, the increased diversity of fatty acids in FA2/AS were all extended acyl groups, which apparently could not be combined in an acylsugar, therefore limiting a commensurate impact on diversifying the acylsugar profile. Because the characterized ASAT genes involved in attachment of acyl groups to the sugar residues (Schilmiller et al 2012, 2015; Fan et al. 2016) do not reside in any of the FAQTL introgressions, additional genes, or pleiotropic effects of the FAQTL and epistatic interactions between them could also help explain the differential effects on fatty acid and acylsugar richness. In general, addition of FAQTL increased the evenness of both the fatty acid and acylsugar profiles, particularly in lines containing FA8QTL. An explanation for the increase in profile evenness in lines containing FA8QTL could involve accumulation of both i-C4 and i-C5 fatty acids, and when FA8QTL is combined with FA2QTL, an increase in extended even and odd branched chain fatty acids. The impact of FA8QTL on fatty acid and acylsugar evenness likely contributed significantly to the

increased profile diversity of lines containing FA8QTL, since profile evenness is a component of H' calculation. An important observation from these analyses was that we have not yet identified a limit on the number of moderately accumulated acylsugars a line can accumulate, as evinced by the substantial richness of acylsugars in the FA2/FA7/FA8/AS line.

Additionally, FA7/FA8/AS plants demonstrated low compound richness and yet high fatty acid and acylsugar diversity. This disparity between the relatively low numbers of fatty acids and acylsugars accumulated in FA7/FA8/AS and high H' diversity contrasted with the other FAQTL lines and CU071026, where low compound richness was associated with lower profile diversity. The negative effect of FA7QTL/FA8QTL in combination in FA7/FA8/AS on acylsugar richness is likely due to the observed increase in acylsugar tradeoffs in FA7/FA8/AS. The loss of moderate accumulation of acylsugars in lines containing FA8QTL, predominantly from the non FA8QTL genotype(s), is supported by the genotype clustering in Figure 2.4 showing all FA8QTL genotypes clustering together. This suggests that the FA8QTL has a profound influence on acylsugar richness, and that presence of FA8QTL affects a greater number of acylsugars than FA2QTL or FA7QTL. The high fatty acid and acylsugar profile diversity of FA7/FA8/AS, despite low compound richness, could be due to the high measures of evenness for fatty acids and acylsugars in FA7/FA8/AS, which likely had a large impact on the H' calculation. The compound profile of FA7/FA8/AS, in particular, revealed that the richness and evenness metrics provide different information.

Further work beyond the scope of this paper would be required to elucidate whether the QTL(s) affecting acylsugar level are pleiotropically regulating fatty acid profile, or whether separate QTL, contained in the FAQTL introgressions, are governing the level and profile changes. Information about the FAQTL interactions that can increase total acylsugar level is useful and can be utilized for breeding objectives.

# CONCLUSIONS

In light of the growing desire to breed for metabolites involved in resistance to insects, this study provides an example of the combination and comprehensive evaluation of QTL involved in mediating the chemistry of a specialized metabolite with known involvement in insect resistance in tomato. The development and characterization of these multiple FAQTL lines complements and improves the current platform of knowledge concerning acylsugar biosynthesis and chemistry and can be used to help elucidate remaining knowledge gaps in acylsugar biosynthesis. In particular, the specific effect of the FAQTL to modify fatty acid and acylsugar composition, without affecting acylsugar moiety, will help facilitate identification of the genes underlying FA2QTL and FA7QTL. A detailed understanding of acylsugar biochemistry is necessary for the deployment of these QTL into breeding lines for cultivar development, as there have been many epistatic interactions observed that affect acylsugar moiety, level, and fatty acid and acylsugar production/diversity (Leckie et al. 2012, 2014). Development of these FAQTL lines with variation for acylsugar level, and fatty acid and acylsugar profile will facilitate future studies to determine if deployment of these lines and hybrids derived from this material can reduce insect feeding, damage and virus incidence, as well as potential to reduce or eliminate spray regimes in field trials. The identification of differential acylsugar isomer accumulation from LC-MS analysis raises questions about the biological significance of the acylsugar isomers, and if they are important in breeding for resistance. Future experiments beyond the scope of this study could combine NMR and bioassays to evaluate the significance of differential isomer accumulation for insect resistance.

Leckie et al. (2016) suggest that increased acylsugar diversity could lead to greater insect efficacy through increased synergistic interactions. Current understanding of acylsugar biosynthesis suggests there are two ways to increase acylsugar profile diversity. The first way is to accumulate both acylsucrose and acylglucose acylsugars, and the second way is to increase

the number of unique acyl groups that are incorporated, either within or across sugar moiety (acylsucrose or acylglucose). The most efficacious acylsugars among those tested in Leckie et al. 2016 have a profile that is more complex due to both mechanisms, but there was no indication if the acylsugars from those samples were more effective due to the fatty acids, the sugar moiety, or a combination of both. Since all of the FAQTL lines in this paper only accumulate acylsucroses, use of these lines in future bioassays and field trials can help determine whether increased diversity of acylsucrose acylsugars alone is sufficient for increased insect efficacy. Similarly, this germplasm also provides an ideal platform to test the more general hypothesis that a greater diversity of fatty acids/acylsugars will correspond with an increase in insect resistance. Finally, lines can also be used to help elucidate how insects interact with and are deterred by acylsugars and further clarify and inform the components of acylsugar chemistry that mediate the synergistic mechanism of insect resistance governed by acylsugars observed in Leckie et al. (2016).

The diversity of acylsugars in the combination FAQTL lines do not include diversity for sugar moiety, as is seen in some wild Solanum accessions. Higher levels of diversity could likely be attained by combining the FAQTL and the QTL necessary for acylglucose accumulation (Leckie et al. 2013), which could be due to the ASAT genes from *S. pennellii* LA716 (Schilmiller et al 2012, 2015; Fan et al. 2016) that co-localize with some of the acylglucose QTL; breeding has begun to combine the FAQTL lines and acylglucose QTL. The high heritabilities of the fatty acid and acylsugar compounds evaluated in this study are promising for breeding efforts, and understanding of the environmental impact on acylsugar traits is valuable for selection of target environments for deployment of these lines. Evaluation of this germplasm in bioassays and field trials will inform selection of optimal breeding lines for the creation of elite hybrids with improved insect resistance.

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## CHAPTER 3

## Introgression of Acylsugar Moiety QTL Modifies the Composition of Acylsugars Produced by High-accumulating Tomato Lines


### ABSTRACT

Acylsugars are important insect defense compounds produced at high levels by glandular trichomes of the wild tomato, *Solanum pennellii*. The ability to produce acylsucrose acylsugars at elevated levels was previously bred into tomato using *S. pennellii* (Correll) D'Arcy accession LA716 which led to the creation of the acylsugar benchmark line CU071026. Our study utilized marker-assisted backcrossing and selfing approaches to transfer and combine two acylglucose quantitative trait loci (AGQTL) named AG3QTL, and AG11QTL, which were previously associated with changes in acylsugar moiety, from mono-introgression lines into CU071026. Additionally, marker assisted selection was used to select for plants containing the three AGQTL named AG3QTL, AG4QTL and AG11QTL from selfed populations derived from an interspecific backcross population of CU071026 x (CU071026 x *S. pennellii* LA716). High acylglucose accumulating lines were selected from these populations that possess the AGQTL and few extraneous *S. pennellii* LA716 introgressions. Incorporation of the three AGQTL in the presence of the five standard *S. pennellii* introgressions of CU071026 altered acylsugar level and moiety, demonstrating epistatic interactions between the AGQTL on both of these traits. Comparison of the lines generated from the two breeding techniques indicated the three AGQTL are essential but not necessarily sufficient for the production of elevated levels of acylglucose acylsugars. Fine-mapping of AG3QTL, AG4QTL and AG11QTL resulted in less than 1 Mbp intervals for the locations for AG4QTL/AG11QTL; proposals of the causal genes underlying these AGQTL are discussed. The acylglucose accumulating lines containing the AGQTL were characterized for acylsugar level through a spectrophotometric assay, and acylsugar chemistry

through gas chromatography/mass spectrometry and liquid chromatography/mass spectrometry. Characterization of the fatty acid profile of lines selected out of the interspecific backcross populations revealed an increase in the proportion of acylsugar n-C10 fatty acid acyl chains, suggesting that additional changes to the acylsugar composition beyond the sugar moiety could be governed by one or more AGQTL or linked QTL. Characterization of the acylsugar profile of acylglucose lines selected from the interspecific backcross populations also demonstrated interactions between the AGQTL and other QTL to further modulate the diversity of acylsugars accumulated. The novel acylsugar moiety lines created by this work could be used to elucidate of the mechanism of insect resistance mediated by acylsugars and can be combined with the FAQTL discussed in chapter one and two to help generate high acylsugar accumulating tomato lines with increased acylsugar profile diversity.

## INTRODUCTION

Plants accumulate a seemingly inexhaustible diversity of specialized metabolites, many of which are involved in mediating interactions with the environment. Many of the specialized metabolites produced by plants function in resistance to pathogens and herbivores and interest in utilization of these metabolites over traditional practices, such as pesticides, has substantially grown. Durable resistance to insect herbivores and the diseases and viruses they vector has long been a primary goal of plant breeders, and a promising group of specialized metabolites, known as acylsugars, has received considerable attention for their potential as a source of resistance to a myriad of insect herbivores and the diseases and viruses they vector. Numerous species in the nightshade family (Solanaceae) accumulate acylsugars, such as *Solanum pennellii*, *Solanum galapagense, Solanum habrochaites, Solanum berthaultii,* and *Nicotiana tabacum* (Fobes et al. 1985; Severson et al. 1985; Burke et al. 1987; King et al. 1986, 1988; Shapiro et al. 1994; Kim et al. 2012; Schilmiller et al. 2015). Acylsugars are secreted from glandular trichomes and act as

a direct (feeding and oviposition deterrents) and indirect defense to a variety of insect herbivores (Severson et al. 1985; Goffreda and Mutschler 1989; Hawthorne et al. 1992; Rodriguez et al. 1993; Juvik et al. 1994; Liedl et al. 1995; Fancelli et al. 2005; Weinhold and Baldwin 2011; Leckie et al. 2016). The acylsugars accumulated by tomato species are composed of a sugar backbone, either sucrose or glucose, to which are esterified several aliphatic acids, ranging from 4 to 14 carbons in length. These fatty acid acyl chains can be straight-chained or branched (iso or anteiso) (Fobes et al. 1985; Burke et al. 1987; Shapiro et al. 1994; Schilmiller et al. 2010, 2012, 2016; Fan et al. 2016). A two carbon extension method has been proposed and demonstrated in tomato to explain the production of medium chain length fatty acid acyl chains from the short chain (C4/C5) precursors (Slocombe et al. 2008; Walters and Steffens 1990). *Solanum pennellii* (Correll) D'Arcy accession LA716 accumulates elevated levels of acylsugars that are associated with resistance to many insects and is a promising source of insect resistance (Goffreda and Mutschler 1989; Hawthorne et al. 1992; Rodriguez et al. 1993; Juvik et al. 1994; Shapiro et al. 1994; Liedl et al. 1995 that can be transferred to tomato (Mutschler and Wintermantel 2006; Leckie et al. 2012; Dias et al. 2013).

Breeding efforts to transfer increased acylsugar accumulation QTL into tomato from *S. pennellii* LA716 led to the generation of the Cornell benchmark line, CU071026, which accumulates moderate levels of acylsugars. CU071026 contains five introgressions from *S. pennellii* LA716 on chromosomes 2, 3, 7, and 10 (called AS2, AS3, AS7, AS10.1, and AS10.2, respectively) (Smeda et al. 2016) (See Supplementary Table S1 of Leckie et al. 2012 for markers and map positions of the *S. pennellii* LA716 introgressions in CU071026). The moderate levels of acylsugars in CU071026 are sufficient to reduce silverleaf whitefly (*Bemisia tabaci)* oviposition (Leckie et al. 2012) and similar levels in acylsugar hybrids reduced incidence of tomato infectious chlorosis virus in fields with heavy greenhouse whitefly (*Trialeurodes vaporariorum*) pressure (Mutschler and Wintermantel 2006).

*Solanum pennellii* accessions accumulate diverse acylsugar chemotypes that vary with geographical location (Shapiro et al. 1994; Ning et al. 2015), which suggests the possibility of co-evolution of specific metabolic profiles with local herbivore populations. *S. pennellii* LA716 accumulates predominantly acylglucoses with a characteristic array of fatty acids, including 2-Methylpropanoate (i-C4), 2-Methylbutanoate (ai-C5), 3-Methylbutanoate (i-C5), 8-Methylnonanoate (i-C10), n-Decanoate (n-C10), and n-Dodecanoate (n-C12) (Burke et al. 1987; Shapiro et al. 1994; Blauth et al. 1999; Schilmiller et al. 2012; Ning et al. 2015). In contrast, the profile of CU071026 is exclusively acylsucroses with predominantly ai-C5, i-C5 and n-C12 fatty acids, and only trace or undetectable levels of i-C4, i-C10 and n-C10 (Leckie et al. 2014; Smeda et al. 2016), despite having been bred using *S. pennellii* LA716. The fatty acid profile of CU071026 is similar to that of cultivated tomato, which predominantly accumulates i-C5, ai-C5 and n-C12 fatty acids as well (Schilmiller et al. 2010 and Ghosh et al. 2014). A series of recent studies identified four BAHD acyltransferases (ASAT1-4), named according to the first four characterized enzymes of the family: BEAT, AHCT, HCBT, DAT (St-Pierre and Luca 2000; D'Auria, 2006), which together function to generate most of the acylsucroses accumulated by cultivated tomato and several wild relatives (Schilmiller et al 2012, 2015; Fan et al. 2016).

A study that utilized purified acylsugars from CU071026 and several *S. pennellii* accessions, including *S. pennellii* LA716, indicated that the purified acylsugars from CU071026 are less effective at equimolar levels than purified acylsugars of some *S. pennellii* accessions at controlling silverleaf whitefly (*Bemisia tabaci*) and western flower thrips (*Frankliniella occidentalis*) oviposition in laboratory assays (Leckie et al. 2016). Furthermore, the Leckie et al. (2016) study revealed synergistic interaction between acylsugar fractions, which led to improved insect resistance. These results suggest that the insect control of CU071026 or derived lines could be improved by altering their acylsugar and or fatty acid profiles to increase diversity, which could lead to greater synergistic interactions. This hypothesis about the benefits of an increasingly diverse metabolic profile is in agreement with a number of other studies evaluating

plant-herbivore interactions with a focus on specialized metabolites that suggested chemical diversity could decrease insect feeding, adaptation and survival (Duffey and Stout 1996; Castellanos and Espinosa-Garcia 1997; Akhtar and Isman 2003).

QTL that affect acylsugar moiety (acylglucose vs acylsucrose) have been identified and shown to alter the sugar moiety in both inter and intra-specific mapping populations (Mutschler et al. 1996; Blauth et al. 1998; Leckie et al. 2013). Recently, Leckie et al. (2013) identified three acylglucose QTL (AG3, AG4, and AG11) that largely controlled acylglucose accumulation in a BC1F1 population of CU071026 x (CU071026 x *S. pennellii* LA716) and a subsequent BC1F2 population. One objective of our study was to introgress several previously identified acylglucose QTL into CU071026 to generate a series of tomato lines closely related to CU071026 that accumulated high levels of acylsugars that were primarily acylglucoses. Another objective of our study was to select high acylglucose accumulating lines out of selfed populations derived from a backcross population of CU071026 x (CU071026 x *S. pennellii* LA716) (Leckie et al. 2013) and reduce extraneous *S. pennellii* introgressions. A final objective was to extensively characterize these acylglucose lines for alterations in the level of acylsugars accumulated through a spectrophotometric assay and for alterations in fatty acid and acylsugar composition through gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS). The mono-introgression lines (ILs) created by Eshed and Zamir (1994, 1995) were identified as an optimum source of the desired *S. pennellii* LA716 acylsugar chemotype QTL for transfer to CU070126. Two acylglucose QTL: acylglucose 3 (AG3QTL) and acylglucose 11 (AG11QTL), previously shown to alter acylsugar level and moiety (Leckie et al. 2013) were introgressed into CU071026 to test the effect of these QTL in an acylsugar-producing tomato line, both to confirm function, and to create tomato lines for testing against insects. Simultaneously, selection of high acylglucose accumulating acylsugar lines was conducted out of BC1F3, BC1F4 and BC1F5 populations derived from a BC1F2 plant generated by Leckie et al. (2013) and identification of plants with recombinations within the

acylglucose QTL introgressions during selection allowed fine mapping of the QTL within them. The acylsugars accumulated by the selected acylglucose accumulating lines were characterized by a spectrophotometric acylsugar assay, which measures acylsugar level, by GC-MS analysis, which determines relative proportions of the fatty acids present, and by LC-MS characterization, which determines the relative proportions of accumulated acylsugar molecules with information concerning the number and length of fatty acids esterified to the sugar backbone. The implications of these data are discussed, including how addition of the acylglucose QTL affected the level, moiety and composition of acylsugars accumulated, and whether addition of the acylglucose QTL lead to alteration of the fatty acid profile.

## MATERIALS AND METHODS

### Plant materials

CU071026 is the acylsugar-producing tomato benchmark line bred by the Cornell University tomato breeding program using *S. pennellii* LA716. Seeds from *S. pennellii* LA716 were produced by the Cornell University tomato breeding program, derived from seed originally obtained from the Tomato Genetics Resource Center (TGRC) at the University of California at Davis.

The interspecific populations at the BC1F3, BC1F4 and BC1F5 generations used to select for high acylglucose accumulating plants were derived from a single BC1F2 plant which was selected from the BC1F1 population CU071026 x (CU071026 x LA716) used in a prior study to map acylsugar QTL (Leckie et al. 2013, 2014).

A series of tomato lines with individual introgressions of *S. pennellii* LA716 DNA in the processing tomato M82 (a sub-selection of UC82-B) were produced by Eshed and Zamir (1994, 1995). Based on prior QTL analysis the introgression lines (Leckie et al. 2013), IL3-5 and IL11-3 were used by the Cornell University tomato breeding program as a source of AG3QTL and

AG11QTL, respectively for the introgression line breeding strategy for transfer of QTL. The seed of IL3-5 and IL11-3 were produced at Cornell University, derived from seed obtained from D. Zamir (Hebrew University of Jerusalem, Rehovot, Israel). Seed of M82 was produced by the Cornell University tomato breeding program, derived from seed originally obtained from the Tomato Genetics Resource Center (TGRC) at the University of California at Davis.

**Plant growth conditions**

Seed were germinated in 32 cell flat cups with LM1 (Lambert, Rivière-Ouelle, Quebec, Canada) mix until *ca.* five weeks of age, during which time any necessary marker based genetic analysis could be completed. Selected plants were transplanted to eight in. plastic pots of LM111 (Lambert, Rivière-Ouelle, Quebec, Canada) mixed with turface (Turface Athletics, Buffalo Grove, IL) in a 1:1.8 ratio, with 0.3% unimix (10-5-10) and calcium sulfate additive. Plants for all populations and experiments were grown in a greenhouse in the Guterman Bioclimatic Laboratory and Greenhouse Complex at Cornell University in Ithaca NY, and were typically maintained at 29°C: 20°C day night temperatures with a 16:8 hr light:dark photoperiod.

**Introgression line breeding strategy for transfer of QTL and selection of acylglucose lines**

Two introgression lines (IL lines), each of which putatively contained either AG3QTL or AG11QTL, were selected for transfer of these two AGQTL. The selected IL lines were intermated and selfed to generated a line containing both AG3QTL and AG11QTL in the homozygous condition. The double introgression IL line was then crossed as the female parent to CU071026, and a resulting F1 plant backcrossed to CU071026 to create the BC1F1 population (CU071026 x (IL line x CU071026). Selection of plants from the BC1F1 population was based on markers within the five *S. pennellii* LA716 introgressions possessed by CU071026 and markers within the additional introgressions being introduced into CU071026.

The original introgressions of CU071026 were selected for homozygosity and markers within the new IL introgressions were utilized to select for presence of the IL introgressions, as well as for recombinations. Markers within the IL introgressions were used in the BC1F2 population to select for homozygosity and recombinations within the IL introgressions.

**Genotypic screening**

Molecular markers utilized in all populations to select for the five standard *S. pennellii* introgressions in CU071026 are provided in Table 3.1. The identity and location of the markers used to introgress the AG3QTL and AG11QTL from the IL 3-5 and 11-3 lines to create lines with them and also the five CU071026 introgressions are provided in Table 3.2. The identity and location of markers used to select for and fine-map AG3QTL, AG4QTL, and AG11QTL out of the BC1F3, BC1F4 and BC1F5 populations are provided in Table 3.3. Genotyping by sequencing (GBS) was used on selections from the BC1F4 population to fine-map AG11QTL and to define the introgressions contained in the final acylglucose line selections. Genomic DNA was isolated with a DNeasy® Plant Mini Kit (Qiagen). GBS was performed as described by Elshire et al. (2011) [11] and submitted to the Weill Cornell Medical College Genomics Resources Core Facility for 101-cycle single-end sequencing on one lane of a 16-lane flow cell of an Illumina HiSeq 2000 instrument (Illumina Inc., San Diego, CA, USA). The sequencing reads were processed with the GBS Discovery Pipeline for species with a reference genome implemented in TASSEL version 3.0 (Bradbury et al. 2007) and following the pipeline documentation (Glaubitz et al. 2014). The sequence tags for our GBS library were aligned to the EXPEN SL2.50 ITAG2.4 release of the *Solanum lycopersicum* genome (Fernandez-Pozo et al. 2014).

Table 3.1. Markers and map locations delineating CU071026 introgressions based on Tomato-EXPEN SL2.50 ITAG2.4

| Marker[a] | Chromosome | Start Position (Bp) | End Position (Bp) |
|---|---|---|---|
| C2_At4g37300 | 2 | 53,834,679 | 53,835,655 |
| C2_At3g26900 | 2 | 54,947,728 | 54,950,298 |
| solcap_snp_sl_63290[b] | 3 | 1,390,271 | 1,390,304 |
| TG130 | 3 | 1,755,716 | 1,756,224 |
| C2_At5g24120 | 3 | 1,914,316 | 1,920,336 |
| C2_At3g02420 | 3 | 11,509,743 | 11,514,318 |
| C2_At5g23060 | 3 | 64,448,262 | 64,451,474 |
| C2_At3g15430 | 7 | 65,800,017 | 65,803,497 |
| TG303 | 10 | 1,773,625 | 1,774,114 |
| C2_At5g60990 | 10 | 1,853,562 | 1,864,123 |
| SSR85 | 10 | 61,580,912 | 61,581,577 |
| C2_At3g12290 | 10 | 62,141,901 | 62,147,562 |

Table 3.2. Markers and map locations used for delineating AG3QTL and AG11QTL introgressions based on Tomato-EXPEN SL2.50 ITAG2.4

| Marker[a] | QTL | Chromosome | Start Position (bp) | End Position (bp) |
|---|---|---|---|---|
| C2_At3g17970 | AG3QTL | 3 | 67,543,166 | 67,547,468 |
| TG244 | AG3QTL | 3 | 70,680,223 | 70,680,913 |
| C2_At2g14260 | AG11QTL | 11 | 33,041,080 | 33,046,140 |
| C2_At5g04590 | AG11QTL | 11 | 51,140,318 | 51,144,951 |
| C2_At4g01560 | AG11QTL | 11 | 52,008,134 | 52,014,923 |
| C2_At3g44600 | AG11QTL | 11 | 52,835,120 | 52,845,120 |
| C2_At2g27730 | AG11QTL | 11 | 53,208,464 | 53,216,010 |
| C2_At2g27290 | AG11QTL | 11 | 53,417,233 | 53,420,233 |
| C2_At5g60600 | AG11QTL | 11 | 53,978,721 | 53,987,402 |
| C2_At5g25760 | AG11QTL | 11 | 54,793,551 | 54,800,368 |

[a] Full marker information provided by The Sol Genomics Network (SGN; http://solgenomics.net/)

Table 3.3. Markers and map locations used to select for AGQTL based on Tomato-EXPEN SL2.50 ITAG2.4

| Marker[a] | QTL | Chromosome | Start Position (bp) | End Position (bp) |
|---|---|---|---|---|
| C2_At2g42110 | AG3QTL | 3 | 69,187,816 | 69,189,787 |
| C2_At3g13700 | AG3QTL | 3 | 69,556,297 | 69,563,346 |
| C2_At3g19895 | AG4QTL | 4 | 2,774,629 | 2,787,443 |
| C2_At5g50720 | AG4QTL | 4 | 4,663,508 | 4,665,167 |
| C2_At1g28120 | AG4QTL | 4 | 33,670,018 | 33,681,978 |
| C2_At3g55010 | AG4QTL | 4 | 55,199,449 | 55,207,322 |
| C2_At5g04590 | AG11QTL | 11 | 51,140,978 | 51,144,287 |
| C2_At4g01560 | AG11QTL | 11 | 52,008,134 | 52,014,923 |
| C2_At5g60600 | AG11QTL | 11 | 53,978,721 | 53,987,402 |

[a] Full marker information provided by The Sol Genomics Network (SGN; http://solgenomics.net/).

**Phenotypic screening**

Acylsugar level and acylglucose concentration: Levels of acylsugar for plants of the controls and of populations in the development of the acylglucose-accumulating acylsugar lines were measured on 9-10 weeks of age plants using the method of Leckie et al. (2012), which is a modification of the prior method described by Goffreda et al. (1990), replacing the Nelson reaction originally used to measure sugar (Goffreda et al. 1990) with a modified peroxidase/glucose oxidase assay (Setter et al. 2001) that measures glucose. Evaluation of single plants from segregating populations entailed collecting two-four samples of two primary lateral leaflets from leaves two to three nodes from the apex of the plant. Evaluation of the replicated acylglucose lines, hybrids, and controls entailed collecting four samples per plant from four plants of two lateral leaflets from leaves that were two to three nodes from the apex of plants. Each two leaflet sample was placed in wide mouth plastic scintillation vials and completely dried in a seed dryer at 290 C. Fully dried leaflets were rinsed with 3 ml of methanol, which contained methyl heptanoate (30 mg L-1), an internal standard for fatty acid analysis if gas chromatography (GC-MS) was desired. The assay uses 100ul of each rinsate. Leaflets were re-dried immediately after rinsing and weighed, so that acylsugar level could be expressed

per weight dried leaf. Dried leaf weights ranged from about 50 to 90 mg. Percent of acylsugars that were acylglucoses was calculated by dividing the concentration of glucoses by the concentration of total sugars. Acylsugar level data were analyzed using ANOVA in JMP Pro 12 (SAS Institute Inc. 2015), and means were separated by Tukey-Kramer HSD ($p < 0.05$). Prior to analysis, acylsugar level data were often $Log_{10}(x)$ or Cube-root(x) transformed to improve normality. Concentration of acylglucoses and percent acylglucose data were often $Log_{10}(x)$ or Cube-root(x) transformed prior to analysis to improve normality.

Fatty acid characterization: Percentages of each type of fatty acids from each sample were ascertained by collecting pairs of young, fully expanded primary lateral leaflets, rinsing leaflets with 3 ml of methanol containing methyl heptanoate (30 mg $L^{-1}$) as an internal standard, and then utilizing transmethylation/GC-MS analysis, as described in Leckie et al. (2014). Peak areas of the resulting chromatograms were calculated using Varian MS Workstation Version 6.9.1 (Agilent Technologies, Santa Clara, CA) and levels of respective fatty acids were determined through comparison with levels of the internal standard to generate relative proportions of each fatty acid. Percent fatty acid GC data was analyzed using ANOVA in JMP Pro 12 (SAS Institute Inc. 2015), and means separated by Tukey-Kramer HSD ($p < 0.05$). Prior to analysis, data for a number of fatty acids were $log_{10(x+1)}$ or cube-root transformed to improve normality.

Acylsugar composition characterization: Liquid chromatography mass spectrometry (LC-MS) was utilized to analyze the composition of acylsugars accumulated in some of the acylglucose BC1F5 selections and controls. Three samples of a single primary lateral leaflet per genotype were taken and extracted with a buffer consisting of isopropanol:acetonitrile:water (3:3:2 v/v/v) containing 0.1% formic acid and 10 uM of propyl-4- hydroxybenzoate, an internal standard, and processed as described in Schilmiller et al. (2015). Acylsugar results from the LC-MS analysis are described using the nomenclature of Schilmiller et al. (2010), in which the acylsugar name G3:19 indicates a glucose backbone sugar, with three fatty acid acyl chains that have a total of

19 carbons. LC-MS data were analyzed by hierarchical clustering with a Pearson correlation using a pairwise average-linkage clustering method for both genotypes and acylsugars using the hierarchical clustering tools provided by GenePattern Reich et al. (2006).

## RESULTS AND DISCUSSION

### Use of *S. pennellii* Introgression lines to transfer AG3QTL and AG11QTL into CU071026

One attempt to generate high acylglucose-accumulating acylsugar lines was made by introgressing the additional *S. pennellii regions* predicted to contain QTL previously associated with acylglucose accumulation into the acylsugar benchmark line CU071026, utilizing the mono-introgression lines created by Eshed and Zamir (1994, 1995) as sources of these additional regions. Based on the markers used for QTL analysis of Leckie et al. (2013), the mono-introgression lines IL3-5 and IL11-3 were selected as those likely to contain the acylglucose QTL AG3QTL and AG11QTL, respectively. Line IL3-5 contained an introgression of *ca.* 3.4 Mbp from *S. pennellii* LA716 and IL11-3 contained an introgression of *ca*. 44.4 Mbp from *S. pennellii* LA716 (Long et al. 2013). IL3-5 and IL11-3 were inter-mated, the resulting F1 was selfed to generate an F2 population from which a plant homozygous for both the IL3-5 and IL11-3 introgressions was selected to create an IL3-5/IL11-3 line. This line was used to create a (CU071026 x (CU071026 x IL3-5/IL11-3)) BC1F1 population. From this 380 plant BC1F1 population, three plants were identified through marker assisted selection (MAS) that were both homozygous for the five CU071026 *S. pennellii* introgressed regions and also heterozygous for the IL3-5 and IL11-3 introgressions. These three plants accumulated slightly lower total acylsugar levels (69.0%) than CU071026 (BC1F1 section of Table 3.4) and accumulated trace levels of acylglucose acylsugars (0.73% of the total acylsugars), comparable to CU071026. Prior identification and evaluation of the AG3QTL and AG11QTL (Leckie et al. 2013) suggested that in a CU071026 x (CU0701026 *S. pennellii*) BC1F1 population, selections heterozygous for

the two acylglucose QTL (AG3QTL/AG11QTL accumulated higher levels of acylglucoses (about 20% of total acylsugars; however, in that BC1F1 population it was likely that additional regions of the *S. pennellii* genome would be present in most plants.

Table 3.4. Initial characterization of the heterozygous and homozygous combination of AG3QTL and AG11QTL in the presence of the CU071026 introgressions in a BC1F1 and BC1F2 population

| Population | QTL[ab] | | n[c] | Acylsugar as percent of CU071026 (%)[de] | Amount of acylglucoses[df] | Percent acylsugars that are AG (%)[dg] |
|---|---|---|---|---|---|---|
| | AG3 | AG11 | | | | |
| BC1F1 | CU071026 | | 4 | 100.0 a | 0.02 a | 0.05 a |
| | 2 | 2 | 3 | 69.0 b | 0.11 a | 0.73 a |
| BC1F2 | 1 | 1 | 2 | 103.8 a | 0.0 bc | 0.0 b |
| | 1 | 2 | 3 | 92.6 ab | 0.09 b | 0.73 b |
| | 2 | 2 | 6 | 83.2 b | 0.11 b | 0.86 b |
| | 1 | 3 | 5 | 1.9 d | 0.0 c | 0.0 c |
| | 2 | 3 | 3 | 3.5 c | 0.34 a | 64.22 a |
| | 3 | 3 | 10 | 1.6 d | 0.06 b | 3.37 b |

[a] 1 = locus homozygous for *S. lycopersicum* alleles; 3 = locus homozygous for *S. pennellii* LA716 alleles

[b] Plants with haplotype "1 1" for AG3/AG11 in BC1F2 are selections comparable to CU071026. All plants in the BC1F1 and BC1F2 lack the AG4QTL.

[c] Number of plants with respective haplotype that were identified and averaged for acylsugar level and fatty acid profile

[d] Means followed by different letters within a column and within each population are significantly different at $P < 0.05$

[e] % CU071026 acylsugar level transformed in BC1F1 ($Log_{10}$) and BC1F2 (Cube-root) prior to analysis to improve normality

[f] umol of acylglucoses $g^{-1}$ leaf weight, transformed in BC1F2 ($Log_{10}(0.5+x)$) prior to analysis to improve normality

[g] Percent acylsugars that are acylglucoses, transformed in BC1F2 (Cube-root) prior to analysis to improve normality

Self seed of one of the three (CU071026 x (CU071026 x IL3-5/IL11-3)) BC1F1 plants (121265-56) that were homozygous for the five CU071026 *S. pennellii* introgressed regions and also heterozygous for the IL3-5 and IL11-3 introgressions was used to produce a BC1F2 population to obtain the plants homozygous for AG3QTL/AG11QTL that were needed to ensure the QTL were contained within the IL3-5 and IL11-3 introgressions, respectively, and to observe whether these QTL in the homozygous condition would lead to higher levels of acylglucoses in

the presence of the CU071026 introgressions. The 184 plant BC1F2 population was characterized using multiple markers both to determine that the AG3QTL and AG11QTL regions were present and to screen for pertinent recombinations to fine map the AG3QTL and AG11QTL. Ten BC1F2 plants were identified that were homozygous for both the IL3-5 and the IL11-3 introgressions and these, as well as other selections with contrasting genotypes, were evaluated for acylsugar level and acylglucose accumulation (BC1F2 section Table 3.4).

Two BC1F2 plants, identified as lacking both the IL3-5 and IL11-3 introgressions, accumulated total levels of acylsugars comparable to CU071026 (103.8%) and only trace levels of acylglucoses (<0.05 umol $g^{-1}$ leaf weight), which amounted to <0.05% of the acylsugars detected (BC1F2 Table 3.4). Similarly, the plants heterozygous for just IL11-3 or heterozygous for both IL3-5 and IL11-3 accumulated total levels of acylsugars comparable or slightly lower than CU071026 with trace levels of acylglucoses that were no more than 0.11 umol $g^{-1}$ leaf weight (0.73% to 0.86% of the acylsugars detected). In contrast, all three of the classes of plants homozygous for the IL11-3 introgression accumulated greatly reduced levels of acylsugars. Specifically, plants lacking IL3-5 and homozygous for IL11-3 accumulated acylsugars at 1.9% of CU071026, and plants homozygous for IL11-3 and heterozygous and homozygous for IL3-5 accumulated acylsugars at 3.5% and 1.6% of CU071026, respectively. These three classes of plants homozygous for IL11-3 accumulated low levels of acylglucoses similar to the other classes; however, the class of plants heterozygous for IL3-5 and homozygous for IL11-3 accumulated slightly more acylglucoses at 0.34 umol $g^{-1}$ leaf weight. Interestingly, the class of plants heterozygous for IL3-5 and homozygous for IL11-3 accumulated

The combination of the AG3QTL and AG11 QTL into plants that possess the five *S. pennellii* introgressions of CU071026 did result in an increase in acylglucose production, which is almost absent in CU071026, however the levels of acylglucose were quite low, suggesting that while these two QTL might be needed for acylglucose production, they are not sufficient for

accumulation of higher levels of acylglucoses. The impact of homozygosity for the introgression containing AG11QTL resulted in trace total acylsugar accumulation, which is a complicating factor. This problem might be a secondary effect of the AG11QTL itself or to a different QTL also contained within the introgression. A similar situation was observed with the transfer of a fatty acid QTL, FA5QTL, which was expected to alter the chain length of fatty acid constituents of acylsugars, but also severely reduced acylsugar accumulations in plants either heterozygous or homozygous for FA5QTL (Smeda et al 2016). It is feasible that if two or more QTL interact to create a different form of acylsugar, the lack of one or more of the QTL would result in a block in acylsugar biosynthesis. A selection from the BC1F2 that set seed well and was homozygous for AG3QTL and AG11QTL was selected to establish the new line, designated AG3/AG11/AS. A schematic depiction of the size and location of *S. pennellii* LA716 introgressions in the AG3/AG11/AS line are displayed in Figure 3.1.

The practical implication of the failure of the plants homozygous for AG4QTL, AG11QTL and the acylsugar introgressions of CU071026 to accumulate high levels of acylsugars and acylglucoses is that one or more additional regions are probably necessary for these traits, but it was not clear how many additional QTL were needed, and where they were located. One candidate would be the AG4QTL also identified by Leckie et al (2013), but the data from the QTL analysis were not sufficient to indicate if only that additional QTL would be needed to support high levels of acylglucose production. Therefore, the data from the IL strategy and prior QTL analysis together result in uncertainty of the likelihood of successfully generating high-accumulating acylglucose germplasm even if AG4QTL was combined with the BC1F2 selections homozygous for the CU071026, IL3-5 and IL11-3 introgressions. With this in mind, the second approach to producing tomato lines with accumulation of acylglucoses at high levels was pursued using the progenies of selected plants from the interspecific (CU071026 x (CU071026 x *S. pennellii*) BC1F1 population used by Leckie et al (2013, 2014) for mapping acylsugar QTL.

**Selection of high-accumulating acylglucose lines out of progenies of an interspecific backcross population**

This alternative strategy was based on selecting plants out the BC1F2 population generated by Leckie et al. (2013) that produced seed and also were noted for having high acylglucose production and whose genomes had relatively low number and size of additional *S. pennellii* LA716 introgressions. Based on marker data from Leckie et al. (2013), plant 111205-235 of the BC1F2 population was selected to generate a BC1F3 population. This plant accumulated high levels of total acylsugars and acylglucoses, was homozygous for the five *S. pennellii* introgressions of CU071026, heterozygous for the AG3QTL, AG4QTL and AG11QTL introgressions, and was homozygous for small introgressions on the ends of chromosomes 4 and 10, as well as heterozygous for four additional introgressions on chromosomes 1, 3, 9, and 12. A schematic depiction of the size and location of introgressions in 111205-235 is displayed in Figure 3.1.

*BC1F3 Population* The purpose of the BC1F3 population was to identify high acylglucose selections in which the relatively small AG3QTL introgression was homozygous and to select for recombinations in the large AG4QTL and AG11QTL introgressions. Additionally, we would select against the segregating additional introgressions on chromosomes 1, 3, 9, and 12 in pursuit of our goal of selecting a homozygous line that accumulated high levels of acylglucoses with minimal additional introgressions other than those containing AG3QTL, AG4QTL, and AG11QTL. Seeds from the BC1F2 selection 111205-235 were used to generate a 353 individual BC1F3 population. Through MAS, a number of plants were identified that were homozygous for combinations of the AG3QTL, AG4QTL and AG11QTL introgressions, and a number of plants that had lost several of the additional *S. pennellii* LA716 segregating introgressions on chromosomes 1, 3, 9, and 12 were also identified. A single plant that was homozygous for all three introgressions containing AG3QTL, AG4QTL and AG11QTL was also identified.

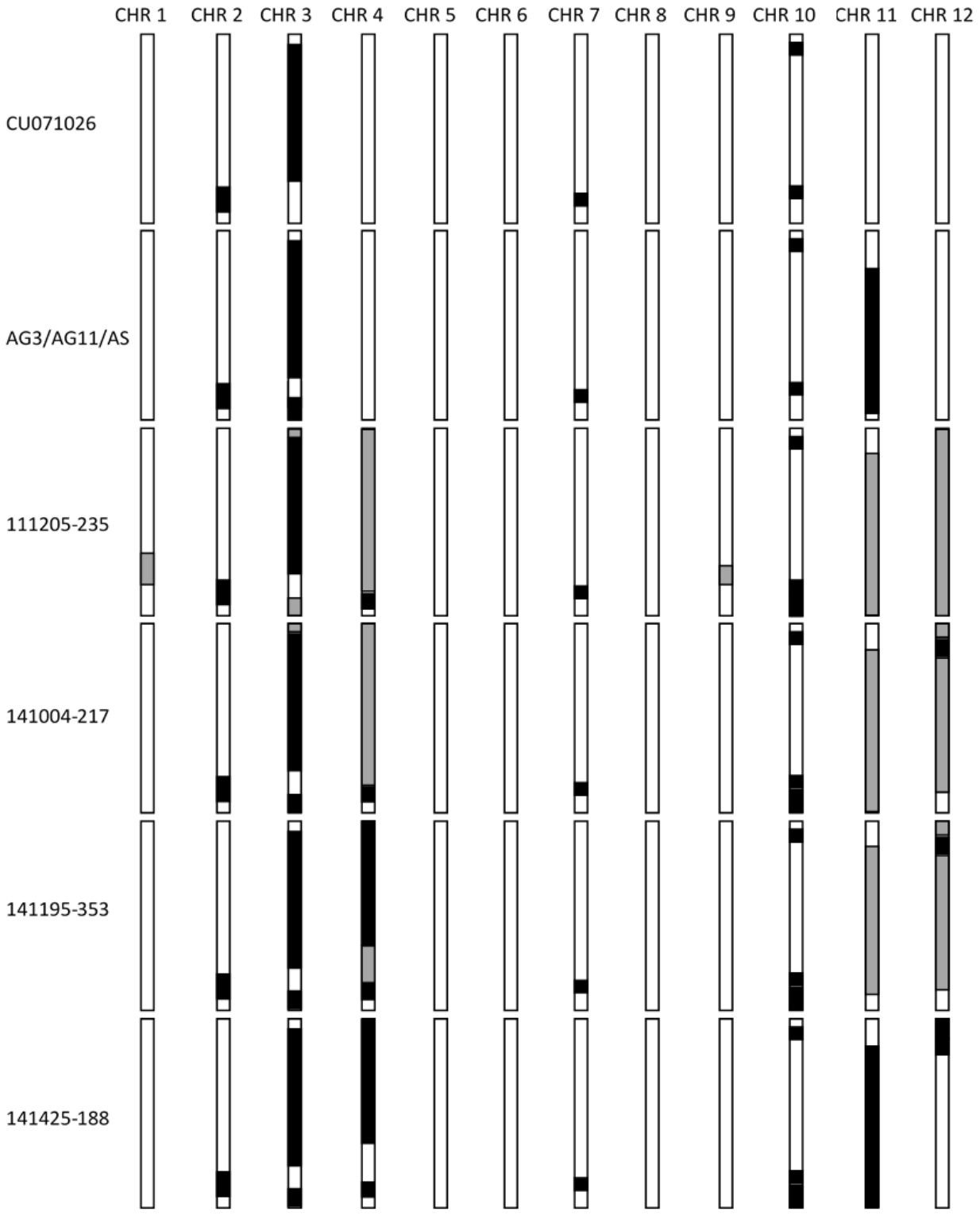



Figure 3.1. Depiction of the size and location of *S. pennellii* LA716 introgressions on each chromosome, based on physical distance, in CU071026 and the acylglucose selections. White is tomato, black is LA716, and grey is heterozygous

The BC1F3 plants varied for both for total acylsugar level and for the amount and relative proportion of acylglucoses. CU071026 and plants lacking the AG3QTL, AG4QTL and AG11QTL accumulated low to trace levels of acylglucoses (0.1 and 1.3 umol $g^{-1}$ leaf weight, respectively) despite high levels of total acylsugars (BC1F3 Table 3.5). Plants heterozygous for all three acylglucose QTL accumulated high levels of acylsugars (140.9% the level of CU071026), with high levels of acylglucoses (11.7 umol $g^{-1}$ leaf weight), which amounted to 55.7% of the total acylsugars. As expected, the CU071026 x *S. pennellii* LA716 F1 plants grown for comparison accumulated higher levels of acylsugars (254.6% of CU071026) and higher levels of acylglucoses (20.1 umol g-1 leaf weight) than the BC1F3 selections heterozygous for the three acylglucose QTL, but the two types of plants were similar in producing *ca.* 50% acylglucoses. The selections homozygous for the AG11QTL and lacking the AG4QTL accumulated low levels of acylsugars (<40% the level of CU071026), and selections lacking the AG4QTL accumulated lower levels of acylglucoses compared to plants heterozygous for the three acylglucose QTL or homozygous for AG3QTL and heterozygous for AG4QTL and AG11QTL.

Table 3.5. Selected haplotype summary of the effect of the AG3QTL, AG4QTL, and AG11QTL on total acylsugar level, acylglucose level and percent acylglucose in BC1F3, BC1F4, and BC1F5 selections

| | QTL[ab] | | | n[c] | Acylsugar as percent of CU071026 (%)[de] | Amount of acylglucose accumulated[df] | Percent of acylsugars that are acylglucoses (%)[dg] |
|---|---|---|---|---|---|---|---|
| | AG3 | AG4 | AG11 | | | | |
| BC1F3 | CU071026 x LA716 | | | 4 | 254.6 a | 20.1 a | 50.8 b |
| | CU071026 | | | 3 | 100.0 bc | 0.1 f | 0.5 d |
| | 1 | 1 | 1 | 4 | 110.2 bc | 1.3 e | 7.2 d |
| | 2 | 1 | 2 | 7 | 72.6 cd | 3.9 d | 35.9 c |
| | 3 | 1 | 2 | 5 | 102.8 bc | 4.1 d | 24.7 c |
| | 2 | 1 | 3 | 7 | 36.4 e | 5.2 d | 91.1 a |
| | 3 | 1 | 3 | 3 | 39.5 de | 5.2 cd | 84.2 a |
| | 2 | 2 | 2 | 40 | 140.9 b | 11.7 b | 55.7 b |
| | 3 | 2 | 2 | 18 | 121.8 b | 10.2 bc | 54.8 b |
| BC1F4 | CU071026 | | | 4 | 100.0 bc | 0.0 d | 0.0 c |
| | 3 | 2 | 1 | 2 | 223.3 a | 0.6 c | 0.5 c |
| | 3 | 3 | 1 | 2 | 124.3 b | 1.6 c | 2.9 c |
| | 3 | 2 | 2 | 7 | 112.5 b | 17.1 b | 36.1 b |
| | 3 | 2 | 3 | 10 | 81.4 cd | 26.0 a | 71.9 a |
| | 3 | 3 | 2 | 10 | 71.5 de | 23.0 ab | 73.1 a |
| | 3 | 3 | 3 | 5 | 61.5 e | 20.1 ab | 73.1 a |
| BC1F5 | CU071026 | | | 4 | 100.0 b | 0.0 c | 0.0 c |
| | 3 | 3 | 1 | 14 | 117.5 a | 0.7 b | 2.3 b |
| | 3 | 3 | 2 | 7 | 51.8 c | 11.1 a | 77.1 a |
| | 3 | 3 | 3 | 11 | 57.9 c | 13.2 a | 82.2 a |

[a] 1: locus homozygous for *S. lycopersicum* alleles; 3: locus homozygous for *S. pennellii* LA716 alleles; 2: locus heterozygous

[b] Haplotype 1 1 1 are selections lacking the ACYLGLUCOSE QTL, comparable to CU071026 for acylglucose accumulation

[c] Number of plants identified/tested with respective haplotype

[d] Means followed by different letters within a column are significantly different at $P < 0.05$

[e] % CU071026 data transformed in BC1F3/BC1F5 (cube-root), and in BC1F4 ($Log_{10}$) prior to analysis to improve normality

[f] Glucose level data transformed in BC1F3/BC1F4 (cube-root), and in BC1F5 ($Log_{10}$) prior to analysis to improve normality

[g] % Acylglucose data transformed in BC1F5 ($Log_{10}$) prior to analysis to improve normality

Data from the BC1F3 selections demonstrated the importance of the presence of all of the AG3QTL, AG4QTL and AG11QTL for plants to generate high levels of acylglucoses. The selections lacking the AG4QTL and heterozygous or homozygous for the AG3QTL/AG11QTL showed that AG4QTL was not necessary to generate levels of acylglucoses above that of CU071026 or selections lacking all three acylglucose QTL. However, when AG11QTL was homozygous, lack of the AG4QTL resulted in greatly reduced levels of accumulated acylsugars. These results largely match those observed in Leckie et al. (2013) in the QTL analysis using an interspecific BC1F2 population. The negative interaction between AG4QTL and AG11QTL on total acylsugar level observed in our study is also supported by the epistatic interaction between these QTL reported in Leckie et al. (2013). While the data from our study largely support previous studies concluding acylglucose accumulation is governed by AG3QTL, AG4QTL, AG11QTL, and their epistatic interaction, there is some evidence from the BC1F3 selections that supports the hypothesis of an additional region(s) involved in acylglucose accumulation. For example, selections heterozygous or homozygous for the AG3QTL and AG11QTL, but lacking the AG4QTL, accumulated acylglucoses at a concentration of at least 3.9 umol $g^{-1}$ leaf weight (BC1F3 Table 3.5). In comparison, the selections from the BC1F1 and BC1F2 populations from the IL breeding strategy that were heterozygous or homozygous for the AG3QTL/AG11QTL combination accumulated acylglucoses at a concentration of at most 0.34 umol $g^{-1}$ leaf tissue (Table 3.4). Additionally, within the BC1F3 population, the plants lacking AG3QTL, AG4QTL, and AG11QTL accumulated a higher concentration of acylglucoses than CU071026 (1.3 vs 0.1 umol $g^{-1}$ leaf weight), despite a comparable level of total acylsugars (BC1F3 Table 3.5), suggesting that these plants might contain an unidentified acylglucose QTL absent in CU071026. Also, the plants heterozygous for the AG3QTL/AG4QTL/AG11QTL accumulated less total acylsugar and a lower concentration of acylglucoses than the CU071026 x *S. pennellii* LA716 plants. An explanation for the higher acylglucoses in the CU071026 x LA716 hybrid is that these plants contain several QTL previously identified to increase total acylsugar levels

(Blauth et al. 1998; Leckie et al. 2012). However, if the top four highest total acylsugar accumulating plants are evaluated from the class of plants heterozygous for the three acylglucose QTL, these plants accumulate total acylsugar levels comparable to CU071026 but concentrations of acylglucoses that are only 16.0 umol $g^{-1}$ leaf weight, about 25% less than the F1 plants (data not shown). The relative percent of acylglucoses in the CU071026 x *S. pennellii* LA716 plants, though, is comparable to the plants heterozygous for the three acylglucose QTL. A recent study by Ranjan et al. (2016) showed that chromosomes 4, 6 and 8 in tomato have a high abundance of trans active expression QTLs (eQTLs), QTLs that function to affect gene expression from a distance; therefore, it is possible the homozygous/heterozygous introgressions on chromosome 4 could contain eQTL(s) that improve the accumulation of acylsugars/acylglucoses by interacting with QTL on other chromosomes, like AG3QTL/AG11QTL. Together, the data from the BC1F3 plants suggest that a region or regions with minor effect on acylglucose accumulation could have been brought to homozygosity in the 111205-235 plant and universally improved acylglucose accumulation in selections from the BC1F3. This region(s) could be recessive in function, which could help explain why the CU071026 x *S. pennellii* LA716 plants did not have increased percent acylglucose over the selections heterozygous for the three acylglucose QTL.

Due to the presence of *S. pennellii* LA716 introgressions still segregating in the BC1F3 population, we used MAS to select the BC1F3 plant 141004-217 to generate a BC1F4 population. Plant 141004-217 accumulated high levels of total acylsugars and acylglucoses. Plant 141004-217 was homozygous for the five introgressions of CU0170226, and for the small introgression containing AG3QTL, but was heterozygous for a large introgression on chromosome 4 containing AG4QTL and a large introgression on chromosome 11 containing the AG11QTL introgression. Regarding additional introgressions, Plant 141004-217 was homozygous for a small introgression on the top of chromosome 12, was heterozygous for an additional small introgression on chromosome 3, and a large introgression on chromosome 12,

and had lost the additional introgressions on chromosomes 1 and 9. A schematic depiction of the size and location of introgressions in 141004-217 is displayed in Figure 3.1.

*BC1F4 population* Seeds from the 141004-217 BC1F3 selection were used to generate a 380 individual BC1F4 population. The purpose of this BC1F4 population was to bring to homozygosity part of the introgression on chromosome 4 containing the AG4QTL and part of the introgression on chromosome 11 containing the AG11QTL, and to select for recombinations on these chromosomes. Additionally, we wanted to select against the segregating introgressions on chromosomes 3 and 12 in pursuit of our goal of selecting a homozygous line that accumulated high levels of acylglucoses with minimal additional introgressions besides the AG3QTL, AG4QTL, and AG11QTL introgressions. From the 380 BC1F4 plant population, a number of plants were identified that were homozygous for both the AG4QTL and AG11QTL introgressions, and a number of potentially important recombinations on chromosomes 4 and 11 near the AG4QTL and AG11QTL, respectively, were also identified.

Evaluation of the BC1F4 selections again demonstrated the importance of the interaction between AG4QTL and AG11QTL to increase accumulation of acylglucoses. Specifically, selections heterozygous for the AG4QTL and AG11QTL accumulated high concentrations of acylglucoses (17.1 umol $g^{-1}$ leaf tissue), which amounted to 36.1% of the acylsugar profile (BC1F4 Table 3.5). When AG4QTL, AG11QTL, or both was homozygous, the concentration of acylglucoses increased in some of these classes versus the plants heterozygous for both AG4QTL/AG11QTL, up to 26.0 umol $g^{-1}$ leaf tissue. The more striking difference though was that the relative proportion of acylglucoses increased, up to 73.1% of the total acylsugars when one, the other, or both of the AG4QTL/AG11QTL were homozygous, but the total accumulation of acylsugars decreased compared to the plants heterozygous for both AG4QTL/AG11QTL. The effect of homozygosity for AG4QTL alone can be seen by comparing the class of plants homozygous for AG3QTL and heterozygous for AG4QTL/AG11QTL with the class of plants homozygous for AG3QTL/AG4QTL and heterozygous for AG11QTL. Specifically, the plants

homozygous for AG4QTL accumulated lower levels of acylsugars (71.5% of CU071026) and a higher proportion of acylglucoses (73.1%) compared to the plants heterozygous for AG4QTL which accumulated levels of acylsugars at 112.5% of CU071026 and a proportion of acylglucoses at 36.1% (BC1F4 Table 3.5). Similarly, the effect of the AG11QTL alone can be seen by comparing the class of plants homozygous for AG3QTL/AG11QTL and heterozygous for AG4QTL with the class of plants homozygous for AG3QTL and heterozygous for AG4QTL/AG11QTL. Specifically, the plants homozygous for AG11QTL and heterozygous for AG4QTL accumulated lower levels of acylsugars (81.4% of CU071026), yet higher levels of acylglucoses (26.0 umol $g^{-1}$ leaf tissue) and a higher proportion of acylglucoses (71.9%) than plants heterozygous for AG4QTL/AG11QTL, which accumulated total acylsugars at 112.5% of CU071026 with accumulation of acylglucoses at (17.1 umol $g^{-1}$ leaf tissue) which constituted 36.1% of the acylsugar profile (BC1F4 Table 3.5).

The effect of the AG4QTL and AG11QTL on acylglucose accumulation and percent acylglucose was comparable to that seen in Leckie et al. (2013), but the effect on total acylsugar was different. Specifically, the class of plants in Leckie et al. (2013) homozygous for the AG3QTL, AG4QTL, and AG11QTL was comparable in total acylsugar level to the plants homozygous for the AG3QTL, but heterozygous for the AG4QTL and AG11QTL. In our study, comparison of those classes of plants demonstrated that the plants homozygous for AG4QTL/AG11QTL accumulated about half the level of acylsugars compared to plants heterozygous for AG4QTL/AG11QTL (BC1F4 Table 3.5). This incongruity could be explained by selection against additional *S. pennellii* LA716 introgressions in the BC1F3 and BC1F4 populations, some of which could have as of yet unidentified acylsugar QTL that have interactive effect with the AG3QTL, AG4QTL, and AG11QTL to control total acylsugar levels or specifically acylsucrose accumulation. There is considerable evidence for epistatic interactions in acylsugar biosynthesis, both for total acylsugar and acylglucose accumulation (Blauth et al. 1998; Leckie et al. 2012, 2013). The general trend observed in Leckie et al. (2013) and our

study could suggest that homozygosity for the acylglucose QTL leads to greater relative proportions of acylglucoses, partly through increased acylglucose accumulation and partly through lower total acylsugar levels. However, *S. pennellii* LA716 accumulates mostly acylglucoses, and accumulates levels of acylsugars at least six times that of CU071026 (Leckie et al. 2013), demonstrating that homozygosity for the acylglucose QTL does not necessitate lower acylsugar levels, and suggesting that additional QTL are necessary to combine with the acylglucose QTL to achieve even higher acylsugar levels.

Due to *S. pennellii* LA716 introgressions still segregating in the BC1F4 population, we used MAS to select a plant 141195-353 which accumulated high levels of total acylsugars and acylglucoses. Plant 141195-353 was homozygous for the five introgressions of CU071026, and for the small introgression containing AG3QTL, and was homozygous for a large part of the AG4QTL introgression (likely homozygous for the AG4QTL) and had a recombination to lose the end of the chromosome 4 introgression (ca. 9 Mbp). Additionally, 141195-353 had a recombination on chromosome 11 such that it was homozygous for a small part of the end of the AG11QTL introgression (ca. 2.8 Mbp); it was uncertain if this region contained AG11QTL, however since the rest of chromosome 11 was heterozygous, the plant would still possess AG11QTL. Plant 141195-353 had also lost the small introgression on the top of chromosome 3 (ca. 1 Mbp), but was heterozygous for a small introgression on the end of chromosome 4, and a large introgression on chromosome 12. A schematic depiction of the size and location of introgressions in 141195-353 is displayed in Figure 3.1.

*BC1F5 population* Seeds from the 141195-353 BC1F4 selection were used to generate a 313 individual BC1F5 population. The purpose of the BC1F5 population was to bring to homozygosity, if necessary, part of the introgression on chromosome 11, and to select against the segregating introgressions on chromosomes 4 and 12 in pursuit of our goal of selecting a homozygous line that accumulated high levels of acylglucoses with minimal additional introgressions besides the AG3QTL, AG4QTL, and AG11QTL introgressions. Six plants were

identified that had lost the introgressions on chromosome 4 (ca. 9 Mbp) and 11 (ca. 50.5 Mbp) and were homozygous for the top of chromosome 12 (ca. 2.9 Mbp) but had lost the vast majority of the large chromosome 12 introgression (ca. 40 Mbp). Three additional plants were identified that were homozygous for the chromosome 11 introgression and the top of the chromosome 12 introgression, but lost the end of the chromosome 4 introgression and the bulk of the chromosome 12 introgression.

Evaluation of the acylsugar level and acylglucose accumulation in BC1F5 selections further supported the essential role of the AG11QTL and revealed that the recombination on chromosome 11 in the BC1F4 selection did not bring the AG11QTL into homozygosity. Specifically, the 14 selections that lost the chromosome 4 and 11 introgressions accumulated levels of acylsugars slightly higher than CU071026 (118.9%), but accumulated acylglucoses at a concentration of only 0.7 umol $g^{-1}$ leaf weight (BC1F5 Table 3.5). The plants heterozygous for AG11QTL accumulated reduced levels of acylsugars (51.8% of CU071026), but with high levels of acylglucoses (11.1 umol $g^{-1}$ leaf weight), which amounted to 77.1% of the acylsugar profile. Similarly, the plants homozygous for the AG11QTL accumulated lower levels of acylsugars (57.9% of CU071026) with high levels of acylglucoses (13.2 umol $g^{-1}$ leaf weight), which equated to 82.2% of the acylsugar profile. The AG11QTL effect in the BC1F5 to control acylglucose accumulation was consistent with the BC1F4 and BC1F3 populations and the BC1F2 population from Leckie et al. (2013). Again, however, the effect of the AG11QTL on total acylsugar level was not consistent between the BC1F5 in our study and the BC1F2 population in Leckie et al. (2013). Specifically, Leckie et al. (2013), observed that the total acylsugar levels accumulated were comparable between the classes of plants homozygous for AG3QTL/AG4QTL, but lacking, heterozygous or homozygous for the AG11QTL. Conversely, in our study, the same classes of plants in the BC1F5 population demonstrated that the total levels of acylsugars in the plants lacking the AG11QTL were at least twice as high as the plants heterozygous for or homozygous for the AG11QTL. An explanation for this could be that

additional segregating introgressions, from *S. pennellii* LA716, present in the BC1F2 from Leckie et al. (2013), were lost or selected against in the BC1F3, BC1F4, and BC1F5. These introgressions could have contained QTL that interacted with the AG11QTL to affect total acylsugar level, or specifically acylsucrose accumulation.

Based on the acylglucose phenotype and the loss of several extraneous introgressions, one of the selections (141425-188) that was homozygous for the CU071026 introgressions and the AG11QTL introgression, and that set seed well, was designated as the initial plant for the new line, AG3/AG4/AG11/AS. A schematic of the *S. pennellii* LA716 introgressions, contained within 141425-188 is displayed in Figure 3.1. Identity of the boundaries of the introgressions in 141425-188, outside of those from CU071026, and defined by SNPs obtained by GBS from the BC1F4 selection, 141195-353, are displayed in Table 3.6. The selection (141425-042), lacking the AG11QTL introgression was selected as the initial plant for the new line, AG11/AG4/-AG11/AS, which provides a closely related high-acylsucrose accumulating line to compare with the AG3/AG4/AG11/AS line.

Table 3.6. Names, chromosome location and physical ranges of additional *Solanum pennellii* LA716 introgressions contained within 141195-353, the source of the AG3/AG4/AG11/AS line

| | | Tomato-EXPEN SL2.50 ITAG2.4 | | Tomato-EXPEN SL3.0 ITAG3.1 | |
|---|---|---|---|---|---|
| Introgression[a] | Chr. | Start Position (Bp) | End Position (Bp) | Approx Start Position (Bp) | Approx End Position (Bp) |
| Add-int-3 | 3 | 67,785,747 | 70,755,827 | 69,273,871 | 72,261,603 |
| Add-int-4a | 4 | 2,567,686 | 53,483,733 | 2,565,794 | 53,424,590 |
| Add-int-4b | 4 | 62,648,875 | 64,694,133 | 62,734,276 | 64,780,930 |
| Add-int-10 | 10 | 60,498,688 | 65,501,089 | 60,623,704 | 65,628,087 |
| Add-int-11 | 11 | 2,980,642 | 56,274,540 | 2,977,714 | 56,569,478 |
| Add-int-12 | 12 | 19,246 | 2,919,236 | 18,295 | 2,920,288 |

[a] Introgressions defined by Genotyping by Sequencing (GBS) single nucleotide polymorphisms (SNPs)

**Characterization of acylsugar levels, fatty acid profile and acylsugar profile of acylglucose lines and hybrids**

Acylsugar biosynthesis in tomato is a complicated system that relies on interaction among a number of genes scattered across the genome. Complicating understanding of acylsugar biosynthesis and the phenotypes observed in the BC1F1-BC1F5 populations is environmental interaction. For example, Shapiro et al. (1994) observed that acylsugar accumulation in *S. pennellii* accessions was affected by the environment, such that the same accessions grown simultaneously in a greenhouse or in the field in Ithaca, NY had two or three times the total acylsugar levels accumulated in the field. Percentage of acylglucoses also varied by location for some accessions, but the degrees of difference were modest, with the differences being 10% or less. Previous studies that introgressed and evaluated QTL affecting composition of fatty acids esterified to the sugar backbone (Smeda et al. 2016, in review), evaluated the heritability of a number of traits, including acylsugar level, profile and fatty acid profile, and showed that despite their high heritabilities many of these traits were still significantly influenced by the environment. Due to evaluation of single plants in the BC1F1-BC1F5 a replicated experiment growing a series of acylglucose lines, hybrids, and controls was necessary to properly characterize the effect of the acylglucose QTL on acylsugar level, acylglucose accumulation, and fatty acid profile. The entries in a replicated evaluation experiment included the CU071026 control, the AG3/AG11/AS line, the AG3/AG4/-AG11/AS line, the AG3/AG4/AG11/AS line, and a CU071026 x AG3/AG4/AG11/AS hybrid.

*Total acylsugar level* Analysis of the replicated entries clearly demonstrated that the AG3QTL, AG4QTL and AG11QTL and their interaction significantly influenced acylsugar level. The AG3/AG11/AS line accumulated trace levels of acylsugar (0.2% of CU071026) (Table 3.7), which agreed with initial evaluation of selections from our IL BC1F2 population. Similarly, the AG3/AG4/AG11/AS line accumulated lower levels of acylsugars compared to CU071026 (62.6%), similar to initial evaluation of plants from the BC1F5 population. The CU071026 x

AG3/AG4/AG11/AS F1 hybrid was observed to accumulate levels of acylsugars intermediate to CU071026 and the AG3/AG4/AG11/AS line at 84.3% of CU071026. The AG3/AG4/-AG11/AS line was observed to accumulate higher levels of acylsugars (118.8% of CU071026), which is consistent with initial evaluation in the BC1F5 selections lacking the AG11QTL The level of acylsugars accumulated in the AG3/AG4/AG11/AS line were about half the level observed in the AG3/AG4/-AG11/AS line, which demonstrates the pronounced impact of the AG11QTL, when homozygous, to decrease total acylsugar levels. The CU071026 x AG3/AG4/AG11/AS F1 hybrid accumulated an intermediate level of acylsugars to the parental lines, which highlights the incomplete dominance effect of the AG11QTL on acylsugar level. The extremely low levels of acylsugars observed in the AG3/AG11/AS line also support the role of AG11QTL, when homozygous to decrease acylsugar levels, but also indicates the necessity of AG4QTL to raise acylsugar/acylglucose levels. The trace levels of acylsugars in the AG3/AG11/AS line are much lower than that observed in similar haplotype selections from the BC1F3, which accumulated levels of acylsugars at 39.5 % of CU071026. This again suggests that a region or regions from *S. pennellii* LA716, necessary for raising acylsugar levels of acylglucose producing lines, and which were present in the BC1F3 population are missing in the AG3/AG11/AS line.

Table 3.7. Total acylsugar and acylglucose level, percent acylglucose and fatty acid profile characterization of related acylglucose entries in greenhouse summer 2016

| Acylglucose Entries | Acylsugar as percent of CU071026 (%)[ac] | Amount of Acylglucoses[ab] | Percent Acylglucose[ac] | Selected fatty acid profile %[ac] | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | *i*-C4 | *ai*-C5 | *i*-C5 | *n*-C10 | *i*-C11 | *n*-C12 |
| CU071026 | 100.0 b | 0.0 d | 0.0 d | 0.74 a | 10.15 a | 58.26 ab | 1.37 d | 0.05 a | 28.80 b |
| AG3/AG4/AG11/AS | 62.6 c | 14.1 a | 84.8 a | 0.37 b | 6.69 b | 55.22 b | 5.62 b | 0.0 b | 31.47 b |
| CU071026 x AG3/AG4/AG11/AS | 84.3 b | 9.8 b | 43.8 b | 0.39 b | 8.83 a | 62.59 a | 1.47 d | 0.01 b | 26.11 b |
| AG3/AG4/-AG11/AS | 118.8 a | 0.3 c | 1.0 c | 0.21 c | 6.25 b | 56.95 ab | 9.05 a | 0.05 a | 27.19 b |
| AG3/AG11/AS | 0.2 d | 0.0 d | 0.0 d | 0.0 d | 7.45 b | 30.32 c | 2.24 c | 0.0 b | 51.08 a |

[a] Means followed by different letters within a column are significantly different at $P < 0.05$

[b] uMol of acylglucose acylsugars $g^{-1}$ leaf weight

[c] i-C4, i-C5, ai-C5, n-C10, acylsugar as % of CU071026 log10(x) transformed and percent acylglucose cube-root(x) transformed to improve normality

*Acylglucose accumulation* Analysis of the replicated entries also demonstrated that AG3QTL, AG4QTL, and AG11QTL profoundly impact acylglucose accumulation and the relative proportion of acylglucoses (Table 3.7). CU071026 did not accumulate appreciable levels of acylglucoses, consistent with prior evaluation, whereas the AG3/AG4/AG11/AS line accumulated high levels of acylglucoses (14.1 umol $g^{-1}$ leaf weight) which amounted to 84.8% of the acylsugar profile. The CU071026 x AG3/AG4/AG11/AS F1 hybrid accumulated high levels of acylglucoses (9.8 umol $g^{-1}$ leaf weight), which were slightly lower than that observed in AG3/AG4/AG11/AS. The percent of acylsugars that were acylglucoses in the CU071026 x AG3/AG4/AG11/AS hybrid was only 43.8%, which was much less than the percent of acylglucose acylsugars in AG3/AG4/AG11/AS (84.8%). Conversely, the AG3/AG4/-AG11/AS and AG3/AG11/AS lines accumulated trace (0.3 umol $g^{-1}$ leaf weight) and no acylglucoses, respectively. The lack of acylglucose accumulation in the AG3/AG11/AS line and the trace acylglucose accumulation in the AG3/AG4/-AG11/AS line demonstrate clearly that both AG4QTL and AG11QTL are necessary for accumulation of high levels of acylglucoses. The high levels of acylglucoses in the CU071026 x AG3/AG4/AG11/AS F1 hybrid, were only slightly less than that of the AG3/AG4/AG11/AS line, indicating that heterozygosity for all three acylglucose QTL is sufficient to generate significant acylglucose accumulation. The much lower relative percent of acylglucose acylsugars in the CU071026 x AG3/AG4/AG11/AS F1 hybrid compared to that of the AG3/AG4/AG11/AS line, though, matches well with the BC1F4 and BC1F5 selections which illustrated that homozygosity for either AG4QTL or AG11QTL or both is necessary for a large proportion of the accumulated acylsugars to be acylglucoses. The lack of detectable acylglucoses in the AG3/AG11/AS line was again in contrast to the BC1F3 selections homozygous for AG3QTL and AG11QTL, but lacking AG4QTL which accumulated detectable levels of acylglucoses. This contrast suggests that a region or regions from *S. pennellii* LA716, in addition to AG4QTL, are missing in the AG3/AG11/AS line and necessary to generate high levels of acylglucoses.

*Fatty acid profile* Analysis of the entries revealed an unexpected impact on the fatty acid profile of the acylsugars produced, potentially due to the acylglucose QTL. Generally, the entries had fatty acid profiles that were similar to that of CU071026, as expected, but two entries (AG3/AG4/AG11/AS and AG3/AG4/-AG11/AS) displayed a decrease in ai-C5 fatty acids, and an increase in n-C10 fatty acids (Table 3.7). Specifically, the proportion of ai-C5 and n-C10 fatty acids in AG3/AG4/AG11/AS and AG3/AG4/-AG11/AS was 6.69%/5.62% and 6.25%/9.05%, respectively. Conversely, CU071026 displayed a higher proportion of ai-C5 (10.15%) and a lower proportion of n-C10 (1.37%). In addition, the AG3/AG11/AS line displayed a much lower proportion of i-C5 fatty acids, and higher proportion of n-C12 fatty acids than CU071026. However, because the AG3/AG11/AS line has very low acylsugar levels, which can result in the GC-MS derived fatty acid profile being unreliable, as discussed in Smeda et al. (2016). This suggests LC-MS profiling would be the preferable means of characterization of the AG3/AG11/AS acylsugars. The FA7QTL has also been shown to generate an increase in n-C10 fatty acids (Leckie et al. 2014; Smeda et al. 2016, in review), however, an introgression containing FA7QTL was not present in any of these entries, which suggests that one of the additional introgressions in the AG3/AG4/AG11/AS and AG3/AG4/-AG11/AS lines is involved in this trait. Because the AG3/AG4/-AG11/AS line displays the increased n-C10 phenotype, the AG11QTL region is not likely contributing to the increase in n-C10. The AG3/AG11/AS line does not accumulate an increase in n-C10, which again suggests the AG11QTL introgression is not involved, and that the AG3QTL introgression is not involved. Because the CU071026 x AG3/AG4/AG11/AS hybrid does not display an increase in n-C10 fatty acids, the *S. pennellii* LA716 alleles involved in the increased proportion of n-C10 are likely recessive, and increased n-C10 would require homozygosity for the LA716 alleles. Based on the other introgressions contained within the AG3/AG4/AG11/AS line (Table 3.6), the most likely remaining introgressions that might contribute to the increased n-C10 are the large introgression on chromosome 4, the introgression on the end of chromosome 10, or the introgression on the top

of chromosome 12. Additionally, the AG3/AG4/-AG11/AS line contains a small introgression (ca. 2.8 Mbp) on the end of chromosome 11 that is also contained in AG3/AG4/AG11/AS, and not in AG3/AG11/AS, and therefore could also be involved in the increased n-C10 fatty acids.

*LC-MS Characterization* An additional level of characterization, LC-MS, was implemented on some of the selections from the BC1F5 to more fully evaluate the effect of the AG11QTL on the acylsugar composition of the plants. LC-MS provided a way to corroborate the spectrophotometric assay evaluation which revealed the accumulation of acylglucoses, and also enabled a detailed comparison of the specific acylsugars accumulated between entries. The three BC1F5 plants identified as homozygous for the AG11QTL introgression (141425-120, 141425-185, 141425-188), as well as a plant lacking the AG11QTL introgression (141425-042) and a plant that was heterozygous for the introgression (141425-112) were included in the LC-MS analysis with the CU071026 control. Representative LC-MS chromatograms for each entry are displayed in Figure 3.2. A total of 71 acylsugars were identified as being accumulated by at least one of the entries (Figure 3.3). CU071026 was observed to accumulate exclusively acylsucroses, while the 141425-042 samples displayed a profile dominated by acylsucroses, but with accumulation of several acylglucoses. Specifically, the 141425-042 plant accumulated moderate levels of five acylglucoses: G3:22 (ID 1), G3:22 (ID 3), G3:15 (ID 5), G3:20 (ID 6), and an acylsugar annotated as an acylglucose (ID 63), but whose fatty acid acyl group profile was difficult to confirm due to low abundance and peak overlap. The 141425-120, 141425-185, and 141425-188 plants, homozygous for the AG11QTL, exhibited an acylsugar profile that was dominated by acylglucoses, but with significant accumulation of several acylsucroses. Specifically, looking at the peak areas of the acylsugars (data not shown), the most abundant acylsugars in the 141425-120, 141425-185, 141425-188 plants are two acylsucroses: S3:22 (ID 4) and S3:20 (ID 2). The next most abundant acylsugars in these three plants are acylglucoses with ID 5, ID 6, and ID 1, respectively. The 141425-112 plants, heterozygous for the AG11QTL, accumulated a more even mixture of acylsucroses and acylglucoses. Like the 141425-120,

141425-185, and 141425-188 plants, the 141425-112 plants' acylsugar profile was dominated most by the two acylsucroses with ID 2 and ID 4, but the next two most abundant acylsugars were also two acylsucroses with IDs 38 and ID 40, which were accumulated at very low levels in the 141425-120, 141425-185, and 141425-188 plants.

Figure 3.2. Representative LC-MS base peak intensity chromatograms for the BC1F5 acylglucose entries and CU071026. Each chromatogram is scaled according to the most abundant peak in the chromatogram to show differences in acylsugar profiles. The most predominant peaks are labeled with the sugar moiety (S, sucrose or G, glucose) and the number of acyl groups attached with the total number of carbons between the acyl groups.

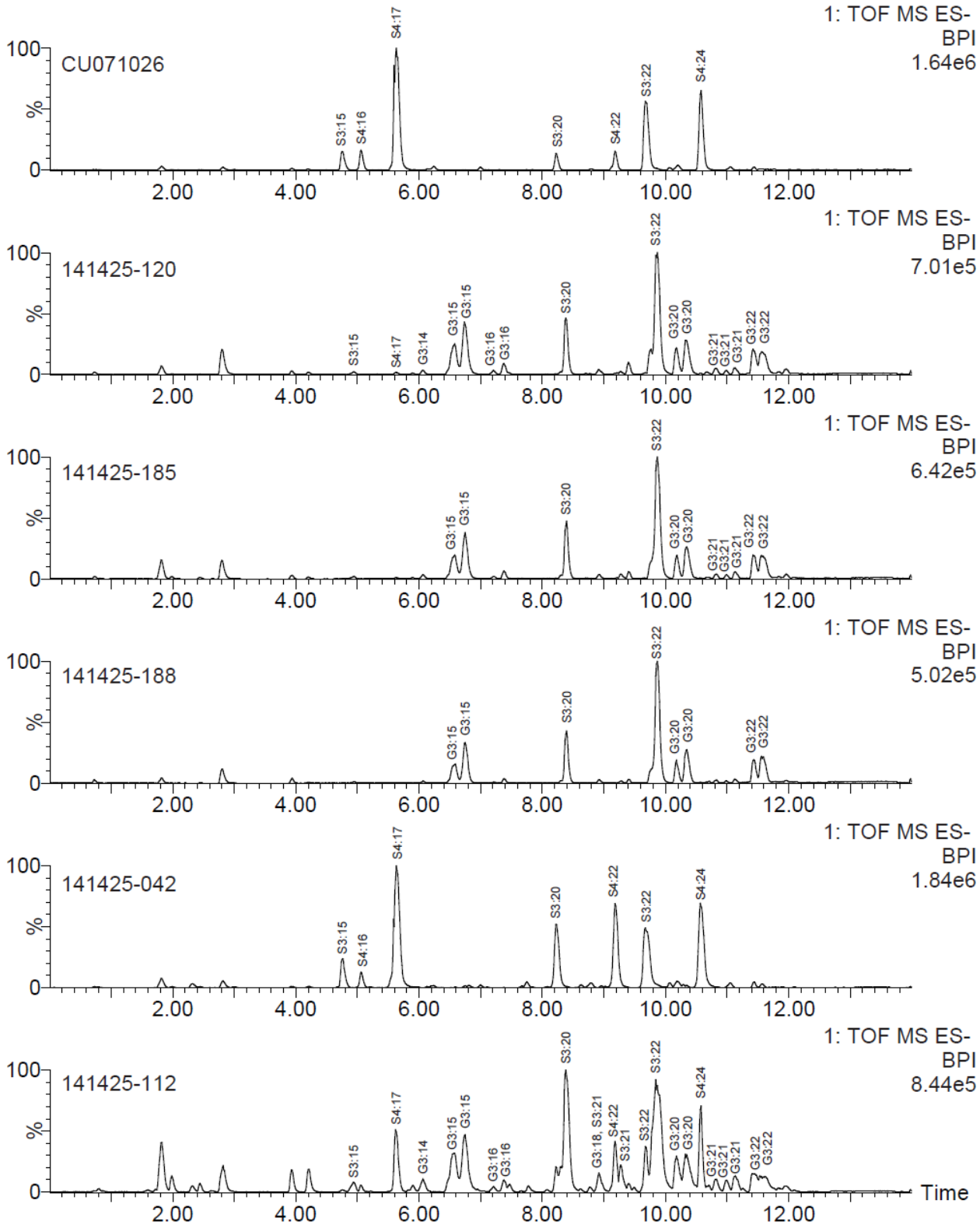
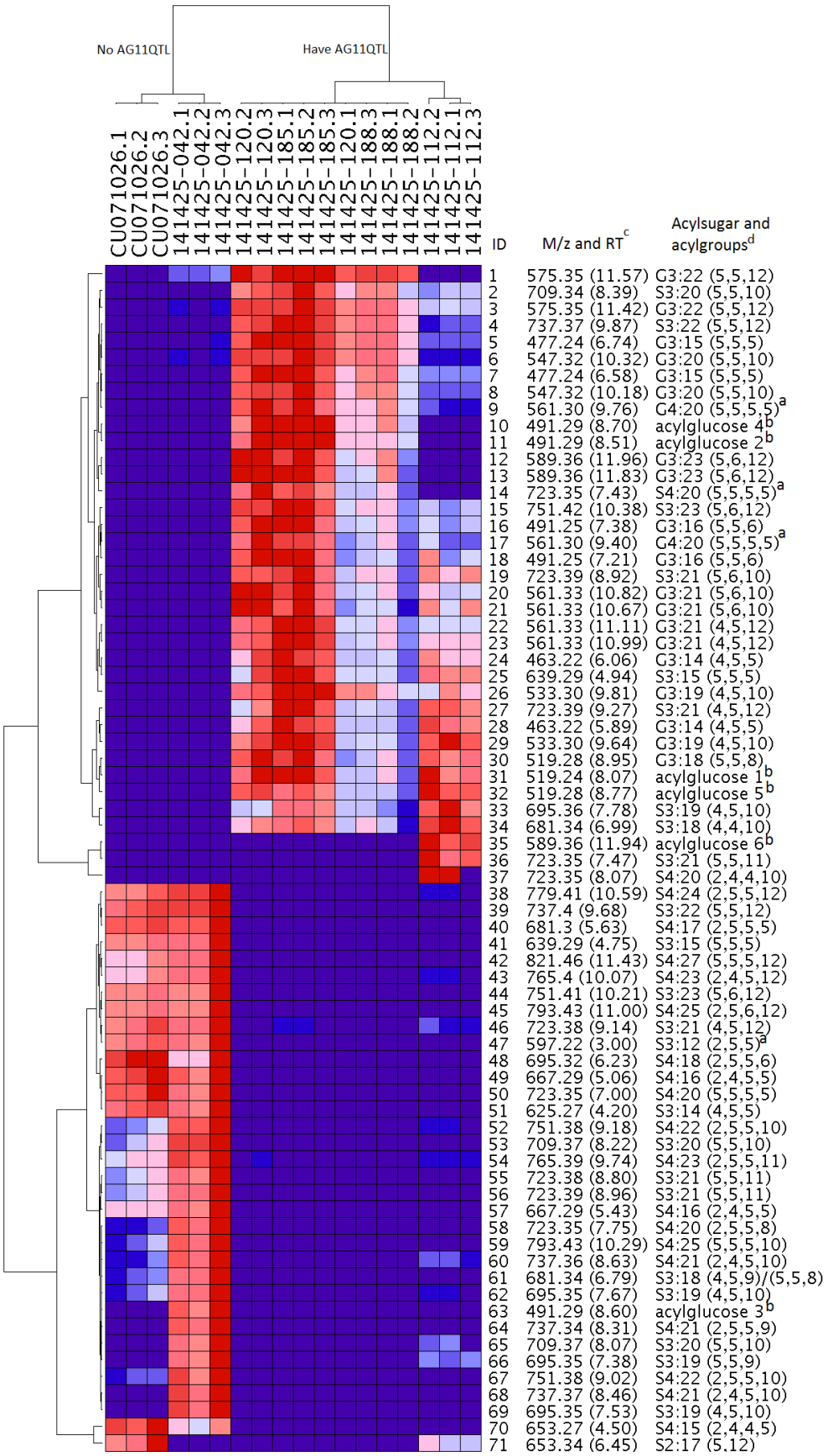

CU071026
1: TOF MS ES-
BPI
1.64e6
S3:15
S4:16
S4:17
S3:20
S4:22
S3:22
S4:24
141425-120
1: TOF MS ES-
BPI
7.01e5
S3:15
S4:17
G3:14
G3:15
G3:15
G3:16
G3:16
S3:20
S3:22
G3:20
G3:20
G3:21
G3:21
G3:21
G3:22
G3:22
141425-185
1: TOF MS ES-
BPI
6.42e5
G3:15
G3:15
S3:20
S3:22
G3:20
G3:20
G3:21
G3:21
G3:21
G3:22
G3:22
141425-188
1: TOF MS ES-
BPI
5.02e5
G3:15
G3:15
S3:20
S3:22
G3:20
G3:20
G3:22
G3:22
141425-042
1: TOF MS ES-
BPI
1.84e6
S3:15
S4:16
S4:17
S3:20
S4:22
S3:22
S4:24
141425-112
1: TOF MS ES-
BPI
8.44e5
S3:15
S4:17
G3:14
G3:15
G3:15
G3:16
G3:16
S3:20
G3:18, S3:21
S4:22
S3:21
S3:22
S3:22
G3:20
G3:20
S4:24
G3:21
G3:21
G3:21
G3:22
G3:22
%
100
0
2.00
4.00
6.00
8.00
10.00
12.00
Time

Figure 3.3. Hierarchical cluster analysis with Pearson correlation using a pairwise average-linkage clustering method, indicating the predominant acylsucroses and acylglucoses accumulated by each plant. Three samples were analyzed per plant. Color across a row indicates relative levels (peak area/g leaf weight) of the respective acylsugar, with red indicating samples with the highest levels detected and blue/purple indicating low or no detection relative to the highest sample for the particular type of acylsugar. [a]The proposed acylgroup number and length for an acylsugar whose identification was hampered by low abundance and peak overlap. [b]An annotated acylglucose based on m/z range but whose acylgroup number and length was hampered by low abundance and peak overlap. [c]The mass to charge ratio for each acylsugar followed by retention time in minutes. [d]Acylsugar nomenclature indicates S for sucrose backbone of the molecule and G for glucose, as well as the number of fatty acid acyl chains (2 to 4) with their cumulative length in carbons that are esterified to the sugar followed by the lengths in carbons of each acyl group in the respective acylsugar.


No AG11QTL
Have AG11QTL
CU071026.1
CU071026.2
CU071026.3
141425-042.1
141425-042.2
141425-042.3
141425-120.2
141425-120.3
141425-185.1
141425-185.2
141425-185.3
141425-120.1
141425-188.3
141425-188.1
141425-188.2
141425-112.2
141425-112.1
141425-112.3
ID
M/z and RT[c]
Acylsugar and acylgroups[d]
1 575.35 (11.57) G3:22 (5,5,12)
2 709.34 (8.39) S3:20 (5,5,10)
3 575.35 (11.42) G3:22 (5,5,12)
4 737.37 (9.87) S3:22 (5,5,12)
5 477.24 (6.74) G3:15 (5,5,5)
6 547.32 (10.32) G3:20 (5,5,10)
7 477.24 (6.58) G3:15 (5,5,5)
8 547.32 (10.18) G3:20 (5,5,10)
9 561.30 (9.76) G4:20 (5,5,5,5)[a]
10 491.29 (8.70) acylglucose 4[b]
11 491.29 (8.51) acylglucose 2[b]
12 589.36 (11.96) G3:23 (5,6,12)
13 589.36 (11.83) G3:23 (5,6,12)
14 723.35 (7.43) S4:20 (5,5,5,5)[a]
15 751.42 (10.38) S3:23 (5,6,12)
16 491.25 (7.38) G3:16 (5,5,6)
17 561.30 (9.40) G4:20 (5,5,5,5)[a]
18 491.25 (7.21) G3:16 (5,5,6)
19 723.39 (8.92) S3:21 (5,6,10)
20 561.33 (10.82) G3:21 (5,6,10)
21 561.33 (10.67) G3:21 (5,6,10)
22 561.33 (11.11) G3:21 (4,5,12)
23 561.33 (10.99) G3:21 (4,5,12)
24 463.22 (6.06) G3:14 (4,5,5)
25 639.29 (4.94) S3:15 (5,5,5)
26 533.30 (9.81) G3:19 (4,5,10)
27 723.39 (9.27) S3:21 (4,5,12)
28 463.22 (5.89) G3:14 (4,5,5)
29 533.30 (9.64) G3:19 (4,5,10)
30 519.28 (8.95) G3:18 (5,5,8)
31 519.24 (8.07) acylglucose 1[b]
32 519.28 (8.77) acylglucose 5[b]
33 695.36 (7.78) S3:19 (4,5,10)
34 681.34 (6.99) S3:18 (4,4,10)
35 589.36 (11.94) acylglucose 6[b]
36 723.35 (7.47) S3:21 (5,5,11)
37 723.35 (8.07) S4:20 (2,4,4,10)
38 779.41 (10.59) S4:24 (2,5,5,12)
39 737.4 (9.68) S3:22 (5,5,12)
40 681.3 (5.63) S4:17 (2,5,5,5)
41 639.29 (4.75) S3:15 (5,5,5)
42 821.46 (11.43) S4:27 (5,5,5,12)
43 765.4 (10.07) S4:23 (2,4,5,12)
44 751.41 (10.21) S3:23 (5,6,12)
45 793.43 (11.00) S4:25 (2,5,6,12)
46 723.38 (9.14) S3:21 (4,5,12)
47 597.22 (3.00) S3:12 (2,5,5)[a]
48 695.32 (6.23) S4:18 (2,5,5,6)
49 667.29 (5.06) S4:16 (2,4,5,5)
50 723.35 (7.00) S4:20 (5,5,5,5)
51 625.27 (4.20) S3:14 (4,5,5)
52 751.38 (9.18) S4:22 (2,5,5,10)
53 709.37 (8.22) S3:20 (5,5,10)
54 765.39 (9.74) S4:23 (2,5,5,11)
55 723.38 (8.80) S3:21 (5,5,11)
56 723.39 (8.96) S3:21 (5,5,11)
57 667.29 (5.43) S4:16 (2,4,5,5)
58 723.35 (7.75) S4:20 (2,5,5,8)
59 793.43 (10.29) S4:25 (5,5,5,10)
60 737.36 (8.63) S4:21 (2,4,5,10)
61 681.34 (6.79) S3:18 (4,5,9)/(5,5,8)
62 695.35 (7.67) S3:19 (4,5,10)
63 491.29 (8.60) acylglucose 3[b]
64 737.34 (8.31) S4:21 (2,5,5,9)
65 709.37 (8.07) S3:20 (5,5,10)
66 695.35 (7.38) S3:19 (5,5,9)
67 751.38 (9.02) S4:22 (2,5,5,10)
68 737.37 (8.46) S4:21 (2,4,5,10)
69 695.35 (7.53) S3:19 (4,5,10)
70 653.27 (4.50) S4:15 (2,4,4,5)
71 653.34 (6.45) S2:17 (5,12)

The hierarchical cluster analysis (HCA) revealed a pattern of acylsugar composition largely defined by AG11QTL, but also by AG3QTL and AG4QTL. For example, the HCA revealed that the entries split into two broad groups, those with and without AG11QTL (Figure 3.3). Within the plants lacking the AG11QTL, the HCA clearly showed the 141425-042 plants clustered versus the CU071026 plants, which is largely due to the moderate accumulation of several acylglucoses in the 141425-042 plant. Within the plants containing the AG11QTL, the three entries homozygous for AG11QTL (141425-120, 141425-185, and 141425-188) were virtually indistinguishable. The 141425-112 plants, on the other hand, clustered separately, likely from the decrease in moderate accumulation of acylglucoses, and increase in accumulation of acylsucroses, indicating the incompletely dominant action of the AG11QTL, or linked QTL. The 141425-112 plants also accumulated moderate levels of three acylsugars with ID 35, ID 36, and ID 37 that are only moderately accumulated in this entry.

For several acylsugars identified in the LC-MS analysis, there were clear chromatographic separation of compounds having identical mass and indistinguishable mass spectra, for example G3:22 (5,5,12) (ID 1) with a retention time of 11.57 min and G3:22 (5,5,12) (ID 3) with a retention time of 11.42 min (Figure 3.3). These acylsugar isomers likely differ in either the position of acyl chain attachment or in the branching of the acyl chains. This is similar to the observations in Smeda et al. (2016, in review) of acylsucroses having clear chromatographic separation despite identical mass and indistinguishable mass spectra. For the two acylglucoses (ID 1 and ID 3) that likely differ in position or orientation of the acyl chains, the abundance of these acylsugars differed between entries. Specifically, the acylglucose with ID 3 was highly abundant in the 141425-120, 141425-185, and 141425-188 plants, but also moderately accumulated in the 141425-112 and 141425-042 entries. Conversely, the acylglucose with ID 1 was abundant in the 141425-120, 141425-185, and 141425-188 plants, which are homozygous for AG11QTL, but was not detectable in the 141425-112 plants, which are heterozygous for AG11QTL. This observation suggested there could be a recessive effect of

the AG11QTL or a linked QTL to affect either acyl chain position or orientation. However, the 141425-042 plants accumulated moderate levels of the ID 1 acylglucose, despite lacking of the AG11QTL. An explanation for this could involve the complex interactions previously detected between the AG3QTL, AG4QTL, and AG11QTL, such that homozygous *S. lycopersicum* or *S. pennellii* AG11QTL alleles confer a similar effect to generate the ID 1 acylglucose isomer through interaction with the AG3QTL and AG4QTL, but that when heterozygous, this interaction is disrupted.

In addition to these acylglucose isomers, several acylsucrose isomers displayed presence/absence variation between entries. For example, a pair of acylsucrose isomers differing in retention time despite identical mass and indistinguishable mass spectra suggests a role for the AG4QTL in the HCA. Specifically, the S3:19 (4,5,10) ID 62 acylsugar is accumulated by both CU071026 and 141425-042, although the levels are 11x higher in 141425-042 (data not shown). Conversely, the S3:19 (4,5,10) ID 69 acylsugar is only accumulated in 141425-042, although at a much lower level (ca. 0.1x) (data not shown) than the ID 62 acylsugar. The presence/absence of the ID 62 and 69 acylsugar in CU071026 and 141425-042 is consistent with data presented in Fan et al. (2016) showing the presence of a recently characterized acyltransferase, Sp-ASAT2, led to accumulation of a few acylsucroses with all acyl chains on the pyranose ring, which were virtually undetectable in plants lacking Sp-ASAT2. Additionally, Fan et al. (2016) observed that the levels of the isomer with all acyl chains on the pyranose ring were much lower than the isomer with an acyl chain on the furanose ring, consistent with the much lower levels of the ID 69 acylsugar in 141425-042 vs the ID 62 acylsugar. Together this data suggests that the ID 62 acylsugar has an acyl chain on the furanose ring while the ID 69 acylsugar has all acyl chains on the pyranose ring. The presence/absence of these acylsucrose isomers likely demonstrates an effect of the AG4QTL, probably through action of Sp-ASAT2, to generate acylsucroses distinct from those of CU071026.

Another pair of acylsucroses (ID 2 and ID 4) are exclusively accumulated in the entries containing the AG11QTL (141425-112, 141425-120, 141425-185, and 141425-188) and not detected in the 141425-042 samples or CU071026. The likely explanation for this pattern is that the AG11QTL introgression overlaps with the previously identified ASAT3 acyltransferase gene (Schilmiller et al. 2015), which controls the location of an acyl chain attachment. Specifically, Schilmiller et al. (2015) showed that Sl-ASAT3 functions in *S. lycopersicum* to esterify an acyl chain to the furanose (fructose) ring in an acylsucrose, whereas in IL11-3 and *S. pennellii* LA716, Sp-ASAT3 has a transposon insertion, which inhibits acylation of the furanose ring. Two acylsucroses (IDs 53 and 65) that are comparable to the ID 2 acylsucrose in mass and mass spectra are highly accumulated in the 141425-042 plants and lowly accumulated in the 141425-112 plants, but not detectable in the 141425-120, 141425-185, and 141425-188 plants. This same pattern holds true for the acylsucrose with ID 4 and an acylsucrose with ID 39. Therefore, it is likely that the difference between the ID 2 and ID 53/65 acylsucroses and the ID 4 and 39 acylsucroses is that the ID 2 and 4 acylsucroses have all acyl chains on the pyranose (glucose) ring. This hypothesis is also supported by the observation that the plants homozygous for AG11QTL (141425-120, 141425-185, and 141425-188) only share trace accumulation of two acylsucroses (ID 46 and ID 54) that are moderately accumulated by CU071026. The accumulation patterns of these acylsugars suggests AG11QTL, likely due to the action of Sp-ASAT3, leads to a profile characterized by acylsucroses with all acyl chains on the pyranose ring.

HCA results also show that the 141425-120, 141425-185, and 141425-188 plants, homozygous for AG11QTL, did not accumulate appreciable levels of tetra-acylsucroses, or acylglucoses/acylsucroses with an acetate (C=2) acyl group. The likely explanation for this phenotype again is that the Sp-ASAT3 acyltransferase acylates the pyranose ring, specifically at the R2 position (Schilmiller et al. 2015), which is the same location that the tomato allele of the ASAT4 acyltransferase (Sl-ASAT4) esterifies an acetate group (Schilmiller et al. 2012). Because

Sp-ASAT3 acylates the R2 position prior to acylation by Sl-ASAT4, plants homozygous for AG11QTL, and therefore likely the Sp-ASAT3 allele, cannot accumulate tetra-acylsucroses, or acylsugars with an acetate group.

Across entries, the LC-MS data demonstrated the major effect of the AG11QTL to control the relative proportion of acylsucroses versus acylglucoses and tetra-acylsucroses vs tri-acylsucroses in the acylsugar profile. This effect was most pronounced when the AG11QTL was homozygous, but also evident when AG11QTL was heterozygous. The data also showed that in the absence of the AG11QTL plants could still accumulate low to moderate levels of a few acylglucoses, highlighting the likelihood that the other necessary QTL (AG3 and AG4) are sufficient for some acylglucose accumulation, but that combination with AG11QTL is required for high levels of acylglucoses. Additionally, the data demonstrated the likely effect of AG4QTL to control accumulation of unique acylsucroses. Finally, the profile of the plant (141425-112) heterozygous for AG11QTL revealed a distinct composition of acylsugars, including three acylsugars unique to 141425-112. This suggests a potential role for hybrid deployment of the AG11QTL, and or other acylglucose QTL, to capitalize on the allelic interactions that convey accumulation of unique acylsugar isomers which could have greater efficacy against insect pests of tomato.

**Fine-mapping of the acylglucose QTL and gene candidates**

Fine-mapping of the AG3QTL was possible using selections and recombinants from the BC1F3 population. Previously, Leckie et al. (2013) had delineated the AG3QTL region to be located between C2_At1g79840 (70,450,223 - 70,457,873) and TG244 (72,179,021 - 72,184,054) (Tomato SL3.0 ITAG3.1 Solgenomics.net). A selection from our study allowed the location of AG3QTL to be further defined as a ca. 1.5 Mbp region between markers C2_At2g42110 (70,672,281 - 70,673,549) and TG244 (Figure 3.4). There are 273 annotated genes within this

region, none of which have been implicated in acylsugar biosynthesis (Golden Helix 2016 using SL2.5 ITAG 2.4).

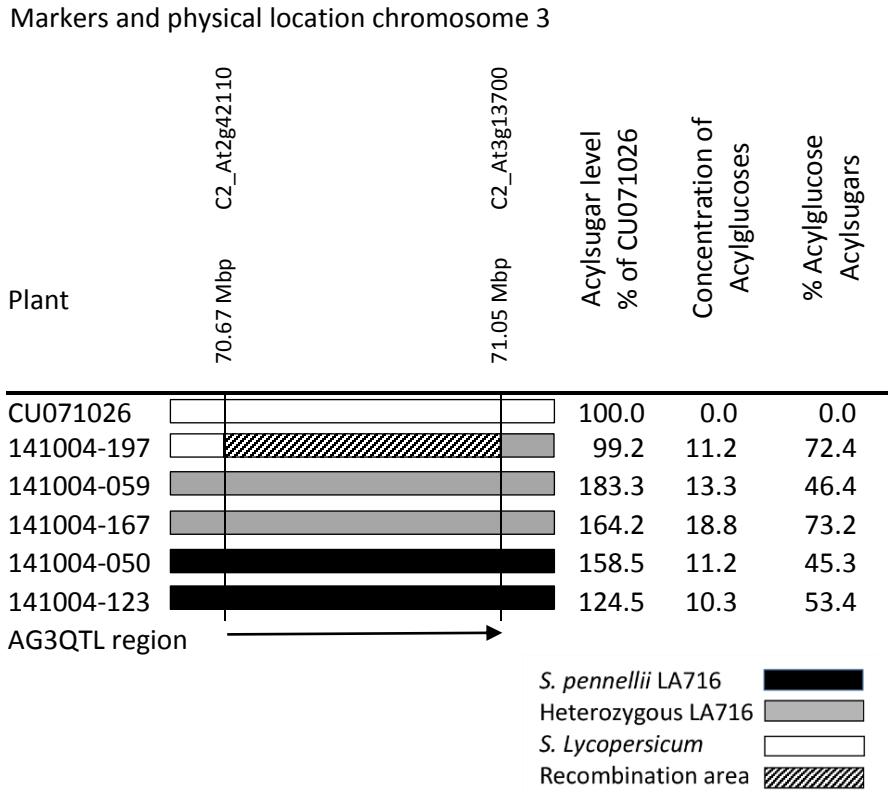



Figure 3.4. Markers, acylsugar level and acylglucose accumulation diagnostic for the presence of AG3QTL, on chromosome 3, in CU071026 and selected individuals out of a BC1F3 population showing the relative location of AG3QTL is between C2_At2g42110 and C2_At3g13700. Concentration of acylglucoses is in umol $g^{-1}$ dry leaf weight.

Fine-mapping of the AG4QTL was also possible using recombinant and non-recombinant plants from the BC1F3 population. Previously, Leckie et al. (2013) had delineated the AG4QTL region to be located between C2_At2g20390 (3,961,733 - 3,968,830) and TG182 (4,833,026 - 4,838,070) (Tomato SL3.0 ITAG3.1 Solgenomics.net). The recombinant plants from our study allowed the location of AG4QTL to be further defined as a 1.9 Mbp region between C2_At3g19895 (2,776,549 - 2,785,771) and C2_At5g50720 (4,663,049 - 4,664,350) (Figure 3.5) (Tomato SL3.0 ITAG3.1 Solgenomics.net) overlapping that from Leckie et al.

(2013). Together the recombinants from this study and data from Leckie et al. (2013) allowed delineation of the AG4QTL down to ca. 700 kb region between C2_At2g20390 and C2_At5g50720. The recently characterized (ASAT2) (Fan et al. 2016) acyltransferase, Solyc04g012020, is located between 4,353,166 - 4,354,506 bp (Tomato SL3.0 ITAG3.1 Solgenomics.net) on chromosome 4, which is within the fine-mapped location of AG4QTL, and could be the gene underlying this acylglucose QTL.

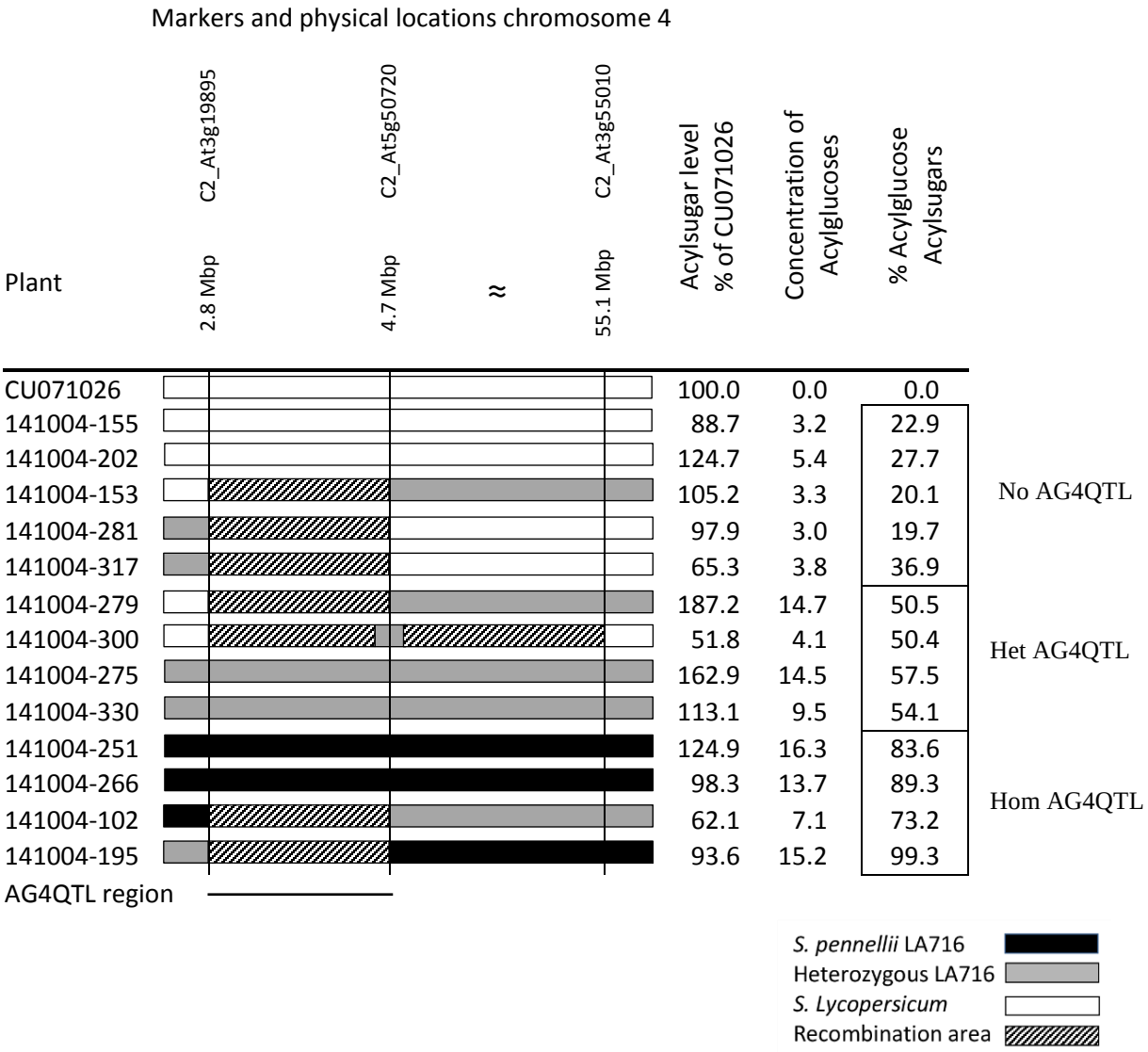



Figure 3.5. Markers, acylsugar level and acylglucose accumulation diagnostic for the presence of AG4QTL, on chromosome 4, in CU071026 and selected individuals out of a BC1F3 population showing the relative location of AG4QTL is between C2_At3g19895 and C2_At5g50720. Concentration of acylglucoses is in umol $g^{-1}$ dry leaf weight. ≈ represents a large physical distance.

Fine-mapping of the AG11QTL was possible using GBS analysis of recombinant and non-recombinant plants from the BC1F4 population. Previously, Leckie et al. (2013) had delineated the AG11QTL region to be located between C2_At4g01560 (52,303,644 - 52,309,660) and C2_At2g27290 (53,712,003 - 53,714,416) (Tomato SL3.0 ITAG3.1 Solgenomics.net). Coordination of GBS, acylsugar level and acylglucose accumulation data from BC1F4 plants in our study allowed finer resolution of AG11QTL to a ca. 500 Kbp region between GBS SNP 3 (ca. 53.2 Mbp) and GBS SNP 4 (ca. 53.7 Mbp) (Figure 3.6). The recently identified ASAT3 acyltransferase gene, Solyc11g067270, (Schilmiller et al. 2015), is contained in the IL11-3 introgression; Sp-ASAT3 has been shown to lower acylsugar levels in IL11-3 and to acylate the pyranose (glucose) ring of acylsucroses in IL11-3 and *S. pennellii* LA716 (Schilmiller et al. 2015). ASAT3 is located between 53,307,619 - 53,308,911bp (Tomato SL3.0 ITAG3.1 Solgenomics.net) on chromosome 11, which is within the fine-mapped location of AG11QTL, and could be the gene underlying the AG11QTL.

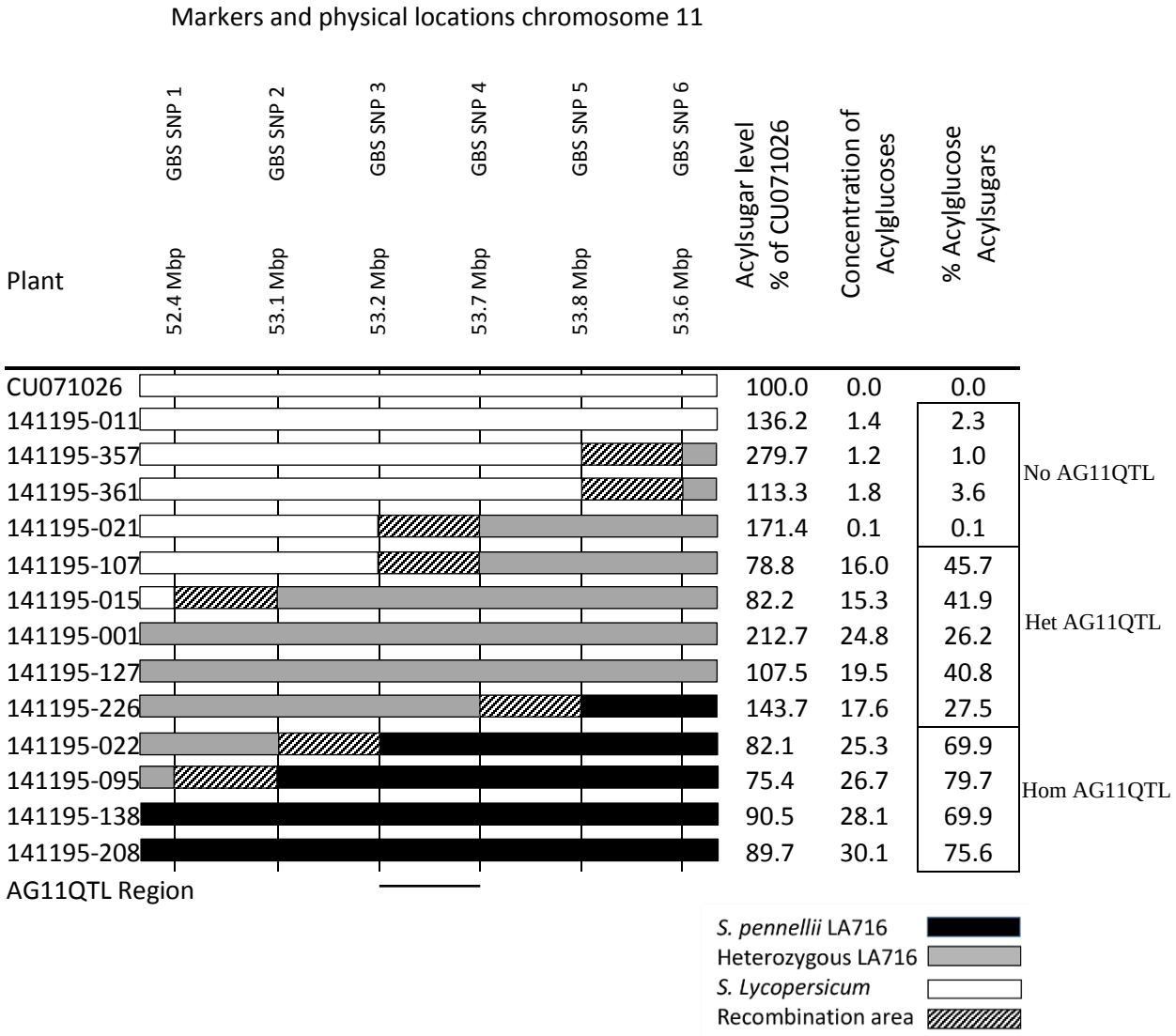



Figure 3.6. Markers, acylsugar level and acylglucose accumulation diagnostic for the presence of AG11QTL, on chromosome 11, in CU071026 and selected individuals out of a BC1F4 population showing the relative location of AG11QTL is between GBS SNP 3 and GBS SNP 4. Concentration of acylglucoses is in umol $g^{-1}$ dry leaf weight. All selections used for fine-mapping were heterozygous/homozygous for AG3QTL and heterozygous for AG4QTL. Het and Hom AG11QTL indicate groups of plants heterozygous and homozygous for AG11QTL, respectively. Physical distances are estimates from converting SL2.5 build locations to SL3.0 build locations.

**Potential for ASAT1 to ASTA4 and other acylsugar genes to affect acylglucose biosynthesis and the phenotypes of BC1F1 to BC1F5 plants**

A series of recent studies have elucidated four BAHD acyltransferases (ASAT1 to ASAT4) that largely control the acylation of acylsucroses in tomato and wild relatives (Schilmiller et al 2012, 2015; Fan et al. 2016). Data from our study in conjunction with knowledge of the ASAT genes helps clarify the likely impact of these acyltrasferases on the acylsugar and acylglucose accumulation observed in the BC1F1 to BC1F5 populations.

*ASAT1* ASAT1, identified in Fan et al. (2016) is on the top of chromosome 12, and controls the first acylation step in acylsucrose biosynthesis, but could be a candidate for an additional QTL involved in acylsugar or acylglucose biosynthesis. Fan et al. (2016) showed that the tomato (Sl-ASAT1) and *S. pennellii* (Sp-ASAT1) alleles have a similar function to acylate the R4 position on the pyranose ring of an acylsucrose with either a short or longer chain fatty acid. The Sl-ASAT1 allele was shown to have no affinity for glucose in vitro, which could contribute to the lack of acylglucoses accumulated in cultivated tomato, such as M82. However, they did not find variation for ASAT1 across the tomato clade, suggesting that Sp-ASAT1 does not have the ability to acylate acylglucoses, and is not likely the first committed step in acylglucose biosynthesis. The IL BC1F1 and BC1F2 populations in our study carried the Sl-ASAT1 allele, while Sp-ASAT1 was segregating in the interspecific BC1F3 and BC1F4 populations, and brought to homozygosity in the BC1F5 selections. The effect of the Sp-ASAT1 versus the Sl-ASAT1 allele on the acylsugar level and acylglucose accumulation in the BC1F3, BC1F4 and BC1F5 populations is unknown. However, high acylsugar and acylglucose accumulating entries with either the Sp-ASAT1 or Sl-ASAT1 allele were identified in the interspecific BC1F3, BC1F4, and BC1F5 populations in our study (data not shown), therefore, there was unlikely an ASAT1 allele effect on acylsugar level or acylglucose accumulation.

*ASAT2 and ASAT3* Fan et al. (2016) also identified ASAT2, the second acylation step in acylsucrose biosynthesis in tomato, which is located on chromosome 4 within our fine-

mapped region of AG4QTL. They showed that Sl-ASAT2 allowed acylation of a variety of acyl chain lengths and orientations at the R3 position on the pyranose ring. The results of Fan et al. (2016) also suggested that the Sp-ASAT2 acyltransferase alone or in conjunction with an unknown enzyme(s) could generate acylsucroses with all three acyl chains on the pyranose ring. They observed this pyranose only acylation phenotype in IL4-1 and IL4-2, through accumulation of two unique acylsugars with all three acyl chains on the pyranose ring, a phenotype which was virtually undetectable in tomato. Sp-ASAT2 was also shown to have little activity in vitro with a common mono-acylsucrose substrate utilized by Sl-ASAT2. This could suggest that the Sp-ASAT2 enzyme has an affinity for glucose, although no evidence for the acylation of acylglucoses by Sp-ASAT2 has been shown. Due to limited activity with mono-acylsucroses, it is likely that in *S. pennellii*, this enzyme may not be the second step in acylsucrose biosynthesis. In fact, evidence for a different order of enzymes in *S. pennellii* acylsucrose biosynthesis is provided by Schilmiller et al. (2015), where they demonstrated in vitro that the *S. pennellii* allele of a third ASAT acyltransferase, Sp-ASAT3, on chromosome 11 within the fine-mapped region of AG11QTL, acylates mono-acylsucroses on the pyranose ring at the R2 position. This is in contrast to the tomato allele, Sl-ASAT3, which acylates the R3' position on the furanose ring in acylsucrose biosynthesis.

ASAT2 and ASAT3 together likely explain much of the variation for acylsugar level and could explain some of the variation for acylglucose accumulation seen in Leckie et al. (2013), and our study. Homozygosity for the Sp-ASAT2 allele with the Sl-ASAT3 allele consistently led to lower total acylsugar levels for our study in the BC1F4 selections homozygous for the AG4QTL and lacking the AG11QTL. This same trend was observed in a BC1F2 population from Leckie et al. (2013), and in the IL4-1/IL4-2 plants tested in Schilmiller et al. (2010). Similarly, homozygosity for Sp-ASAT3 with Sl-ASAT2 led to significant decline in total acylsugar levels, for our study in the BC1F2 and BC1F3 plants homozygous for AG11QTL and lacking the AG4QTL.

This trend was also observed in the BC1F2 selections in Leckie et al. (2013) as well as in evaluation of IL11-3 in Schilmiller et al. (2010, 2015).

There are a few possible explanations for these changes in acylsugar levels. One explanation for when a plant is homozygous for Sl-ASAT2 and Sp-ASAT3 is that there could be competition for the order of acylation. Sl-ASAT2 is the second acylation step in acylsucrose biosynthesis, and Sp-ASAT3 is the second step in the proposed biosynthesis of acylglucoses (Schilmiller et al. 2015). Furthermore, if Sp-ASAT3 acylates the R2 position first, the Sl-ASAT2 activity could be inhibited. Evidence for ASAT activity being affected by acylation location was shown in Schilmiller et al. (2015) where they observed that pre-existing acylation at the R2 position in a di-acylsucrose inhibited Sl-ASAT3 acylation of the R3' position on the furanose ring. If Sl-ASAT2 is inhibited, then it is likely more di-acylsucroses would result. Di-acylsucroses are not commonly observed in LC-MS profiles (Schilmiller et al. 2010, 2015; Fan et al. 2016; Smeda et al. 2016, in review), which could suggest di-acylsucroses are unstable. Evidence for the instability of acylsucroses with less than three acyl chains is demonstrated by Fan et al. (2016) where they showed a mono-acylated sucrose hydrolyzed to sucrose in the absence of an acyl coenzyme A (acylCoA) donor. Therefore, Sl-ASAT2 combined with Sp-ASAT3 could contribute to lower acylsugar through production of di-acylsucroses instead of tri-acylsucroses/tetra-acylsucroses. This same mechanism could explain why acylglucose accumulation is decreased in plants homozygous for Sl-ASAT2 and Sp-ASAT3 *if* Sl-ASAT2 is capable of acylating acylglucoses. Specifically, if Sp-ASAT3 acylation occurs before and inhibits Sl-ASAT2 acylation, then a proportion of the acylsugars synthesized will be di-acylglucoses. No di-acylglucoses were observed in characterization of the BC1F5 plants in our study, which suggests di-acylglucoses are not stable, and would likely be hydrolyzed prior to secretion from trichomes.

An explanation for the decreased total acylsugar and more specifically the reduced acylglucose levels observed in plants homozygous for Sp-ASAT2 and Sl-ASAT3 involves

inhibition of Sl-ASAT3 and lack of acylation of acylglucoses. IL4-1 and IL4-2 were shown to accumulate moderate levels of acylsucroses with three acyl chains on the pyranose ring (Schilmiller et al. 2010), which could suggest Sp-ASAT2, homozygous in these lines, or other unknown enzymes, are able to acylate the R2 and R3 positions on the pyranose ring. Inhibition of the Sl-ASAT3 activity to acylate the R3' position was shown to occur in vitro if the R2 position was acylated first (Schilmiller et al. 2015). If Sp-ASAT2 or another acyltransferase acylates the R2 position of an acylsucrose before the Sl-ASAT3 acylation step, then it is likely that Sl-ASAT3 activity would be inhibited, and a greater proportion of di-acylsucroses would result, which are likely unstable and would be hydrolyzed. Data from the BC1F4 and BC1F5 populations in our study clearly demonstrated that lack of AG11QTL, (likely presence of the Sl-ASAT3 alleles) greatly reduced the accumulation of acylglucoses. The simple explanation for the necessity of Sp-ASAT3 for acylglucose accumulation is that Sl-ASAT3 acylates the furanose ring, which is not present in an acylglucose. Therefore, the acylglucoses detected when Sl-ASAT3 is combined with AG3QTL and AG4QTL (likely Sp-ASAT2), like in 141425-042 (Figure 3.3), likely result from Sp-ASAT2 or an unknown enzyme(s) acylating the R2 and R3 positions. Since Sp-ASAT2 or the unknown enzyme(s) do not appear to readily acylate both the R2 and R3 position, when Sl-ASAT3 is present, it is likely that mostly mono or di-acylglucoses would be produced, which were not observed in our study and are likely unstable and hydrolyzed.

*ASAT4* The ASAT4 acyltransferase (Schilmiller et al. 2012) likely interacted with Sp-ASAT3 in our study to affect the LC-MS profile of the BC1F5 selections. While all plants in our study were homozygous for the tomato allele (Sl-ASAT4), it is possible that the interaction between Sl-ASAT4 and Sp-ASAT3 limited the ability of plants with this combination to accumulate tetra-acylsucroses, such as 141425-120, 141425-185, and 141425-188 (Figure 3.3). The inability to accumulate tetra-acylsucroses in plants with Sp-ASAT3 suggests that interaction between Sp-ASAT3 and Sp-ASAT4 could also contribute to the lower total acylsugar levels observed in our study for plants carrying AG11QTL (likely Sp-ASAT3) through lower

diversity of acylsucroses. This is supported by transformation of IL11-3 plants with Sl-ASAT3 that demonstrated increased acylsugar levels, presumably due to the novel accumulation of tri and tetra-acylsucroses with furanose ring acylation (Schilmiller et al. 2015).

*Additional genes* In addition to the ASATs, other studies have elucidated components of acylglucose biosynthesis that could play a role in the phenotypes observed in our study and that of Leckie et al. (2013). An alternative to the initially proposed thioester acyl-CoA mediated transfer of acyl groups to acylglucoses was shown in work with *S. pennellii* LA716 tissue, which suggested glucosyl transferase activity could form 1-O-acylglucose from a free isobutyrate group and UDP-glucose (Ghangas and Steffens 1993). This was followed by a study demonstrating separation and partial purification of two UDP-glucose:fatty acid glucosyl transferases from *S. pennellii* LA1376 that could catalyze the proposed UDP-Glucose-dependent activation of fatty acids as 1-O-acyl-*β*-glucoses (Kuai et al., 1997). Additionally, a serine carboxypeptidase-like acyltransferase was identified and shown in vitro to catalyze the in vitro disproportionation of two molecules of 1-O-acyl-b-glucose to generate diacylglucose and a free glucose (Li et al., 1999; Li and Steffens, 2000). The serine carboxypeptidase, termed a glucose acyltransferase, was located on chromosome 10 (Ghangas and Steffens 1993; McNally and Mutschler 1997), but was not segregating in the BC1F2 population from Leckie et al. (2013). This glucose acyltransferase was not present in the BC1F1 and BC1F2 populations in our study, and would not have been segregating in the BC1F3, BC1F4, and BC1F5 populations from our study. Therefore, any variation for acylsugar level, acylglucose accumulation, and LC-MS profile observed in the populations and selections in our study is not likely due to the glucose acyltransferase. The additional *S. pennellii* LA716 introgressions in the AG3/AG4/AG11/AS line, beyond those present in CU071026, contain a number of genes annotated with glycosyltransferase-like activity; these genes are found in additional introgressions Add-int-4a, Add-int-4b, Add-int-10, Add-int-11, and Add-int-12 (Table 3.6). However, only one of the additional introgressions, Add-int-10, contained genes annotated with

UDP-glucosyltransferase-like activity, the same activity as the enzymes identified in Kuai et al. (1997) that were associated with the ability to generate mono-acylglucoses. A series of six genes with this annotation are located on chromosome 10 within the Add-int-10 introgression and could play a role in acylglucose biosynthesis.

Slocombe et al. (2008) identified genes integral to another portion of acylsugar biosynthesis in *S. pennellii* LA716, demonstrating that the branched-chain keto acid dehydrogenase (BCKD) enzyme complex, which catalyzes the decarboxylation of keto acids to generate acyl-CoAs, is implicated in the biosynthesis of branched chain fatty acids (BCFAs) destined for acylsugars. Slocombe et al. (2008) demonstrated the importance of the BCKD complex for acylsugar accumulation in *S. pennellii* LA716, but did not provide elucidation of the synthesis of tri-acylglucoses, and still implicated glucosyltransferase activity to generate mono-acylglucoses. The ASAT genes utilize acyl-CoAs as donors for the acyl groups that are esterified to acylsucroses, and therefore support an acyl-CoA mediated system for acylsucrose synthesis, but the presence of acyl-CoA dependent acylglucose acylation has not been elucidated. Slocombe et al. (2008) identified through microarray analysis a number of genes that were preferentially expressed in *S. pennellii* LA716 leaf trichomes. Among the identified genes, several are contained within the *S. pennellii* LA716 introgressions in the AG3/AG4/AG11/AS line, particularly within Add-int-10. The presence and segregation of the *S. pennellii* alleles for these genes in the BC1F3, BC1F4 and BC1F5 populations in our study indicates that these genes, previously associated with preferential accumulation in *S. pennellii* LA716 leaf trichomes, could have played a role in the accumulation of acylsugars and acylglucoses in our study. Together, the previously discovered gene candidates, especially the ASAT genes, likely explain much of the observed variation for acylsugar levels and acylsucrose phenotypes. Additionally, ASAT3 likely explains much of the variation for acylglucose accumulation in our study, however, the biosynthesis of acylglucoses and the genes responsible for additional variation in acylglucose accumulation seen in this work are still largely unknown.

**Utility of generated acylglucose germplasm to address acylsugar efficacy and biosynthesis hypotheses**

A prior study using purified acylsugars collected from CU071026 and four accessions of *S. pennellii* demonstrated the differential efficacy of acylsugars to control insect oviposition, which has led to various hypotheses about the structure and function relationships of acylsugars as insect deterrent compounds. Leckie et al. (2016) showed that the purified acylsugars from several accessions of *S. pennellii* were more effective at equimolar concentrations than those of CU071026 at reducing oviposition of *Bemisia tabaci*, Middle East-Asia Minor 1 Group (MEAM1), and *Frankliniella occidentalis*, western flower thrips (WFT). Purified acylsugars of two of the accessions that were composed an even mixture of acylglucoses and acylsucroses were generally more effective than purified acylsugars of the other two *S. pennellii* accessions composed of mostly acylglucoses. Leckie et al. (2016) separated the purified acylsugars of one of the accessions (LA1376), which has an even mixture of acylsucroses and acylglucoses, using column chromatography into polar and less polar fractions, which corresponded to acylsucroses and acylglucoses, respectively. Tests using these two acylsugar fractions showed that the less polar fraction (acylglucoses) was more effective at reducing oviposition of both *B. tabaci* (MEAM1) and *F. occidentalis* than the more polar fraction (acylsucroses), but that an even blend of both fractions exhibited synergism in that the blend was more effective than the summed effect of either fraction alone. While the data from the Leckie et al. (2016) study could suggest that acylglucoses are more effective than acylsucroses, the accessions and acylsugar fractions used in that study also differed in fatty acid composition of the acyl chains esterified to the sugar molecules. Additionally, acylglucoses are almost exclusively tri-acylated, while acylsucroses can be tri or tetra-acylated, and often exhibit acylation on both the pyranose and furanose rings. These multiple differences between the two fractions confound interpretation of which components of acylsugars contributed to the differential insect efficacy and synergy observed in Leckie et al. (2016); however, the results clearly indicate that increased diversity of

acylsugars could lead to synergy and pursuit of this phenomenon should be prioritized in breeding for optimal insect resistance.

The germplasm generated by our study could facilitate the evaluation of several hypotheses related to the results of Leckie et al. (2016). One hypothesis is that acylglucoses are more effective than acylsucroses in reducing insect oviposition, feeding and virus transmission. This hypothesis is hard to test because of the differences in number and location of fatty acid acyl groups and likelihood of differential fatty acid composition between acylsucroses and acylglucoses. Due to these potential confounding factors we cannot directly answer the hypothesis of whether acylglucoses are more effective than acylsucroses, but one related hypothesis this germplasm could address is whether the location of acyl chains on the sugar backbone contributes to acylsugar efficacy. The best way to address this hypothesis would be to fractionate the purified acylsugars of the AG3/AG4/AG11/AS and AG3/AG4/-AG11/AS lines and utilize the acylsucrose fractions in bio-assays against thrips and whiteflies. Since these two lines have a comparable fatty acid profile (Table 3.7), it is likely the relative proportions of the fatty acid acyl chains esterified to the acylsucroses of the two lines will be analogous. Where the two acylsucrose fractions differ, however, is that the AG3/AG4/AG11/AS line acylsucroses would almost exclusively contain acyl groups on the pyranose ring, with only trace levels of tetra-acylation, whereas the AG3/AG4/-AG11/AS acylsucroses would almost exclusively have furanose ring acylation, and a number would also be tetra-acylated. Comparison of the efficacy of these two fractions in bioassays would help determine if acylation on the furanose ring, in conjunction with tetra-acylation, alters the efficacy of acylsucroses.

A related hypothesis that could be addressed is whether the number of acyl chains and location of acyl chain attachment is integral to the synergism observed between acylglucose and acylsucrose fractions in Leckie et al. (2016). Specifically, the purified acylglucose fraction from AG3/AG4/AG11/AS could be individually combined with the acylsucrose fractions from AG3/AG4/AG11/AS, AG3/AG4/-AG11/AS and a mixture of the acylsucrose fractions of

AG3/AG4/AG11/AS and AG3/AG4/-AG11/AS. These would only be useful comparisons if the acylsucrose fractions from these two genotypes had similar fatty acid compositions, which is likely, as indicated above. The combined acylsucrose and acylglucose fractions from AG3/AG4/AG11/AS would likely all have acyl chains only on the pyranose ring, and the acylsucroses would be almost exclusively tri-acylated. The combination of acylglucoses from AG3/AG4/AG11/AS and the acylsucrose fraction from AG3/AG4/-AG11/AS would have the same acylglucose composition as the first combination, but almost all acylsucroses would have an acyl group on the furanose ring, with some being tetra-acylated, most likely with an acetate group. The final combination of acylglucoses from AG3/AG4/AG11/AS and the blended acylsucrose fractions from AG3/AG4/AG11/AS and AG3/AG4/-AG11/AS would have the same acylglucose composition as the other two fractions, but would have an acylsucrose composition that was a blend of the acylsucrose acylation composition from the first two fractions. Evaluation of the relative efficacy of the acylsugar fractions and blends and for synergistic effects of the blends could elucidate whether the location and number of acyl groups has a discernable impact on efficacy and synergism. Testing these blends would also provide data to help address the hypothesis that increased diversity of acylsugars leads to greater efficacy against insects.

A final hypothesis that could be tested using this germplasm is that combination of the mostly dominant FA5QTL (Leckie et al. 2014; Smeda et al. 2016) from *S. pennellii* LA716 with the AG3QTL/AG4QTL/AG11QTL will result in amelioration of the negative acylsugar level phenotype due to FA5QTL. This is predicted because the ASH genes (Schilmiller et al. 2016), likely underlying the FA5QTL, preferentially hydrolyze acyl groups from acylsucroses with furanose ring acylation. An F1 between FA5/AS (Smeda et al. 2016) and AG3/AG4/AG11/AS would allow initial evaluation of this hypothesis, and F2 selections homozygous for FA5QTL and AG3QTL/AG4QTL/AG11QTL would allow further evaluation of amelioration of the low acylsugar level phenotype, potentially through limiting acylsucrose substrate availability for ASH gene hydrolysis.

# CONCLUSIONS

This study presents the result of the combination of QTL involved in mediating the sugar moiety of a specialized metabolite, acylsugars, with known involvement in insect resistance in tomato, to create new acylsugar lines and a comprehensive evaluation of the action of these QTL in the resulting lines to mediate the sugar moiety of acylsugars. The development and characterization of these acylglucose lines and hybrids complements and builds upon the current platform of tomato lines that produce different types of acylsugars at moderately high levels and increases our knowledge concerning acylsugar biosynthesis and chemistry. This platform of germplasm could be used to elucidate remaining gaps in our understanding of acylsugar biosynthesis, such as the gene or genes underlying the AG3QTL, and the missing enzymatic steps in acylglucose biosynthesis. Two of the recently identified ASAT genes (ASAT2 and ASAT3) co-localized with the fine-mapped locations of the acylglucose QTL. Together with ASAT4, ASAT2 and ASAT3 could largely explain the acylsucrose acylation patterns and total acylsugar level phenotypes in our study, however, the potential ability of the ASAT genes to also control acylation of acylglucoses and the levels of acylglucoses accumulated has not yet been elucidated. The acylglucose germplasm developed in our study could also be used to facilitate the determination of whether the acylation patterns and acylglucose accumulation trait are pleiotropically controlled by interaction between ASAT2 and ASAT3. Additionally, this platform of germplasm will help with identification of the gene or genes controlling the increase in n-C10 fatty acids and additional regions involved in acylsugar accumulation and acylglucose biosynthesis.

This platform of germplasm could also be utilized for research in entomology, virology and ecology to elucidate the functionality of acylsugars as insect deterrents, which is still largely unknown. Comprehensive knowledge of the genes that control acylsugar level and structure would greatly facilitate the use of the acylglucose producing tomato breeding lines for cultivar development, since there are numerous epistatic interactions known to affect acylsugar moiety,

level, and fatty acid and acylsugar production/diversity (Leckie et al. 2012, 2014; Smeda et al. 2016, accepted). In particular, knowledge of gene identification and location would greatly aid in selecting plants with recombinations that maintain necessary genes while eliminating linkage drag.

Since the results of Leckie et al. (2016) suggest that increased acylsugar diversity could lead to greater insect efficacy through increased synergistic interactions, breeding work has already begun to combine the acylglucose QTL with the fatty acid QTL (FAQTL) from the acylsucrose-accumulating acylsugar tomato lines that possess different sets of FAQTL and produce acylsugars with different fatty acid and acylsucrose profiles (Smeda et al. 2016; in review) to increase the diversity of both acylsugar moiety/acyl chain location and acyl chain length/orientation. Additional QTL can be utilized, if it is necessary to raise to the total acylsugar levels accumulated, that were previously identified on chromosomes 6 and 10, (Blauth et al. 1998; Leckie et al. 2012) which raise acylsugar levels, sometimes through increased trichome density. In addition, since many of the acylsugar QTL are not recessive, hybrids between different acylsugar lines should also allow for creation of different sets of plants with increased acylsugar profile diversity. Seed of such F1 hybrids can be quickly generated for evaluation in bio-assays and field trials. Development of these acylglucose QTL lines with variation for acylsugar level, acylglucose accumulation, and fatty acid and acylsugar profile will facilitate development of elite breeding lines for the creation of hybrids that have the potential to reduce insect feeding, damage and virus incidence, as well as the possibility to reduce or eliminate spray regimes in field trials.

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