UNIVERSITAT DE BARCELONA

Altered Levels of Glycosylated Sterols Affect Tomato Development and Stress Response

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ALTERED LEVELS OF GLYCOSYLATED STEROLS AFFECT TOMATO DEVELOPMENT AND STRESS RESPONSE

Memòria presentada per Ángel de Jesús Chávez Martínez per optar al títol de doctor per la Universitat de Barcelona

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''Equipped with his five senses, man explores the universe around him and calls the adventure Science''

E. Hubble

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Si pudiera viajar al pasado y contarle a mi yo de hace 10 años lo que le tiene preparado el destino ¿por dónde empezaría?, tal vez le contaría que pasará una pandemia escribiendo una tesis sobre tomates, o que durante 5 años vivirá a 9083 km lejos de su familia, su perro y sus amigos, ¿le contaría sobre Bali, o de Australia? Probablemente no le diría nada y dejaría que fuera tan feliz como el actual yo lo ha sido descubriéndolo.

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SUMMARY

Sterols are a family of triterpenoid compounds that occur as free form (FS) or conjugated like steryl esters (SE), steryl glycosides (SG) and acylated steryl glycosides (ASG). Glycosylated sterols (SG and ASG) and FS are components of the cell membranes, where in combination with other membrane bound lipids play a key role in modulating their properties and function. Sterol glycosyltransferases (SGTs) catalyze the glycosylation of the free hydroxyl group at C-3 position of FS to produce steryl glycosides (SG). Pervious work in our laboratory has demonstrated that the tomato SGT gene family consists of 4 members (*SlSGT1-4*) that are differentially expressed. Thus, *SlSGT1* is the most highly expressed gene in the different tomato organs, while *SlSGT4* gene expression is barely detectable under basal conditions but is upregulated in response to different stress stimuli. Although the four *SlSGTs* encode functional SlSGT enzymes, the individual contribution of each isoform to the glycosylated sterol profile, as well as the impact of an altered composition of these conjugated sterols in tomato plants, is far from being understood. In this thesis project, we investigated how altered levels of glycosylated sterols, obtained by artificial microRNA-mediated downregulation of *SlSGT1* expression or overexpression of *SlSGT4,* affect tomato growth and development and its response to stress.

At the vegetative stage, silencing of *SlSGT1* resulted in a pleiotropic phenotype characterized by shorter plants with smaller leaf area. A size reduction was also observed in fruits. In both cases, the phenotypical alterations were associated to a decrease in the content of glycosylated sterols, due mainly to a diminution on the SG levels, which was paralleled by the accumulation of FS. On the other hand, the results obtained suggest some preference of SlSGT1 for stigmasterol as substrate for glycosylation, and demonstrate that this tomato SGT isoform is not involved in the synthesis of steroidal glycoalkaloids (SGAs), a kind of specialized metabolites involved in plant defense. We also studied the response of *SlSGT1* silenced tomato plants to biotic (*Botrytis cinerea* infection) and abiotic (cold) stress, observing an increased resistance to *B. cinerea* infection, but reduced tolerance to cold stress. All these results demonstrated that SGs play a role in tomato plant and fruit development, as well as in the response to stress. To gain some insight about the molecular mechanisms underlying these physiological effects, we performed RNA-seq experiments in leaves and fruits of *SlSGT1* silenced lines, which showed misregulation, among others, of some genes involved in developmental processes and responses to different stimuli that might help to explain some of the observed phenotypes.

Additionally, we generated transgenic tomato plants overexpressing constitutively *SlSGT4*. Surprisingly, the levels of glycosylated sterol in these transgenic lines were lower than in wild type plants, probably as a result of the concomitant lower *SlSGT1* transcript levels detected in these lines. The phenotypic characterization of these plants showed that changes in *SlSGT4* expression, like those of *SlSGT1* downregulation, affect the growth of tomato plants and fruits, but also the seed production and germination.

Altogether, the results obtained in this work show compelling evidences that glycosylated sterols play an important role in the growth and development of tomato plants and fruits, as well as in the plant response to biotic and abiotic stresses, and establishes the basis for further studies aimed at understanding in more detail the molecular mechanisms through which glycosylated sterols affect these physiological processes.

RESUMEN

Los esteroles son una familia de compuestos triterpénicos que se presentan en forma libre (FS, por sus siglas en inglés) o conjugada, como ésteres (SE), glicósidos (SG) y acilglicósidos de esteroles (ASG). Los esteroles glicosilados (SG y ASG) y los FS son componentes de la membrana celular, donde en combinación con otros lípidos unidos a la membrana juegan un papel clave en la modulación de sus propiedades y función. Las esterol glicosiltransferasas (SGT) catalizan la glicosilación del grupo hidroxilo en la posición C-3 de los FS para producir SGs. Trabajos previos realizados en nuestro grupo de investigación han demostrado que la familia de genes SGT en tomate consta de 4 miembros (*SlSGT1-4*) los cuales se expresan diferencialmente. Siendo *SlSGT1* el gen más expresado en los diferentes órganos del tomate, mientras que la expresión del gen SlSGT4 es apenas detectable en condiciones basales, pero se regula positivamente en respuesta a diferentes estímulos de estrés. Aunque las cuatro *SlSGT* codifican enzimas *SlSGT* funcionales, la contribución individual de cada isoforma al perfil de esteroles glicosilados, así como el impacto de una composición alterada de estos esteroles conjugados en plantas de tomate, están lejos de comprenderse. En este proyecto de tesis investigamos cómo los niveles alterados de esteroles glicosilados, obtenidos por silenciamiento de la expresión de *SlSGT1* mediada por microARN artificial o por la sobreexpresión de *SlSGT4,* afectan el crecimiento y desarrollo del tomate y su respuesta al estrés.

En el estado vegetativo, el silenciamiento de *SlSGT1* provocó un fenotipo pleiotrópico caracterizado por plantas más cortas y con menor área foliar. También se observó una reducción del tamaño de los frutos. En ambos casos, las alteraciones fenotípicas se asociaron a una disminución en el contenido de esteroles glicosilados, debido principalmente a una disminución en los niveles de SG, la cual fue paralela a una acumulación de FS. Por otro lado, los resultados obtenidos sugieren cierta preferencia de SlSGT1 por el estigmasterol como sustrato para la glicosilación, y demuestran que está isoforma de SGT de tomate no está involucrada en la síntesis de glicoalcaloides esteroideos (SGA), un tipo de metabolitos especializados que participan en la respuesta de defensa de las plantas. También se estudió la respuesta de las plantas silenciadas *SlSGT1* al estrés biótico (infección por *Botrytis cinerea*) y abiótico (frio), y se observó una mayor resistencia a la infección por *B. cinera*, pero una menor tolerancia al estrés por frio. Estos resultados demuestran que los SG juegan un papel en el desarrollo de las plantas y frutos de tomate, así como en la respuesta al estrés. Para entender mejor los mecanismos moleculares que conllevan a estos efectos

fisiológicos, se realizaron experimentos de secuenciación de ARN (RNA-seq) en hojas y frutos de las líneas silenciadas *SlSGT1*, los resultados de este análisis muestran una regulación negativa de varios genes involucrados en los procesos de desarrollo y respuesta a diferentes estímulos que podrían ayudar a explicar algunos de los fenotipos observados.

Además, generamos plantas transgénicas de tomate sobreexpresando constitutivamente *SlSGT4*. Sorprendentemente, los niveles de esteroles glicosilados en estas líneas transgénicas fueron más bajos que en las plantas de tipo silvestre, probablemente como resultado de una reducción en los niveles de *SlSGT1* concomitantes detectados en estas líneas. La caracterización fenotípica de estas plantas mostró que los cambios en la expresión de *SlSGT4*, al igual que los observados en el silenciamiento de *SlSGT1*, afectan el crecimiento de las plantas y frutos de tomate, pero también la producción y germinación de semillas.

En conjunto los resultados obtenidos en este trabajo muestran evidencias contundentes del importante papel que juegan los esteroles glicosilados en el crecimiento y desarrollo de las plantas y los frutos de tomate, así como en la respuesta de las plantas a estreses bióticos y abióticos, y sienta las bases para futuros estudios dirigidos a comprender con más detalle los mecanismos moleculares por los cuales los esteroles glicosilados afectan estos procesos fisiológicos.

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MATERIALS AND METHODOS

LIST OF ABBREVIATIONS

DRM Detergent-resistant membranes

PM Plasma membrane

SGA Steroidal glycoalkaloid

IPP Isopentenyl diphosphate

DMAPP Dimethylallyl diphosphate

MVA Mevalonate pathway

ER Endoplasmic reticulum

MEP Methylerythritol 4-phosphate pathway

HMG-CoA 3-hydroxy-3-methylglutaril coenzyme A

AATC Acetoacetyl-CoA thiolase

HMGS 3-hydroxi-3-methylglutaryl-CoA synthase

HMGR 3-hydroxy-3 methylglutaryl-CoA reductase

MVK MVA kinase

PMK Phospho-MVA kinase

MVAPP MVA 5-diphosphate

MVD MVAPP decarboxylase

IDI IPP isomerase

FPS Farnesyl diphosphate synthase

FPP Farnesyl diphosphate

SQS Squalene synthase

OS 2,3 oxidosqualene

SQE Squalene epoxidase

CAS Cycloartenol synthase

SSR2 Sterol side-chain reductase 2

SMT1/2/3	Sterol-methyltransferase 1, 2 and 3
FS	Free sterols
SE	Steryl esters
SG	Steryl glycosides
ASG	Acyl steryl glycosides
LD	Lipid droplet
ASAT	Acyl-CoA sterol acyltransferases
PSAT	Phospholipid:sterol acyltransferases
SGT	UDP-glucose:sterol glycosyltransferases
SGAT	Sterol glycoside acyltransferase
JA	Jasmonic acid
SA	Salicylic acid
Wt	Wild-type
RH	Relative humidity
FPKM	Fragments per Kilobase Million

1. INTRODUCTION

1. INTRODUCTION

1.1 Structure and function of sterols.

Sterols are isoprenoid-derived lipids that are found in all eukaryotic organisms, including higher plants. The chemical structure of sterols is based on the cyclopentaneperhydrophenanthrene ring system, made up of four rigid rings, with methyl substituents at C4, C10, C13 and C14 in some cases, an hydroxyl group at position C3 and an aliphatic side chain of 8-10 carbon atoms attached to C17 (Ferrer *et al.*, 2017) (Figure 1).

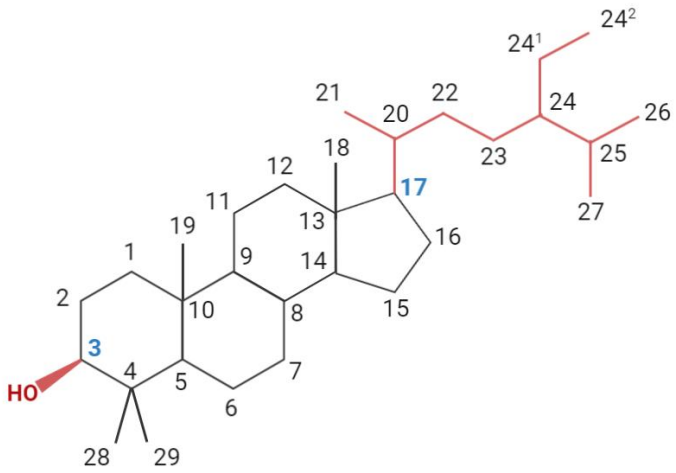

Figure 1. **Sterol basic chemical structure.** Carbon atoms are numbered according to IUPAC nomenclature 1989 (Moreau *et al.*, 2018). The aliphatic side chain at C17 position is shown in red.

While animals only produce one predominant type of sterol, cholesterol, fungi and plants have a more complex sterol composition. There are at least five taxon-specific sterol end-products (mainly ergosterol and fungisterol) in fungi, whereas plants synthesize a complex mixture of more than 250 different sterols, commonly known as phytosterols, which manly differ in the nature of the C17 aliphatic lateral chain and the number and position of double bonds in the side chain and the ring skeleton, being β-sitosterol, stigmasterol and campesterol the most abundant species (Figure 2) (Nes, 2011; Ferrer *et al.*, 2017; Zhang *et al.*, 2020). Cholesterol is also found in plants, although it is usually present in low amounts, representing in most cases less than 1% of the total sterols. However, some plant families, such as Solanaceae, accumulates higher amounts of cholesterol (Benveniste, 2004; Hartmann, 2004; Moreau *et al.*, 2018).

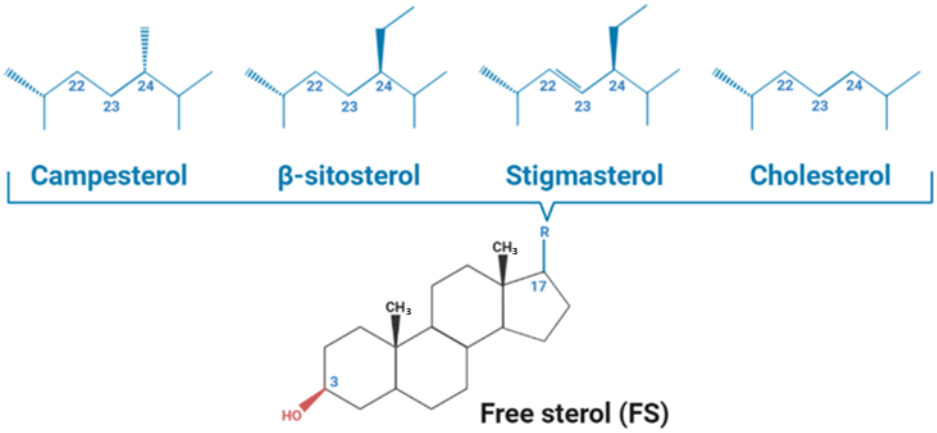

Figure 2. Chemical structure of the main phytosterols. Structural nucleus of free sterols (below) and aliphatic side chains (R) attached to position C17 (above) in the four major phytosterols: campesterol, β-sitosterol, stigmasterol and cholesterol.

Phytosterols can be grouped by the number of methyl substituents at C4 position namely, 4-desmethyl, 4-monomethyl and 4, 4-dimethyl sterols, being 4-desmethyl sterols the most abundant phytosterols. Structural diversity of plant sterols is further increased by alkylation at the C24 position of the aliphatic side chain attached to C17. Of the C24 substitutions, ethylation is more common (>70% of sterols) than methylation (>30% of sterols). Taking into account those variations, sterols can be classified into three groups: (**1**) 24-demethysterols, lacking a C24 substitution, as it is the case of cholesterol (27 carbon atoms), (**2**) 24-methylesterols, which have one methyl group at this position, like campesterol (28 carbon atoms) and (**3**) 24-ethylsterols, with an ethyl group at C24 as occurs in β-sitosterol and stigmasterol (29 carbon atoms). Additionally, the presence of double bonds in the side chain, most frequently between C22 and C23, contribute to the diversification of sterols species found in plant cells (Nes, 2011; Moreau *et al.*, 2018).

Phytosterols, together with glycerolipids (mainly phospholipids) and sphingolipids are essential components of the plant cell plasma membrane (PM), where they interact with the hydrophobic fatty acid chains of the other lipids, restricting their movement (ordering effect) (Hartmann, 2004). Thus, the sterol composition and their relative proportions determine the biophysical properties of cell membranes, as permeability, fluidity and flexibility. Therefore, small changes in the sterol profile or organization can lead to significant differences in the mechanical proprieties of membranes that can modulate the activity of membrane linked proteins, including enzymes, transport channels, receptors and other membrane components involved in signal transduction

pathways (Schaller, 2003; Hodzic *et al.*, 2008). The key role of sterols in determining membrane's physiochemical proprieties also makes sterols play a prominent role in the adaptative response of plants to abiotic stress (Beck *et al.*, 2007; Posé *et al.*, 2009; Mishra *et al.*, 2013; Urbany *et al.*, 2013; Pandey *et al.*, 2014; Kumar *et al.*, 2015; Wagatsuma *et al.*, 2015) and plant-pathogen interactions (Wang *et al.*, 2012; Wang *et al.*, 2012; Kopischke *et al.*, 2013; Castillo *et al.*, 2019).

Sterols are not distributed homogenously in PM. Their interaction with other membrane lipids, mainly sphingolipids, and specific membrane proteins, leads to the formation of specialized microdomains, known as lipid rafts (Figure 3) (Grennan, 2007; Malinsky *et al.*, 2013; Tapken and Murphy, 2015). These microdomains are known as detergent-resistant membranes (DRM) because they display a low solubility in ionic detergents as Triton X-100 (London and Brown, 2000; Peskan *et al.*, 2000). Proteomic analysis of plant DRM have shown differential protein composition when compared to the proteomic profile of the whole PM which may suggest a role in different biological processes such as cell-to-cell interactions, membrane transport, protein trafficking, signal transduction, stress responses and polarized growth (Simon-Plas *et al.*, 2011; Zauber *et al.*, 2014; Tapken and Murphy, 2015; Takahashi *et al.*, 2016).

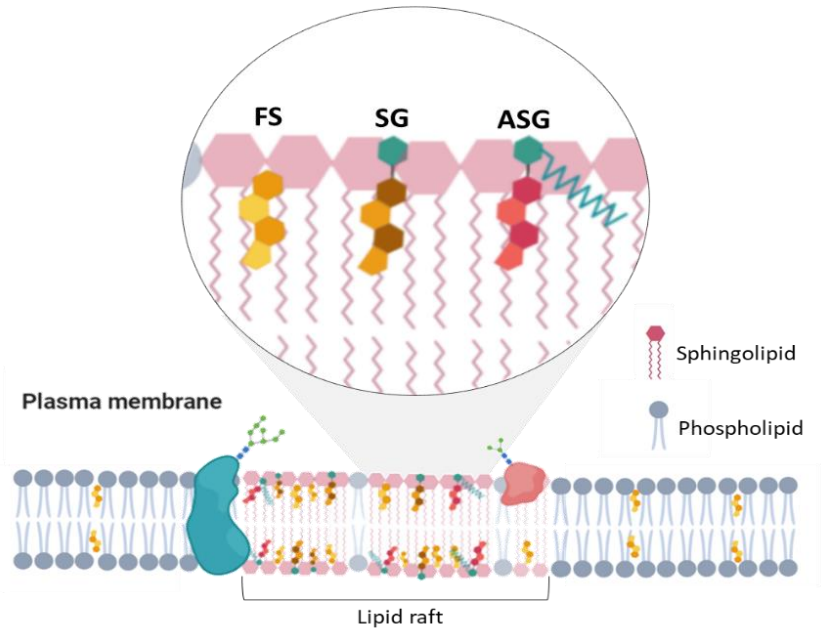

Figure 3. **Distribution of sterols in the PM.** Lipid rafts are specialized domains on the PM enriched on sphingolipids and sterols. The interaction of the different sterols (FS: free sterols, SG: steryl glycosides and ASG: acyl steryl glycosides) with the lipids of the membrane is detailed.

The biological function of phytosterols differs between sterol species. For instance, β-sitosterol and stigmasterol are mainly involved in the maintenance of the structure and stability of the PM, while campesterol is a precursor of brassinosteroids (Schaller, 2003; Boutté and Grebe, 2009), a group of polyhydroxylated steroid hormones that play an essential role in the regulation of plant growth and development, and in some stress responses (Bishop and Koncz, 2002; Planas-Riverola *et al.*, 2019). Finally, cholesterol is not only a structural component of membranes, as in animals, but also a precursor of specialized triterpenoid metabolites as saponins or steroidal glycoalkaloids (SGAs), which are of interest because of their potential pharmacological activity and/or toxicity in animals (Bergenstråhle *et al.*, 1996; Milner *et al.*, 2011; Sonawane *et al.*, 2016). In plants, SGAs are important components for the plant resistance against pests and pathogens (Sonawane *et al.*, 2020).

In addition to their structural function, sterols play an essential role in modulating plant growth and development, not only because campesterol is the precursor of brassinosteroid hormones, but also because changes in the sterol composition directly alters different cellular processes such as stomatal development and patterning (Carland *et al.*, 2002; Qian *et al.*, 2013), cell division, growth and polarity (He *et al.*, 2003; Men *et al.*, 2008), cell to cell connectivity (Grison *et al.*, 2015), hormone signaling (Souter *et al.*, 2002; Kim *et al.*, 2010), cellulose biosynthesis during cell wall formation (Peng *et al.*, 2002; Schrick *et al.*, 2004), pollen viability (Ischebeck, 2016) and plastid development and differentiation (Babiychuk *et al.*, 2008; Manzano *et al.*, 2016).

1.2 Biosynthesis of phytosterols.

As previously mentioned, sterols are isoprenoid-derived lipids and, consequently, are formed from the same five carbon (C5) building blocks, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (known as isoprene units), that all the common precursors of terpenoids. In plants, both isomers are synthesized by two different metabolic pathways that are located in different cell compartments: the mevalonate pathway (MVA) that takes place in the cytosol/ endoplasmatic reticulum (ER), and the methylerythritol 4-phosphate pathway (MEP) that operates in the plastids (Lichtenthaler et al., 1997; Rodríguez-Concepción and Boronat, 2002). Despite their physical separation, there are some interactions between both pathways at molecular and metabolic levels, and in some circumstances there is a limited exchange of IPP and DMAPP between the MVA and MEP pathways (Boronat, 2010), due probably to the need of a coordinated synthesis to produce a high variety of terpenoids from a single common substrate (McCaskill and Croteau, 1998).

Sterols are produced from the MVA-derived IPP/DMAPP (Piironen et al., 2000; Benveniste, 2004; Aboobucker and Suza, 2019). The formation of IPP begins with the condensation of three molecules of acetyl-CoA to form 3-hydroxy-3-methylglutaril coenzyme A (HMG-CoA) catalyzed by two different enzymes: acetoacetyl-CoA thiolase (AATC) mediates the condensation between two molecules of acetyl-CoA to yield acetoacetyl-CoA, and 3-hydroxi-3-methylglutaryl-CoA synthase (HMGS) catalyzes the union of a third molecule of acetyl-CoA through an aldol condensation to produce HMG-CoA (Bach and Benveniste, 1997). These two reactions take place in the cytosol. HMG-CoA is then converted into MVA in a NADPH-dependent reaction catalyzed by the main rate-limiting enzyme of the pathway, the 3-hydroxy-3 methylglutaryl-CoA reductase (HMGR), thus controlling the synthesis of isoprenoid end-products such as sterols. HMGR is anchored to the ER membrane with the catalytic domain facing the cytosol and the association of HMGR to the ER membranes is important to regulate its activity (Pulido *et al.*, 2012; Vranová *et al.*, 2013; Tholl, 2015). Finally, MVA is converted into IPP by three enzymatic reactions: two phosphorylations catalyzed by MVA kinase (MVK) and phospho-MVA kinase (PMK) lead to MVA 5-diphosphate (MVAPP), and the subsequent ATP-dependent decarboxylation of MVAPP catalyzed by MVAPP decarboxylase (MVD) yields IPP, which is isomerized into DMAPP by the action of IPP isomerase (IDI).

Next, the sequential condensation of one molecule of DMAPP with two units of IPP, by the action of the enzyme farnesyl diphosphate synthase (FPS), results in the formation of farnesyl diphosphate (FPP) in the cytosol. FPP is the starting point of the different branches of the cytosol-ER isoprenoid pathway that lead to the formation of a variety of end-products including sesquiterpenes, such as sesquiterpenoid phytoalexins; long chain prenyl-diphosphates such as dolichols and polyprenols and triterpenes such as sterols and brassionesteroids. Furthermore, FPP also serves as a substrate for protein prenylation. Squalene synthase (SQS) catalyzes the condensation of two units of FPP to produce squalene, a polyunsaturated aliphatic C30 hydrocarbon that is the first committed precursor of the sterol triterpene biosynthetic pathway (Benveniste, 2002; Vranová et al., 2013). Squalene oxidation produces 2,3 oxidosqualene (OS) in a reaction catalyzed by the squalene epoxidase (SQE), which is the first oxygenation step in the sterol biosynthesis pathway (Benveniste, 2002). The enzymatic reaction leading to OS from acetyl-CoA are common to all eukaryotic organisms (Piironen et al., 2000; Benveniste, 2004; Aboobucker and Suza, 2019). However, from OS the plant sterol biosynthetic pathway diverges from those of fungi or mammalian, and it is characterized by specific steps

that are restricted to plants. This is the case of cycloartenol synthase (CAS) that catalyzes the cyclization of OS yielding cycloartenol instead of lanosterol, which is the first cyclic intermediate of sterol biosynthesis in fungi and mammals (Hartmann, 1998; Schaller, 2003). The flux of the different branched pathways leading to the main sterol end-products is regulated at specific steps (Figure 4). Cycloartenol is the first metabolite within the pathway that is susceptible to be metabolized by two different branch-point enzymes: sterol side-chain reductase 2 (SSR2) and sterol-methyltransferase 1 (SMT1). The SSR2 converts cycloartenol into cycloartanol, thus diverting the flux of precursors to the cholesterol branch of the pathway, which involves unique but also shared enzymes with other branches of the sterol pathway (Sonawane et al., 2016). On the other hand, SMT1 catalyzes the alkylation of cycloartenol to 24methylenecycloartanol, leading to the generation of precursors for the plant-specific sterols; β-sitosterol, stigmasterol, and campesterol (Benveniste, 2002). Further reactions downstream of SMT1 are essentially linear until reaching 24-methylenelophenol, where another important branching point is found. While 24-methylenelophenol could be considered a specific precursor of campesterol biosynthesis, the branch-point enzymes sterol-methyltransferases 2 and 3 (SMT2/3) transform it into 24-ethylidenelophenol, thus conferring plants the ability of producing 24-ethyl sterols as the major molecular species by directing carbon flux towards β-sitosterol and stigmasterol biosynthesis (Benveniste, 2002; Schaller, 2003; Carland et al., 2010). Stigmasterol is formed from β-sitosterol by the action of C22 desaturase (Schaller, 2004) that catalyzes the final step in this branch of the phytosterol biosynthesis pathway. This last enzymatic reaction plays a key role in the maintenance of PM structure, since as previously mentioned, β-sitosterol and stigmasterol are important elements in the regulation of its dynamic properties (Schuler et al., 1991). Furthermore, it has been reported that the proportion of 24-methyl and 24-ethyl sterols affects plant growth and development (Valitova et al., 2016).

Most of the reactions within the sterol biosynthetic pathway take place in the ER. However, sterols mainly accumulate in PM, which suggests the existence of some transport mechanism between these two membranous systems. In fact, compared with other membrane compartments, the PM shows the greatest sterol content in comparison with the corresponding levels of proteins and phospholipids (Hartmann, 1998).

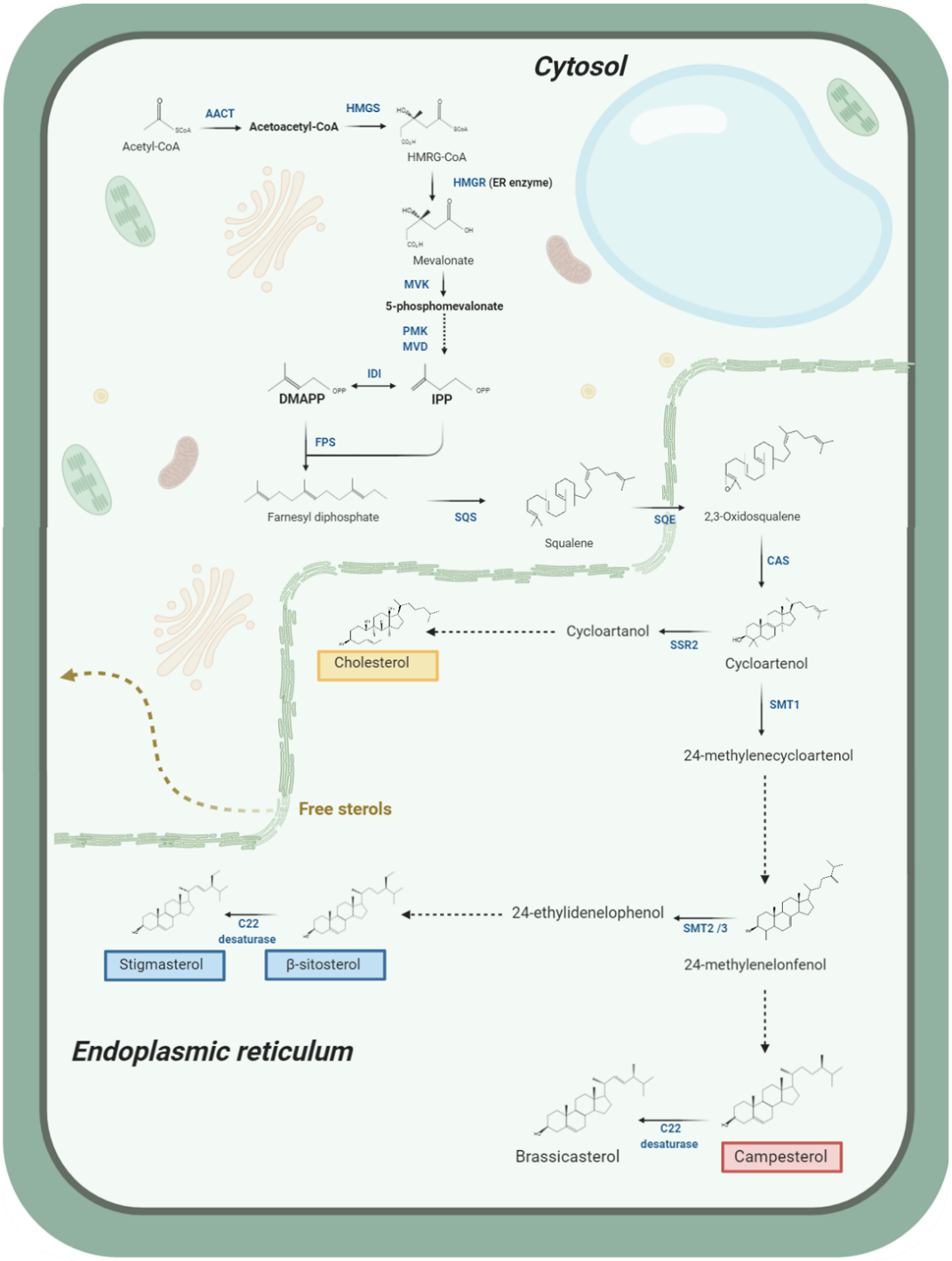

Figure 4. **Simplified phytosterol biosynthesis pathway.** The main enzymes that participate in the sterol biosynthesis pathway are shown in blue. AACT: acetoacetyl-CoA thiolase; HMGS: HMG-CoA synthase; HMG-CoA reductase; MVK: MVA kinase; PMK: phosphomevalonate kinase; MPD: mevalonate pyrophosphate

decarboxylase; IDI: IPP isomerase; FPS: FPP synthase; SQS: squalene synthase; SQE: squalene epoxidase; CAS: cycloartenol synthase; SSR2: sterol side-chain reductase 2; SMT1/2/3: Sterol Methyltransferases 1, 2 and 3; and C22 desaturase. Dotted lines represent multiple enzymatic steps (Adapted from Moreau et al., 2018 and Schaller, 2004).

1.3 Conjugated sterols in plants.

In plants, sterols are found not only as free sterols (FS), which present a β-hydroxyl group at the C3 of the sterol backbone, but also conjugated in form of steryl esters (SE), steryl glycosides (SG) and acyl steryl glycosides (ASG) (Figure 5). In SEs the hydroxyl group at C3 position is esterified with a fatty acid, while in SGs and ASGs the hydroxyl group at C3 is linked to a sugar through a β-glycosidic bond. ASGs are derivatives of SGs in which the hydroxyl group of the C6 position in the sugar moiety is esterified with a fatty acid. While FS, SG and ASG are structural components of the PM, SE are located in storage compartments known as lipid droplets (LD) (Ferrer *et al.*, 2017).

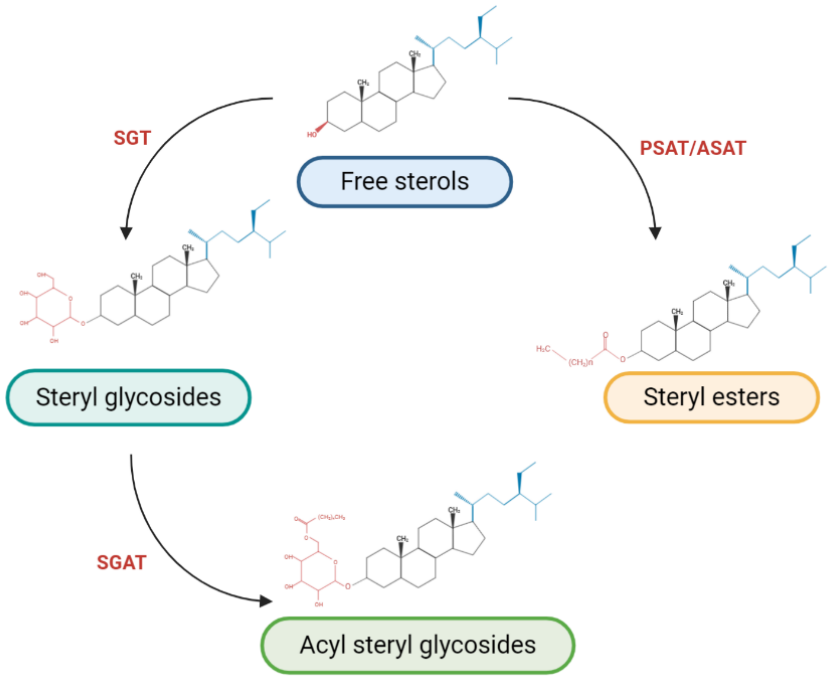

Figure 5. **Biosynthesis of conjugated sterols from free sterols.** Enzymes involved in the synthesis of conjugated sterols are shown in red. SGT: UDP-glucose: sterol glucosyltransferase; SGAT: steryl glucoside acyltransferase; ASAT: acyl-CoA: sterol acyltransferase; PSAT: phospholipid: sterol acyltransferase. (Adapted from Ferrer et al., 2017).

1.3.1 Steryl esters.

Steryl esters are present in all plant tissues, but their amounts vary depending on different factors such as organ or tissue type, as well as the environmental and developmental conditions. The length of the fatty acids found in SE typically range from C12 to C22, being palmitic, stearic, oleic, linoleic and linolenic the most commons species. The sterol species present in the SE fraction are usually the same present in the FS fraction, although some sterol intermediates can also be found probably as a result of regulatory mechanisms acting on the post-squalene portion of the sterol pathway (Ferrer *et al.*, 2017; Lara *et al.*, 2018).

It is generally accepted that SE serve as a storage pool of sterols that plays an important role in maintaining the homeostasis of FS in cell membranes during plant growth and development, preventing that changes in FS levels above or below a certain threshold might have destabilizing effect on membrane integrity and functions (Schaller, 2004; Bouvier-Navé *et al.*, 2010; Silvestro *et al.*, 2013). The regulatory effect of SE on FS content becomes especially evident when the metabolic flux through the sterol biosynthetic pathway increases, as it happens in mutant and transgenic plants overproducing sterols. On these conditions, FS levels remain virtually unchanged whereas sterols produced in excess accumulate mainly as SE in LD (Gondet *et al.*, 1994; Schaller *et al.*, 1994; Bouvier-Navé *et al.*, 2010; Chapman and Mullen, 2011). On the contrary, such LD are absent when SE formation is abolished (Kopischke *et al.*, 2013). The SE pool is also considered as a reservoir of FS needed to sustain an active plasma membrane synthesis during rapid tissue and cell growth. Thus, the high levels of SE found in seeds (Dyas and Goad, 1993; Harker *et al.*, 2003) and pollen grains (Hernández-Pinzón *et al.*, 1999) would be necessary to supply the FS required for seedling growth during the early stages of development and to sustain a very fast pollen tube growth once it lands on the female reproductive organs. The enrichment of SE in senescing tissues (Bouvier-Navé *et al.*, 2010; Li *et al.*, 2016) has been associated to the recycling of the FS and the fatty acids of phospholipids released from cell membranes as senescence progresses, and a role of SE in sterol transport among tissues and organs has also been suggested (Holmer *et al.*, 1973; Chen *et al.*, 2007).

The synthesis of SE is catalyzed by two different types of sterol acyltransferases: (**1**) acyl-CoA sterol acyltransferases (ASAT), that use acyl-CoA as fatty acid donor and (**2**) phospholipid:sterol acyltransferases (PSAT) that use a phospholipid as acyl donor (Korber *et al.*, 2017). In plants, the presence of sterol acyltransferase activity has been reported in a variety of tissues (Garcia and Mudd, 1978; Zimowski and Wojciechowski,

1981; Bouvier-Navé and Benveniste, 1995), but so far the enzymes performing this activity have only been cloned and functionally characterized in *Arabidopsis thaliana:* PSAT1 (Banas *et al.*, 2005) and ASAT1 (Chen *et al.*, 2007), and in *Solanum lycopersicum* (SlPSAT1 and SlASAT1), where the Arabidopsis orthologues where recently identified by our research group (Lara *et al.*, 2018).

1.3.2 Steryl glycosides.

Glycosylated sterols (SG and ASG) are widely distributed in plants, but their total content and relative proportion may change greatly among different plant species, tissues and in response to developmental and environmental conditions (Ferrer *et al.*, 2017). Moreover, the sterol profile of glycosylated sterols does not always reflect the total sterol composition in the same plant tissue, which indicates preferential glycosylation of specific sterol species (Moreau *et al.*, 2002; Nyström *et al.*, 2012). In most plant species SGs and ASGs are minor components of the total sterol fraction, representing about 10-30% of the total sterol content (Wojciechowski, 1992; Nyström *et al.*, 2012). However, plants of the genus *Solanum* are a remarkable exception due to the very high content of glycosylated sterols (Duperon *et al.*, 1984; Furt *et al.*, 2011; Nyström *et al.*, 2012), which in the case of tomato accounts for more than 85% of the total sterols in both leaves and fruits (Duperon *et al.*, 1984; Whitaker, 1988; Whitaker and Gapper, 2008). Moreover, the amount and the profile of FS and conjugated sterols (SE, SG and ASG) change markedly during tomato fruit ripening and also during fruit chilling and after re-warming (Whitaker, 1988; Whitaker, 1991; Whitaker, 1994). The biological significance of these variations and the high content of glycosylated sterols in *Solanum* species are still not completely understood, although it has been suggested that they might be required to maintain the integrity of the cell membranes against the disruptive effect the high levels of SGAs present in these plant species (Steel and Drysdale, 1988; Keukens *et al.*, 1995; Blankemeyer *et al.*, 1997). In fact, the levels of these metabolites are under rigorous transcriptional control (Cárdenas *et al.*, 2016). In the case of tomato, the high levels of glycosylated sterols may explain why tomato tissues are able to withstand the high concentration of α-tomatine (Steel and Drysdale, 1988), a bioactive SGA found in all tomato organs, but especially abundant in the peel of green fruits (Barkai-Golan, 2001; Friedman, 2002; Iijima *et al.*, 2013).

Glycosylation is a mechanism that modifies different compounds as hormones, specialized metabolites, biotic and abiotic chemicals and environmental toxins, to maintain their cellular homeostasis (Chaturvedi *et al.*, 2011). This process not only helps to stabilizes the target compounds, but also alters their physiological activities and control their intracellular distribution (Ullmann *et al.*, 1993). In the case of sterols,

the sugar moiety bound to the OH in SGs, reduces the hydrophobic character of the free sterols (Nyström *et al.*, 2012). Therefore, it has been proposed that the steroid nucleus of SGs is immersed in their hydrophobic core of cell membranes, while the sugar moiety is oriented towards the plane of the polar head groups of the lipid bilayer. In the case of ASGs, both the sterol moiety and the fatty acid chain are probably buried in the hydrophobic phase of the membrane with the sugar oriented to the hydrophilic surface (see Figure 3 in section 1.1) (Furt *et al.*, 2010). Beside their structural function, glycosylated sterols have been proposed to serve as glucose transporters and/or signaling molecules (Grille *et al.*, 2010). SGs could also be used as primers for ceramide glycosylation (Lynch *et al.*, 1997). Additionally, glycosylated sterols have been found to be essential for pollen viability in Arabidopsis by promoting pollen coat maturation (Choi *et al.*, 2014). Furthermore, a significant proportion of glycosylated sterols have been found in phloem being cholesterol and its glycosylated derivative the predominant forms (Behmer *et al.*, 2013), but the biological role of glycosylated sterols in the phloem still is unknown.

1.4 Sterol glycosyltransferases.

Steryl glycosides are synthetized by UDP-glucose:sterol glycosyltransferases (SGT), which catalyze the attachment, through a glycosidic bond, of a sugar residue to the free hydroxyl group at C3 position of the sterol backbone. Although the major sugar donor for plant SGTs is UDP-glucose, other sugars such as galactose, xylose or mannose can be also used for FS glycosylation (Ferrer *et al*., 2017). ASGs are formed from SGs by esterification of the hydroxyl group at C6 position of the sugar moiety with a long-chain fatty acid. This reaction is catalyzed by the activity of enzymes known as sterol glycoside acyltransferase (SGAT). This enzyme activity has been reported in potato tubers (Catz *et al.*, 1985), tobacco seedlings (Frasch and Grunwald, 1977), eggplant leaves (Potocka and Zimowski, 2008), *Calendula officialis* cotyledons (Wojciechowski and Zimowski, 1975), and carrot (Eichenberger and Siegrist, 1975). However, no genes encoding for SGATs have yet been identified in plants, animals, algae, fungi nor bacteria (Ferrer *et al.*, 2017).

The presence of SGT activity has been detected in a number of higher plants, and the reaction has been suggested to be associated primarily with cell membranes (Grille *et al.*, 2010; Chaturvedi *et al.*, 2011; Li *et al.*, 2014; Tiwari *et al.*, 2014; Zauber *et al.*, 2014; Ramirez-Estrada *et al.*, 2017) although the occurrence of soluble cytosolic SGTs has also been described (Madina *et al.*, 2007; Grille *et al.*, 2010; Li *et al.*, 2014; Ramirez-Estrada *et al.*, 2017). However, SGT isozymes have been cloned and functionally characterized in a few number of higher plants: two in *Arabidopsis thaliana*

(Warnecke *et al.*, 1997; DeBolt *et al.*, 2009), four in *Whithania somnifera* (Madina *et al.*, 2007; Chaturvedi *et al.*, 2012), one in *Avena sativa* (Warnecke *et al.*, 1997), two in *Gossypium hirutum* (Li *et al.*, 2014), one in *Gymnea sylvestre* (Tiwari *et al.*, 2014) and four in *Solanum lycopersium* (Ramirez-Estrada *et al.*, 2017). These studies have shown that most plant species contain small gene families coding for SGT isozymes that could perform specialized functions. For instance, in Arabidopsis the two genes encoding SGTs, named as *UGT80A2* and *UGT80B1*, are differentially expressed (DeBolt *et al.*, 2009) and the encoded enzymes display different substrate preferences towards the main phytosterols (Stucky *et al.*, 2015). In cotton, the two *Gh*SGT genes show a differential expression pattern under heat shock stress and the encoded isozymes also display different biochemical properties and subcellular localization (Li *et al.*, 2014). The four members of the *W. somnifera* SGT gene family are also differentially induced by heat and cold stress, as well as by treatment with jasmonic acid (JA) and salicylic acid (SA) (Chaturvedi *et al.*, 2012). In line with these observations, the four tomato *SlSGT* genes were differentially expressed in different plant organs and throughout the different stages of fruit development and ripening. *SlSGT1* was the more highly expressed gene in the different tomato organs, whereas *SlSGT4* expression was barely detectable. In fruits, the expression of *SlSGT1* and SlSGT2 decreased along ripening and an opposite pattern was observed for *SlSGT3*. The *SlSGTs* genes were also differentially regulated in response to biotic and abiotic stress. While the expression of *SlSGT4* and *SlSGT2* increased in response to several abiotic stresses (osmotic, cold and salt) and after treatment with abscisic acid and methyl jasmonate, *SlSGT1* and *SlSGT3* seem to be non-stress responsive genes, since their expression remained almost unaltered under the assayed stress conditions (Ramirez-Estrada *et al.*, 2017).

1.5 Role of sterols in plant growth and development.

Several studies had linked alterations in the sterol composition with abnormalities in plant growth and development (Jang *et al.*, 2000; Schrick *et al.*, 2000; Schaeffer *et al.*, 2001; Clouse, 2002; Boutté and Grebe, 2009; Song *et al.*, 2019). Consistently with their role as brassinosteroid precursors some phenotype alterations observed in mutant plants affected in the early steps of the sterol biosynthetic pathway, including dwarfism and reduced fertility, have been attributed to the deficiency of this hormone, which is necessary for the correct plant development (Valitova et al., 2016). However, in some of these mutants, such as *smt2* (see Figure 4) or *hyd2/fackel* (a gene encoding a sterol C-14 reductase), the observed phenotype alterations cannot be recovered after exogenous addition of brassinosteroids, suggesting that the

growth defects were caused directly by alterations in the sterol composition (Carland *et al.*, 2002; Schaller, 2004; Vriet *et al.*, 2013), which can reduce the cell growth as a result of the changes induced in the physical properties of the PM (Valitova *et al.*, 2016). It is also known than some sterol species, as β-sitosterol and stigmasterol, play a key role in cell differentiation and proliferation (Piironen *et al.*, 2000).

On the other hand, alterations in the PM integrity due to changes in sterol levels may affect the localization of proteins involved in cell polarization that reside in lipid rafts (Carland *et al.*, 2002; Willemsen *et al.*, 2003). Thus, it has been reported that PIN proteins, which belong to a protein family implicated in auxin transport, and therefore in cell polarity, require SMT1 function for correct positioning in Arabidopsis (Willemsen *et al.*, 2003).

Despite all existing studies linking phytosterols with plant development, the information about the role of glycosylated sterols in this process is scarce. At present, it has been reported that an adequate ratio of SGs and FS in the cell membrane seems to be essential for the normal function of plant cells. The Arabidopsis double knock-out mutant *ugt80A2;B1*, which shows highly reduced levels of glycosylated sterols in different plant organs (DeBolt et al., 2009), displays multiple morphological and biochemical seed phenotype alterations, including reduced size, transparent testa, defects in the deposition of flavonoids, loss of the external cuticle and reduction of aliphatic suberin and cutin-like polymers (DeBolt *et al.*, 2009), as well as defects in pollen fitness (Choi *et al.*, 2014), a reduction in the formation of root hairs and an aberrant root epidermal cell pattering (Pook *et al.*, 2017). Moreover, forward- and reverse-genetic approaches have also shown that changes in SGT expression levels are associated to alterations in plant growth and development (Mishra *et al.*, 2015; Singh *et al.*, 2016). Thus, downregulation of *SGT*s in agroinfiltrated *W. somnifera* leaves leads to a reduction in the height and leaf area compared to control plants (Singh *et al.*, 2016), while transgenic *W. somnifera* plants overexpressing *WsSGTL1* were taller than the wt (Saema *et al.*, 2015).

1.6 Sterols and plant stress response.

The PM acts as a physical barrier that protects the cell and, consequently, plays an essential role during stress responses. The lipids integrated in this membrane, and specifically sterols, have dynamic responses to external perturbations on cells, therefore variations in their composition may alter the plant response to environmental stimuli (Valitova *et al.*, 2016). There are several studies supporting the role of sterols in the plant response to different abiotic stresses, including heat shock

(Beck *et al.*, 2007), salt stress (Kerkeb *et al.*, 2001; Mishra *et al.*, 2013; Pandey *et al.*, 2014), drought (Posé *et al.*, 2009; Kumar *et al.*, 2015), cold (Takahashi *et al.*, 2016; Rogowska and Szakiel, 2020) , metal ions (Hossain Khan *et al.*, 2009) and oxidative stress (Wang *et al.*, 2012).

It is widely known that big temperature variations influence the physicochemical properties of cell membranes, consequently sterols may play important roles in the plant response to these temperature fluctuations. A study using liposomes mimicking the PM lipid rafts composition of plants (enriched with β-sitosterol and stigmasterol), mammals (enriched with cholesterol) and fungi (enriched with ergosterol) showed that plant membranes are less sensitive to temperature variations than those of animal and fungi (Beck *et al.*, 2007; Dufourc, 2008). The higher diversity of plant sterol species in plants extend the range of temperatures at which membrane-associated biological processes can take place, which is correlated with the necessity of these sessile organisms to face higher temperature variations and other environmental stress conditions to survive. Regarding glycosylated sterols it has been documented an increase in the expression of *GhSGT1* under heat shock, which may suggest a role for this kind of sterols in the adaptive plant response to temperature changes (Li *et al.*, 2014). On the other hand, the expression of genes encoding enzymes within the sterol biosynthetic pathway (*SMT1*, *SMT2* and *SGT*s) increased after cold exposure (Byun *et al.*, 2014; Mishra *et al.*, 2015; Ramirez-Estrada *et al.*, 2017; Valitova *et al.*, 2019), as well as the synthesis of membrane sterols. Thus, the levels of free sterols, mainly β-sitosterol, increase in the PM of oat (Takahashi *et al.*, 2016), wild and cultivated potato species (Palta *et al.*, 1993),wheat (Valitova *et al.*, 2019) and rye (Uemura and Steponkus, 1994) after cold. It is worth to mention that the time-course of cold-induced changes in the sterol profile of wheat, oat and potato showed a latter decline in the FS amounts, which may be due to modifications such as acylation and glycosylation (Takahashi *et al.*, 2016; Valitova *et al.*, 2019). The increase in ASGs to SGs ratio detected in cold-acclimating potato species (Palta *et al.*, 1993), as well as the upregulation of some *SlSGT* genes in tomato seedlings exposed to cold (Ramirez-Estrada *et al.*, 2017), support the hypothesis that conjugated sterols, mainly the glycosylated fraction, can be involved in the plant response to cold.

A number of studies have also highlighted the role of sterols in improving the biotic stress resistance in plants (Moreau *et al.*, 2018; Aboobucker and Suza, 2019). Pathogens colonize a host and take up nutrients from the apoplast, where they grow. The apoplast usually contains minimal nutrients that came from the exchange with the cytosol via PM permeability (Sattelmacher, 2001). Some enzymes such as SQS, and C22 desaturase are overexpressed after pathogen infections, and regulate nutrient efflux into the apoplast through modification of membrane permeability (Wang *et al.*, 2012). There are several studies showing the relevance of β-sitosterol and stigmasterol in plant-pathogen interactions, since changes in their ratio can influence the biophysical properties of membrane microdomines and thereby modulate plant defense signaling (Griebel and Zeier, 2010; Wang *et al.*, 2012; Wang *et al.*, 2012). Moreover, the antifungal properties of some phytosterols including β-sitosterol, campesterol and stigmasterol have been proven (Choi *et al.*, 2017)

Experimental evidence supporting a role of conjugated sterols in mediating plant responses against biotic stress in far more limited. The enhanced response to *Phytophthora infesntans* showed by the Arabidopsis mutant *psat1*, deficient in the synthesis of SE (Kopischke *et al.*, 2013), suggests the participation of this sterol fraction in the pathogen-inducible response against this oomycete. However, the biochemical analysis showed that not only the SE levels were altered on this mutant, but also those of SG and ASG, making it difficult to attribute the resistance phenotype to the reduced levels of SE or to changes in the glycosylated fraction. It has also been reported that downregulation of SGTs in *W. somnifera* results in increased susceptibility to *Alternaria alternata,* but alteration in the pathogen tolerance could be due not only to changes in the SG and ASG levels but rather to changes in the levels of whitanolides, a type of glycosylated sterol-related specialized metabolites (Singh *et al.*, 2016). Furthermore, the overexpression of *WsSGT1* in Arabidopsis enhanced the plant resistance against the necrotrophic fungi *Alternaria brassicicola*. The resistance phenotype of these transgenic plants was attributed to the accumulation of phenolic compounds (ferulic acid, caffeic acid, chlorogenic acid, protocatechuic acid and rutin) and the over-expression of defense marker gene (*VSP2, PDF1.2, LoX2, PAD3* and *PAL*) after pathogen inoculation (Mishra *et al.*, 2017). Recently, it has been reported that the Arabidopsis double mutant *ugt80A2;B1,* which as mentioned before is severely affected in the biosynthesis of glycosylated sterols, displayed an increased resistance to *Botrytis cinerea* infection, which correlates with increased levels of JA, the up-regulation of defense gene markers (*PDF1.2* and *PR4*) and the accumulation of camalexin, the major Arabidopsis phytoalexin (Castillo *et al.*, 2019). These data

support a role of glycosylated sterols in the plant response to biotic stress. However, the molecular mechanisms beyond this effect remain unclear.

1.7 Tomato, agronomic crop and research plant model.

Solanum lycopersicum, commonly known as tomato, is one of the 1500 species grouped in the *Solanaceae* family. The genus Solanum also includes other important crop plants as potato (*Solanum tuberosum*) and eggplant (*Solanum melongena*).

It has been proposed that tomato was originated and domesticated in America, however, the time and the site of domestication remains unknown. There are two hypothesis, one placed the domestication of tomato in Peru and the other in Mexico (Razifard *et al.*, 2020). Tomato had reached a fairly advanced stage of domestication before its arrival to Europe in the 15th century. Since the 20th century humans have created vast diversity of morphological arrays, cultivars and forms from a single species, *S. lycopersicum*, by plant breeding (Bai and Lindhout, 2007). Nowadays, tomato is one of the world's highest value vegetable crop with a production above 160 million tons worldwide in 2018. That year the production of tomato only in China was higher than 61.5 million tons (Mt), followed by India (19,3 Mt), United States (12.6 Mt), Turkey (12.1 Mt), Egypt (6.6 Mt), Iran (6.5 Mt), Italy (5.7 Mt), Spain (4.7), Mexico (4.5 Mt) and Brazil (4.1 Mt) (FAO, http://www.fao.org/faostat/en/; last accessed May 30, 2020). Through the years, tomato has become a basic ingredient in the diet of most cultures and represent an important source of vitamins, minerals, fiber and antioxidants (Beecher, 1998).

The domestication of tomato has been focused on a wide range of growth habits, such as plant height and self-pruning, and fruit traits, such as size, shape, color, taste and morphology, but also to improve its production under abiotic and biotic stress. Tomato hosts more than 200 species of a wide variety of pests and pathogens that causes important economic losses every year. The control of tomato pests and pathogens using chemical compounds may be not fully effective, require a compliance with chemical-use laws and raise production costs. Since tomato is subtropical origin, its production is sub-optimal over large part of the crop growing areas, due to different unfavorable conditions including high or low temperatures, excessive water or drought, and soil alkalinity or salinity (Bai and Lindhout, 2007). Recently, studies using transcriptomic, proteomic and metabolomic tools are contributing to a better understanding of the biochemical and metabolic processes related to the development and stress tolerance of tomato plants and fruits (Paul *et al.*, 2019).

In addition to its economical and agricultural relevance, tomato is a model system for studies on fruit development and ripening since, unlike other plant models such as *Oryza sativa* or *Arabidopsis thaliana*, tomato produces fleshy fruits (Kimura and Sinha, 2008; Klee and Giovannoni, 2011). Its diploid genome, its relatively short life cycle and the complete genome sequenced makes tomato the most manageable organism for fruits studies. Furthermore, the ripening phenotype in tomato is easily distinguishable, and collections of germplasm (Carvalho *et al.*, 2011) and mutants defective in ripening processes are available (Barry and Giovannoni, 2007).

Tomato plants produce globular or ovoid fruits that exhibits all the common characteristics of berries; fleshy fruits derived from the ovary with enclosed seeds in the pulp (Barry and Giovannoni, 2007). The most external tissue, called pericarp, is covered by the cuticle and inside is the placenta containing the seeds, which includes the embryo surrounded by the endosperm, a tissue that supplies nutrients, and a rigid outer covering, called testa.

The development and ripening process of tomato fruits take around seven to nine weeks (from the time of anthesis to the end of ripening). The most well-defined pathway that mediates fruit ripening is controlled by ethylene, which can be divided into two phases. During phase I or early development, cell divisions and expansion take place to enlarge the final fruit size. During this phase, the biosynthesis of sterols is remarkably active since they are essential for both cell division and subsequent cell expansion (Gutensohn and Dudareva, 2016). During phase II, multiple physiological changes such as color, sugar metabolism, fruit softening and synthesis of volatiles take place. Several changes in the distribution metabolism of steryl lipids have been reported during cv. Rutgers tomato fruit development and ripening, being the increase in conjugated sterols one of the most important events. It has been propose that those changes in the conjugated sterol profile play an important role modulating the activities of membrane-bound enzymes that are involved in the processes of fruit ripening and senescence (Whitaker, 1988).

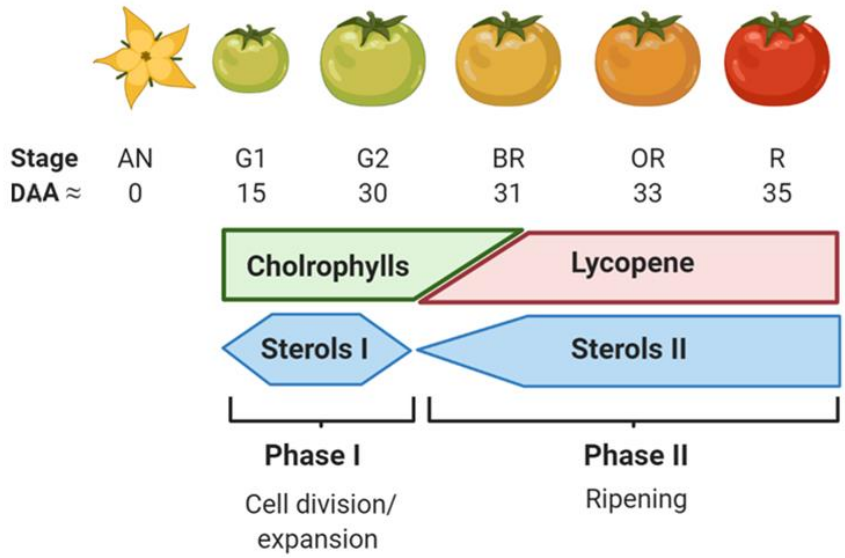

Figure 6. Tomato fruit development. The phase I of fruit development involves cell division and expansion events till reach the final fruit size (approximately 30 days post-anthesis [DAA]). The phase II involves the ripening of the fruit and it is characterized by important changes in organoleptic properties. At the beginning of the second phase, chlorophyll is degraded and lycopene begins to accumulate. G1, Green; G2, mature green; BR, breaker, Or, orange; R, red.

2. OBJECTIVES

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Sterol glycosyltransferases (SGTs) catalyze the glycosylation of the free hydroxyl group at C-3 position of sterols to produce steryl glycosides. Both free and glycosylated sterols are essential components of plant cell membranes, where in combination with other membrane lipids play a key role in modulating their properties and function. The SGT family in tomato (*S. lycopersicum* cv Micro-Tom) is represented by 4 isoforms, SlSGT1 to SlSGT4, and the corresponding tomato *SlSGT* genes show clearly different expression patterns. The *SlSGT1* gene is the most actively expressed in different tomato tissues and organs while expression of the *SlSGT4* gene is barely detectable under basal conditions but is induced in response to different stress signals and conditions (Ramírez-Estrada et al., 2017). Although the four SlSGT isoforms have been recently characterized, their individual contribution to the glycosylated sterol profile as well as the impact of altered composition of these conjugated sterols on tomato plants is far from being understood.

Taking advantage of the above indicated work and the recent availability of two tomato transgenic lines with reduced *SlSGT1* expression levels (Castillo, 2019), we designed this PhD thesis project to extend the current knowledge of the role of glycosylated sterols during tomato plant and fruit growth and development, and in the response to environmental changes. To accomplish this major goal, the following specific objectives were established:

1. To further characterize at the biochemical and molecular levels tomato plants with reduced levels of glycosylated sterols due to the downregulation of *SlSGT1* gene expression.

2. To assess the effects of reduced SG levels in the above mutants on plant and fruit growth and development, and the response to biotic (*Botrytis cinerea* infection) and abiotic (cold) stress conditions.

3. To generate transgenic tomato plants with altered levels of glycosylated sterols due to the overexpression of the *SlSGT4* gene in order to evaluate the effects on plant and fruit growth and development.

3. RESULTS

3. RESULTS

3.1 CHAPTER I

3.1.1 Characterization of tomato SlSGT1 knockdown mutant lines.

In order to elucidate the specific role of *SlSGT1* in tomato plant growth, development and stress response, two independent homozygous lines with reduced levels of *SlSGT1* gene expression, referred to as amiSGT1-31.2 and amiSGT1-61.1, were previously generated in our research group (Castillo, 2019) using artificial micro RNA (amiRNA) technology. Decreased transcript levels of the *SlSGT1* in these two genotypes were confirmed by RT-qPCR using RNA extracted from leaves. As shown in Figure 7, both amiRNAs were highly effective in silencing *SlSGT1* expression since *SlSGT1* mRNA levels in leaves of amiSGT1-31.2 and amiSGT1-61.1 plants decreased to 13.5% and 23.5%, respectively, as compared with leaves of wt plants. Transcriptomic analysis showed that silencing of *SlSGT1* by micro RNA technology does not affect the basal expression of the remaining genes of the tomato *SlSGT* family (*SlSGT2-4*).

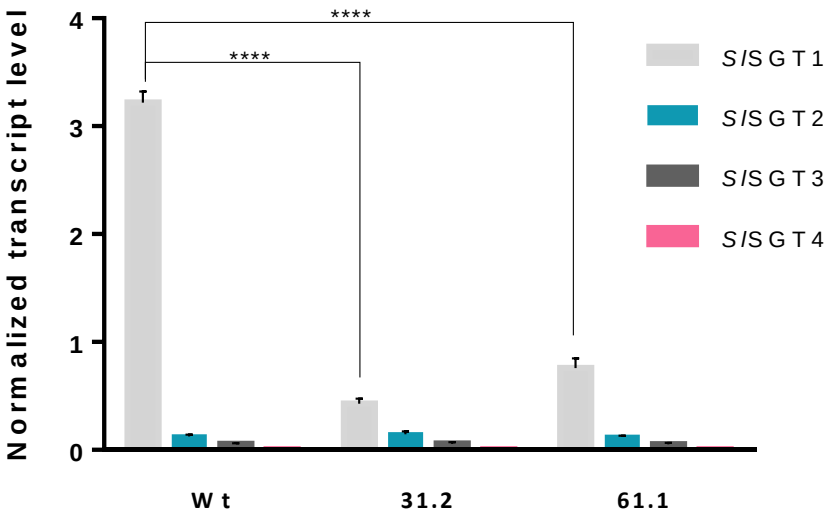

Figure 7. **Silencing of *SlSGT1* gene expression in leaves of tomato plants.** Transcript levels of the *SlSGT* genes in leaves of wt, amiSGT1-31.2 and amiSTG1-61.1 plants were determined by RT-qPCR using RNA extracted from leaves of one-month-old plants. Data are expressed as normalized *SlSGTs* mRNA quantity using the actin gene (Solyc03g078400) as a housekeeping. Values are mean ± SEM from three biological replicates per genotype with three technical replicates each (n=9). Asterisks represented significant differences compared with wt determined by t-test (****P ≤ 0.0001).

3.1.2 Silencing of SlSGT1 results in a reduction of SG levels in leaves of tomato plants.

In order to elucidate the metabolic impact of *SlSGT1* silencing on leaf sterol profile, extracts from the third and fourth leaves of one-month-old wt, amiSGT-31.2 and amiSGT-61.1 plants were subjected to sterol analysis. This analysis showed that the total amount of SG quantified in amiSGT1-31.2 and amiSGT1-61.1 plants was approximately 32% and 46% lower, respectively, than in the wt plants (Figure 8A and S. Table 1). The contents of all four major sterols in the SG fraction were reduced by the silencing of *SlSGT1*, albeit to varying degrees. As shown in Figure 9 and S. Table 2, stigmasterol was the most severely affected sterol species with a reduction of around 57% and 56% in lines amiSGT1-31.2 and amiSGT1-61.1, respectively, compared with the wt, followed by β-sitosterol (46% and 51%), campesterol (44% and 49%) and cholesterol, which was the less affected sterol species (24% and 33%). The reduction of SGs, the precursors for the biosynthesis of ASGs, induced an overall moderate reduction in the total ASG levels in both mutant lines of about 6% and 9%, respectively, compared with wt levels (Figure 8B). This effect is mainly due to a reduction around 24% and 22% in the content of acylated stigmasterol glycoside. Altogether, the reductions of SG and ASG resulted in a decrease of around 13%-17% in the total amount of glycosylated sterols (SG + ASG) in the silenced lines compared to those in the wt (Figure 8C).

On the contrary, total FS levels increased in leaves of both mutant lines. As shown in Figure 8D, line amiSGT1-31.2 accumulates around 37% more FS than the control plants, similar to the enhanced accumulation of 35% found in amiSGT1-61.1 plants. Among the four major FS, cholesterol levels displayed a slight increase of around 15% in both silenced lines, while free campesterol and β-sitosterol levels increased by about 30% and stigmasterol increased by about 48% in amiSGT1-31.2 and amiSGT1-61.1 plants compared with the wt. Total amounts of SE did not show significant differences between silenced plants and the wt plants (Figure 8E), but as shown in Figure 9D, esterified stigmasterol levels increased by 37%-50% in the mutant lines, while esterified cholesterol, campesterol and β-sitosterol did not show differences between the silenced lines and the wt. The increased levels of FS are most likely a consequence of the reduced ability of mutant plants to glycosylate sterols. It is also remarkable that mutant plants transform only a small fraction of the resulting excess of FS into SE, as there is only a slight increase in the content of this storage form of

sterols. Overall, the changes in the different sterol fractions represent a diminution of 7-10% in the total amount of sterols in mutant lines compared with wt plants. (Figure 8E).

Altogether, the results of this analysis showed the relevance of *SlSGT1* in the glycosylation of sterols in leaves of tomato plants, and suggest some preference of *SlSGT1* for stigmasterol as substrate for glycosylation, as both free, esterified and glycosylated stigmasterol are the sterol species whose levels are most drastically altered when *SlSGT1* expression is downregulated. Moreover, the results also indicate that neither *SlSGT2*, *SlSGT3* nor *SlSGT4* can replace the reduced metabolic activity of *SlSGT1*. Surprisingly, the marked reduction of SG did not translate into drastic changes in the content of ASGs, which suggests the importance of maintaining normal amounts of this type of sterols for tomato plant growth and development.

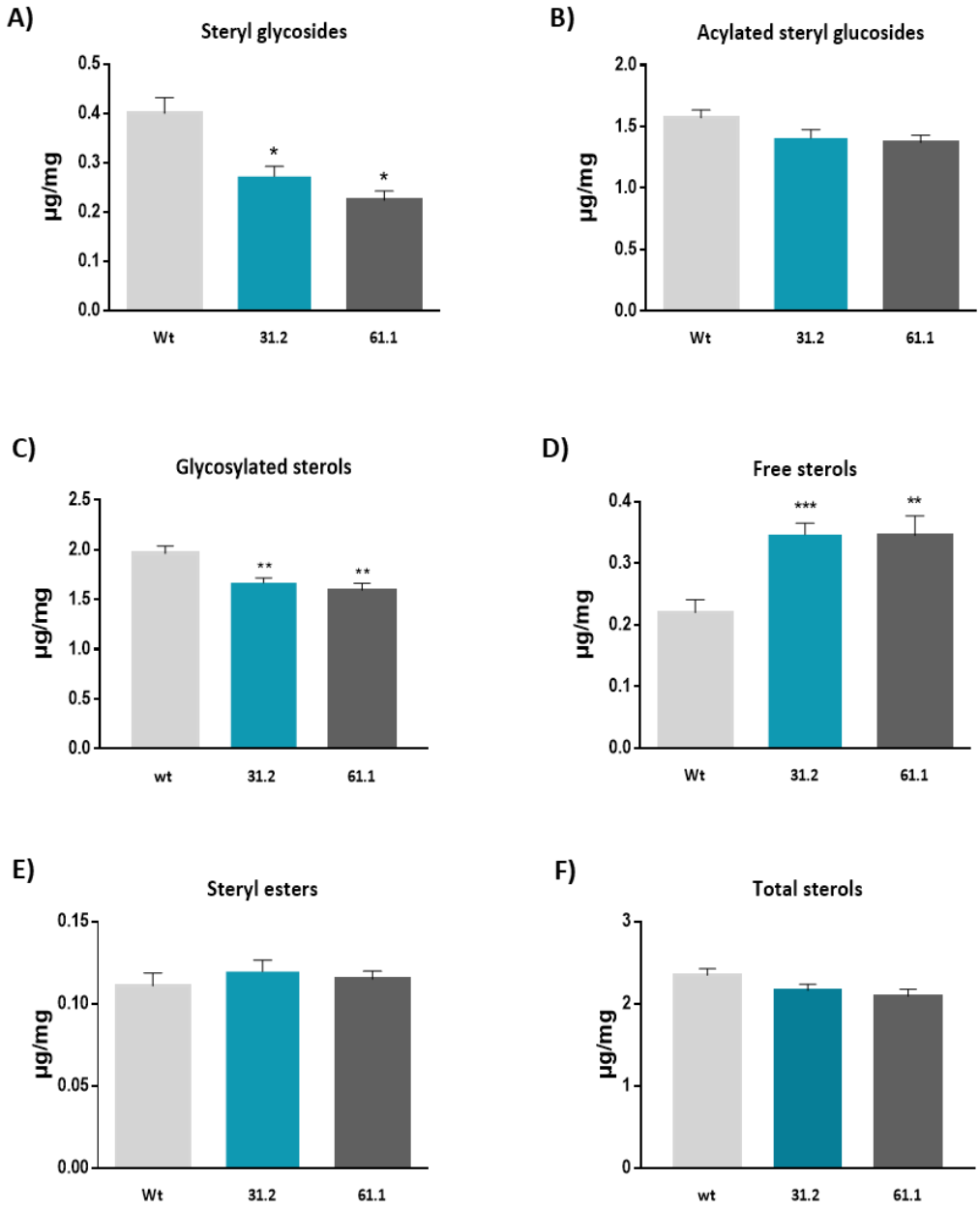

Figure 8. Sterol composition in tomato leaves of amiSGT1 plants. (**A**) Total steryl glycosides, (**B**) acylated steryl glycosides, (**C**) glycosylated sterols (SGs+ASGs), (**D**) free sterols, (**E**) steryl esters, and (**F**) total sterols (four fractions) in wt and silenced lines amiSGT1-31.2 and amiSGT1-61.1. Total content in each fraction includes cholesterol, campesterol, stigmasterol, and β-sitosterol levels. Data are expressed as mean of µg sterol per mg of dry weight of tissue ± SEM from three biological replicates with three technical replicates each (n=9). Asterisks represented significant differences compared with wt determined by t-test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001).

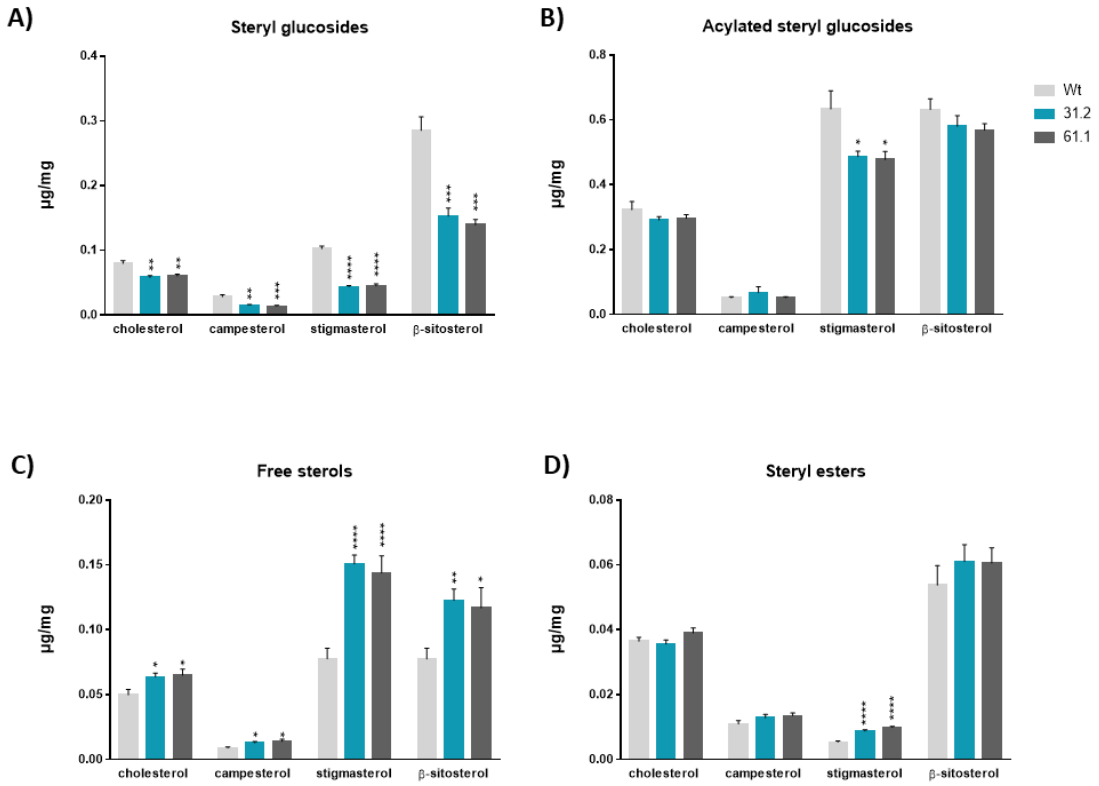

Figure 9. Content of free and conjugated sterols in tomato leaves of amiSGT1 plants. (**A**) steryl glucosides, (**B**) acylated steryl glucosides, (**C**) free sterols, and (**D**) steryl esters. Data are expressed as mean of µg sterol per mg of dry weight of tissue ± SEM from three biological replicates with three technical replicates each (n=9). Asterisks represented significant differences compared with the wt determined by t-test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001).

3.1.3 Silencing of SlSGT1 results in moderate dwarfism in tomato plants.

Morphological phenotype analysis of one-month-old plants from transgenic lines amiSGT1-31.2 and amiSGT1-61.1 revealed a pleiotropic phenotype at the vegetative stage, including shorter plants and smaller leaf area when compared with wt plants. Plants of lines amiSGT1-31.2 and amiSGT1-61.1 were 25.8 % and 19.1% shorter, respectively, than wt plants (Figure 10A-B), which is consistent with the finding that the fifth internode, the most apical internode in one-month-old tomato plants, in amiSGT1-31.2 and amiSGT1-61.1 plants was 56.4% and 45.3% shorter, respectively, that in wt plants (Figure 10C). Similar results were obtained when the diameter of the fifth internode was measured. Reductions of around 37% and 30% were found in amiSGT1-31.2 and amiSGT1-61.1 plants, respectively, compared with the wt (Figure 10D). Both effects could be caused by alterations in cell division and/or expansion. As a first approach to investigate if the observed reduction in length and diameter of the fifth internodal stem displayed by amiSlSGT1 lines is caused by altered cell number and/or size, stem histological sections were prepared and analyzed. The number of cell layers in lines amiSGT1-31.2 and amiSGT1-61.1 was 16% less than in wt internodes, which suggests alterations in cell division. Moreover, cell size was 11-22% smaller in the mutant plants compared to those in the wt. Finally, the leaf area of the third and fourth leaves was also measured, and the results showed a reduction of 35.4% and 31.4% in amiSGT1-31.2 and amiSGT1-61.1 plants, respectively, compared with leaf area of wt plants. Altogether these results suggest that the dwarf phenotype displayed by amiSGT1 plants is the result of anomalies in both cell division and expansion caused by the silencing of *SlSGT1* (Figure 10E-I).

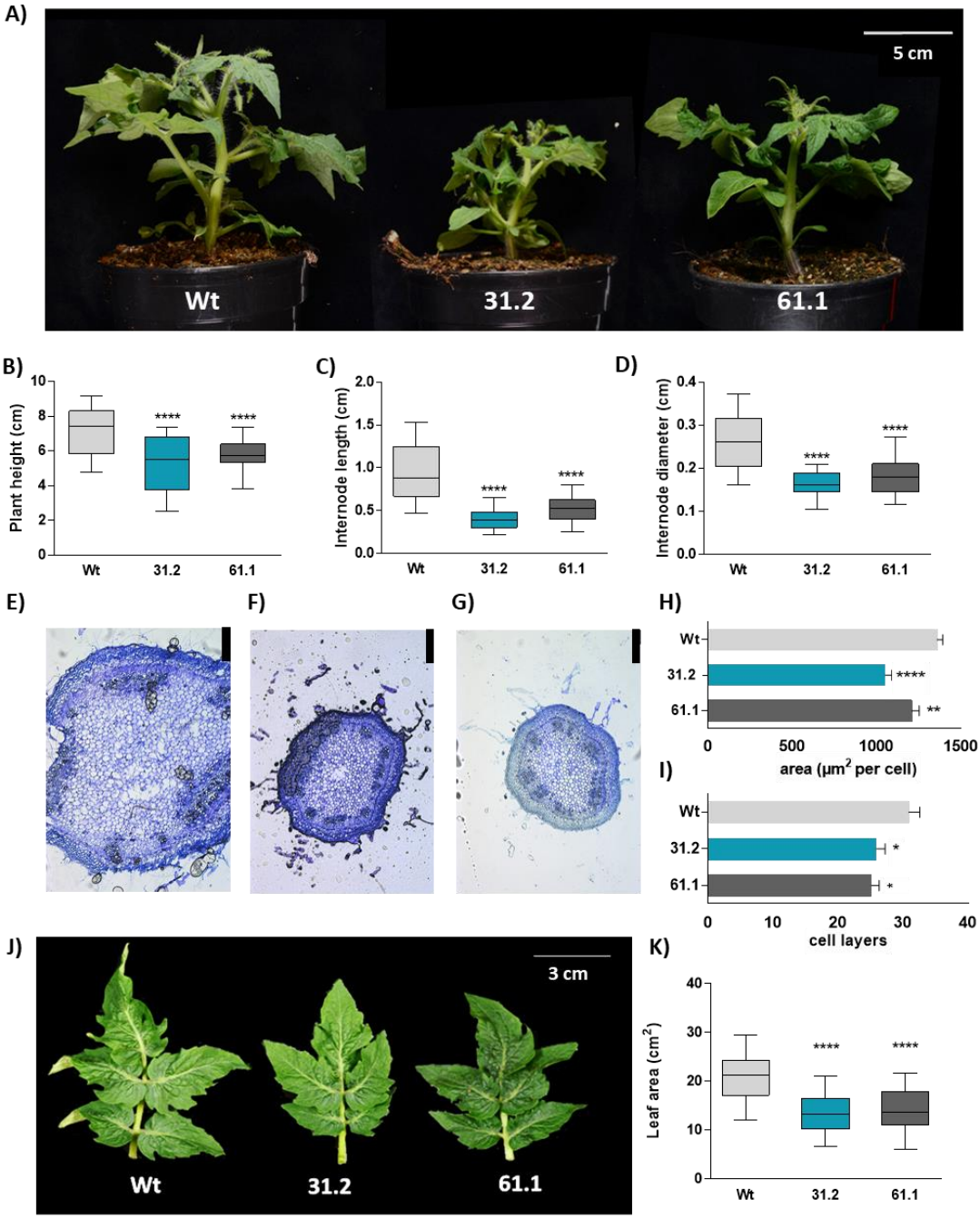

Figure 10. Phenotypic characterization and morphometric analysis of amiSGT1 silenced plants. (A) Representative images of wt, amiSGT1-31.2 and amiSGT1-61.1 one-month-old plants. Quantitative analysis of plant height (B), fifth internode length (C) and diameter (D). Data are the mean ± min and max values from 30 plants per genotype (n=30). (E-G), histological analysis of one-moth-old steams from wt, amiSGT1-31.2 and amiSGT1-61.1 plants. (H-I), quantification of cell area (µm^2) and cell layers in the 5th internode of wt, amiSGT1-31.2 and amiSGT1-61.1 plants. Scale bar equals to 250 µm. Values are mean ± SEM from 5

samples per genotype (n=5). (J) Representative images of the third leaf of wt, amiSGT1-31.2 and amiSGT1-61.1 silenced plants. (K) Quantitative analysis of third and fourth leaf area. Data are the mean ± min and max values of leaves of 30 plants per genotype (n=30). Asterisks represented significant differences compared with wt determined by t-test (*P ≤ 0.05; **P ≤ 0.01; ****P ≤ 0.0001).

3.1.4 Downregulation of SlSGT1 gene expression does not alter tomato glycoalkaloid levels.

Steroidal glycoalkaloids (SGAs) are toxic secondary metabolites that participate in plant defense against different biotic stresses. These compounds have a stereoalkaloid core attached to a sugar side-chain (Sonawane *et al.*, 2020). In *Withania somnifera*, it had been reported that *WsSGTLs* participate in the synthesis of SGAs, and consequently its expression levels alters the biotic stress response of this plant (Singh *et al.*, 2016). To investigate the potential participation of *SlSGT1* in the biosynthesis of the tomato SGAs, in collaboration with Prof. Aharoni's group (Weizmann Institute of Science, Rehovot, Israel), we measured α-tomatine and dehydrotomatine levels in the same tissue samples of wt, amiSGT1-31.2 and amiSlSGT1-61.1 tomato plants used for sterol analysis. In tomato, α-tomatine and dehydrotomatine are the major SGAs in plant and immature fruits, these compounds are the result of the glycosylation of their respective aglycones, tomatidine and dehydrotomatidine (Akiyama *et al.*, 2019). The metabolic analysis showed that neither α-tomatine nor dehydrotomatine levels are affected by silencing of *SlSGT1* in the mutant lines amiSGT1-31.2 and amiSGT1-61.1. These results indicate that *SlSGT1* is not involved in the biosynthesis of the predominant tomato SGAs (Figure 11).

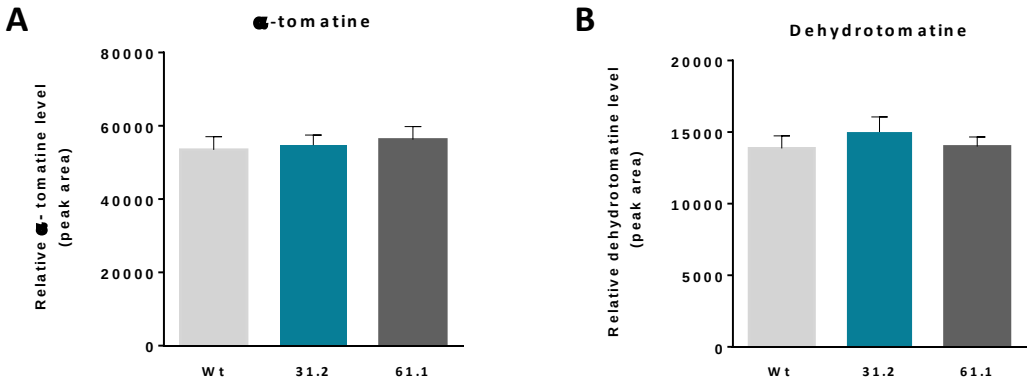

Figure 11. Steroidal glycoalkaloids in leaves of amiSGT1 silenced plants. Relative α-tomatine (A) and dehydrotomatine (B) levels in one-month-old tomato leaves of wt, amiSGT1-31.2 and amiSGT1-61.1 plants were measured by UPLC-qTOF-MS/MS analysis. Dehydrotomatine and α-tomatine were identified by comparison of their retention times and MS/MS fragments to those of the corresponding standards. Data are expressed as mean peak area ± SEM from nine biological replicates (n=9).

3.1.5 Silencing of SlSGT1 modifies expression of genes involved in defense stress responses in leaves.

To investigate the impact of silencing *SlSGT1* on the tomato leaf transcriptome we employed a massive RNA sequencing (RNA-seq) approach using RNA samples from three independent biological replicates of amiSGT1-31.2 and wt plants. Six cDNA libraries were constructed and sequenced with an Illumina HiSeq 2500 platform. After trimming the obtained raw reads to remove adaptor sequences empty reads and low-quality sequences, a total of 13,778,894, 20,315,677 and 21,494,068 high-quality reads, designed as clean reads were generated for wt biological replicates, and a total of 20,397,262, 21,343,654 and 22,040,652 clean reads for amiSGT1-31.2 biological replicates. Clean reads were mapped to the *S. lycopersicum* (SL3.0) reference genome with START aligner (version 2.5.2b). More than 18,900 genes with different abundances were detected. Normalized read counts were obtained from each of the samples and employed for differential gene expression analysis. The resulting list of differential expressed genes (DEGs) was filtered by $\log_2$ (fold change) of 1.5 or greater or -1.5 or less and a statistical value of 0.05 FDR or less. A total of 90 DEGs were identified, 58 upregulated and 33 downregulated (Tables 1-2 and Figure 12). Interestingly, the final list include 24 DEGs (14 upregulated and 10 downregulated, marked as underlined in table X) whose description has been linked with stress response in plants including genes involved in salicylic acid pathway such as pathogenesis-related protein 1 (*PR1*), and synthesis of defense specialized metabolites such as flavonoids, where key genes including chalcone synthase (*CHS*), 4-coumarate:CoA ligase-like (*4CL*), dihydroflavonol 4-reductase family (*DRF*) and two different phenylalanine ammonia-lyase (*PAL*) genes were upregulated by the silencing of *SlSGT1*. Additionally, some DEGs coding for proteins related with fungi-defense-response were found, including leucine-rich repeat (*LRR*), chitinase, and defensin-like proteins. Overall, these results suggest that silencing of *SlSGT1* could alter the defense response of tomato plants.

Additionally, the libraries generated in this analysis were used to search for changes in the transcript levels of genes involved in the sterol biosynthetic pathway. This search included genes involved in the synthesis of sterol precursors such as *AACT*, *HMGS*, *HMGR, MVK, PMK*, *MPD, IDI, FPS, SQS* and *SQE,* genes involved in the synthesis of the different sterol species such as *CAS*, *SMT1*, *SMT2*, *SSR1*, *SMO3* and *C22Des*, and genes involved in the synthesis of conjugated sterols such as *ASAT* and *PSAT* (steryl esters) and *SGT1* to 4 (steryl glycosides) (S. Figure 1). The expression of none of these

genes was affected by the silencing of *SlSGT1,* which according to this analysis showed a 2.5-fold reduction of expression compared with the wt (Table 2, marked in blue).

Table 1. List of upregulated genes in leaves of amiSlSGT1 31.2 plants compared to wt.

Gene ID	Description	log2FC	FDR
Solyc04g039670	ATP-citrate synthase, putative	4.96	0.00E+00
Solyc08g062580	Pentatricopeptide repeat-containing protein family	4.87	0.00E+00
Solyc07g017437	3-oxo-5-alpha-steroid 4-dehydrogenase family protein	4.72	0.00E+00
Solyc07g006380	Defensin-like protein	4.59	0.00E+00
Solyc03g071790	Defective in meristem silencing 3	4.19	0.00E+00
Solyc01g056430	Nodulin / glutamate-ammonia ligase-like protein	4.09	0.00E+00
Solyc01g056450	Glutamate-ammonia ligase-like protein	3.86	0.00E+00
Solyc03g036460	E3 ubiquitin-protein ligase	3.84	0.00E+00
Solyc03g096140	Protein yippee-like	3.79	0.00E+00
Solyc10g038020	Cellulose synthase family protein	3.49	6.99E-15
Solyc05g024230	ATP-dependent 6-phosphofructokinase	3.46	0.00E+00
Solyc03g036470	Phenylalanine ammonia-lyase	3.45	3.55E-05
Solyc02g032910	Glycine rich protein	3.40	1.19E-02
Solyc08g068410	flowering time control protein-related / FCA gamma-like protein	3.40	1.30E-14
Solyc01g108520	Acetyl esterase IPR013094 Alpha_beta hydrolase fold-3	3.30	1.10E-14
Solyc08g066400	Casein kinase I protein	3.29	0.00E+00
Solyc01g090600	Chalcone synthase	3.04	1.62E-02
Solyc03g042560	Phenylalanine ammonia-lyase	3.00	9.83E-03
Solyc06g073750	Beta-glucosidase	2.88	1.64E-02
Solyc10g007800	protodermal factor 1	2.83	1.10E-14
Solyc02g080760	Glycine-rich protein	2.71	1.21E-02
Solyc01g009590	Protease inhibitor/seed storage/lipid transfer protein family protein	2.69	1.22E-02
Solyc05g050700	Leucine-rich repeat protein	2.69	1.22E-02
Solyc11g028040	Defensin-like protein	2.49	1.84E-02
Solyc01g099540	Beta glucosidase 8	2.48	1.82E-02
Solyc02g005340	Oligopeptide transporter, putative	2.27	0.00E+00
Solyc08g066410	Protein kinase family protein	2.24	6.19E-03
Solyc02g089200	TM29	2.15	3.35E-03
Solyc10g081810	MD-2-related lipid recognition domain-containing protein / ML domain-containing protein	2.14	0.00E+00
Solyc01g006390	Extensin-like protein	2.10	1.83E-02
Solyc04g077750	NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mitochondrial	2.08	6.00E-15

(Continued next page 1-2)

Continuation Table 1.

Gene ID	Description	log2FC	FDR
Solyc08g062780	bHLH transcription factor 089	2.06	1.86E-02
Solyc08g067230	MADS box transcription factor	2.06	6.58E-03
Solyc08g079370	Cytochrome P450 family protein	2.00	2.00E-15
Solyc01g086830	Protease inhibitor/seed storage/lipid transfer protein family protein	1.98	2.72E-02
Solyc05g050820	DNAJ	1.87	4.00E-15
Solyc04g077745	NADH-ubiquinone oxidoreductase-like protein	1.87	6.99E-15
Solyc03g053130	Strictosidine synthase family protein	1.85	2.41E-02
Solyc11g042810	Hexosyltransferase	1.83	2.77E-02
Solyc04g025560	ADP-ribosylation factor	1.82	0.00E+00
Solyc08g083500	Cytochrome P450 family protein	1.82	3.00E-15
Solyc11g028070	Defensin-like protein	1.78	2.72E-02
Solyc11g018570	To encode a PR protein, Belongs to the plant thionin family with the following members:, putative	1.77	2.79E-02
Solyc04g064765	exostosin family protein	1.75	0.00E+00
Solyc03g096160	Protein yippee-like	1.74	1.18E-02
Solyc07g065780	ABC transporter G family member	1.71	2.72E-02
Solyc02g088710	4-coumarate:CoA ligase-like	1.71	2.77E-02
Solyc02g069170	Transcription initiation factor TFIID subunit-like protein	1.69	4.00E-14
Solyc09g089780	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	1.68	8.99E-15
Solyc09g075790	Long-Chain Acyl-CoA Synthetase	1.65	0.00E+00
Solyc06g061040	Ribose-phosphate pyrophosphokinase	1.62	9.99E-15
Solyc07g005750	F-box family protein	1.58	5.00E-15
Solyc04g070970	ABC transporter family protein	1.57	1.85E-02
Solyc01g057805	Pyrophosphate--fructose 6-phosphate 1-phosphotransferase subunit beta	1.50	0.00E+00
Solyc04g008780	Dihydroflavonol 4-reductase family	1.50	2.72E-02
Solyc11g018777	Peroxidase	1.50	1.81E-02
Solyc08g079910	P69e protein	1.50	2.11E-02

Table 2. List of downregulated genes in leaves of amiSlSGT1 31.2 plants compared to wt.

Gene ID	Description	log2FC	FDR
Solyc02g005350.3	succinyl-CoA ligase alpha 2 subunit	-1.50	2.00E-15
Solyc07g007250.3	Metallocarboxypeptidase inhibitor	-1.50	0.00E+00
Solyc12g094440.2	High mobility group family	-1.50	1.46E-03
Solyc11g070150.2	Histidine phosphotransfer protein	-1.50	4.52E-03
Solyc08g074910.3	F-box/FBD/LRR-repeat protein	-1.50	2.02E-03
Solyc09g084470.3	Wound-induced proteinase inhibitor 1	-1.50	2.61E-04
Solyc01g091430.2	ATP-dependent DNA helicase 2 subunit KU80	-1.50	9.92E-07
Solyc06g010033.1	MLO-like protein	-1.60	0.00E+00
Solyc00g174340.2	Pathogenesis-related protein 1	-1.69	0.00E+00
Solyc11g011570.1	RING/U-box superfamily protein	-1.78	0.00E+00
Solyc02g088513.1	Saccharopine dehydrogenase	-1.78	2.58E-04
Solyc10g050063.1	Transcription complex	-1.80	2.43E-02
Solyc10g008840.3	Rab family GTPase	-1.91	2.00E-15
Solyc11g007980.2	Cytochrome P450	-1.92	2.26E-02
Solyc03g013440.3	Amino acid transporter, putative	-1.93	0.00E+00
Solyc12g049030.1	Fatty acid desaturase	-1.93	8.09E-03
Solyc04g014830.2	GRAS family transcription factor	-1.94	0.00E+00
Solyc07g009500.2	Chitinase	-2.02	1.51E-02
Solyc07g016210.1	Specific tissue protein	-2.04	5.00E-15
Solyc02g088520.3	integral membrane protein	-2.18	3.53E-03
Solyc09g007010.1	Pathogenesis-related protein 1	-2.22	0.00E+00
Solyc00g006530.1	Calmodulin-binding protein	-2.27	0.00E+00
Solyc00g006540.1	DNA ligase	-2.29	0.00E+00
Solyc12g100250.2	Fatty acid desaturase	-2.30	1.72E-04
Solyc06g007980.3	UDP-glucose:sterol 3-O-glucosyltransferase	-2.49	5.00E-15
Solyc06g034310.3	Myb/SANT-like DNA-binding domain protein	-2.65	6.99E-15
Solyc10g078220.2	Cytochrome P450	-2.72	2.77E-03
Solyc05g043380.1	phosphatidylinositol 4-kinase gamma-like protein	-2.88	2.05E-03
Solyc06g011480.2	Dynamin, putative	-3.50	0.00E+00
Solyc05g023990.2	Leucine-rich receptor-like protein kinase family protein	-4.01	3.71E-13
Solyc12g056690.1	Serine/threonine protein phosphatase 7 long form isogeny	-4.05	1.10E-14
Solyc06g009890.2	PHD finger family protein	-6.08	8.20E-14
Solyc05g018060.1	Transducin/WD40 repeat-like superfamily protein	-6.09	0.00E+00

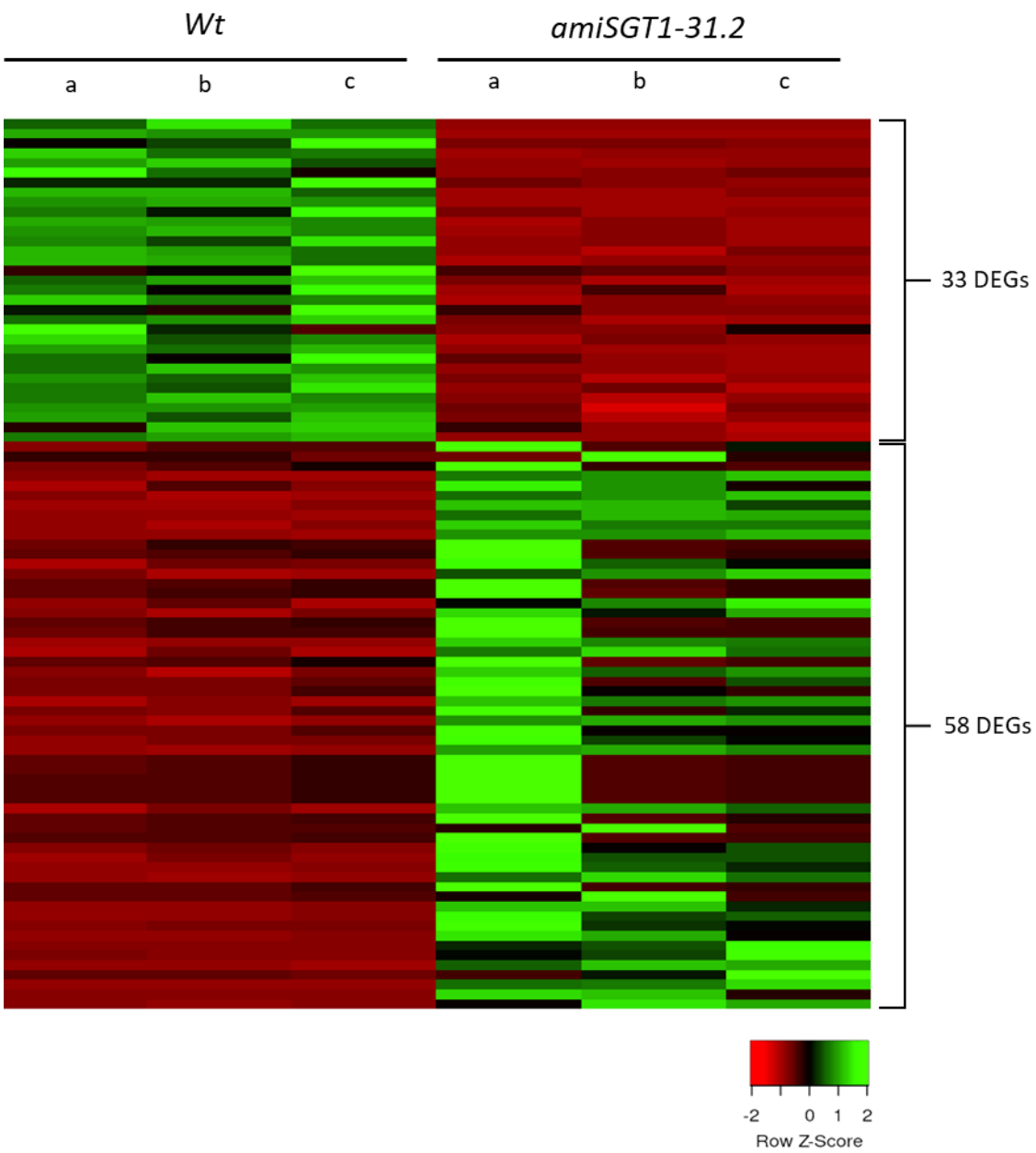

Figure 12. DEGs identified in the amiSGT1 silencing lines. Heatmap represented the normalized expression levels (Z-score) of the identified DEG in the three biological replicates of amiSlSGT1-31.2 and wt leaves from one-month-old tomato plants. FPKM were normalize and plotted with Heatmapper.

3.1.6 Silencing of SlSGT1 negatively affects fruit size.

During tomato fruit development and ripening several metabolic changes take place including changes in sterol composition. The increase in conjugated sterols content has been described as one of the most important events during tomato fruit ripening (Whitaker, 1988). However, the specific role of SGs in this process is far from being understood. As a first approached to investigate the role of SGs in fruit development and ripening, we analyzed the expression level of the *SlSGT* gene family in fruits of wt and silenced lines amiSGT1-31.2 and amiSGT1-61.1 at different stages of development, namely green (15 days after anthesis, DAA), mature green (30 DAA), breaker+1 day (32 DAA), and red (36 DAA) fruits. The results of this analysis showed that *SlSGT1* mRNA levels were drastically depleted to 13% and 9% (green), 28% and 14% (mature green), 13% and 8.5% (breaker+1), and 5% and 7.5% (red), respectively, the mRNA levels in wt fruits at the same developmental stages (Figure 13). These results demonstrate that the selected amiRNAs are also highly efficient in silencing *SlSGT1* expression in fruits. As regards to the expression of the remaining members of the *SlSGT* gene family, the transcript levels of *SlSGT2* and *SlSGT3* remained essentially unaltered, with only a moderate upregulation of their expression at the mature green and red stages, respectively. Transcript levels of *SlSGT4* were barely detectable at any stage of fruit development of any plant line, including wt fruits (Figure 13).

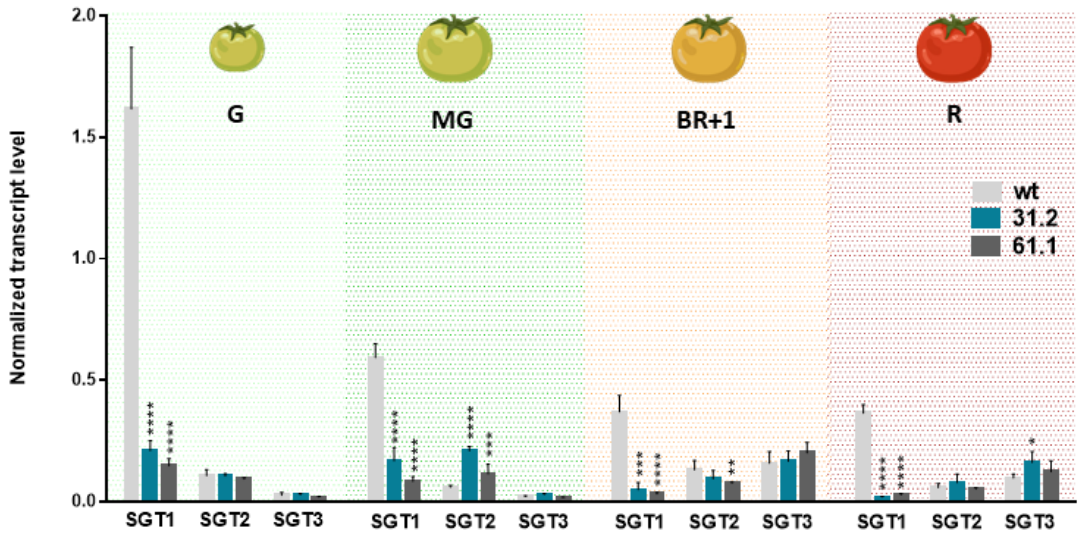

Figure 13. **Silencing of *SlSGT1* in fruits at different developmental stages**. The transcript levels of *SlSGT1*, *SlSGT2* and *SlSGT3* were determined by RT-qPCR using RNA extracted from green (G; 15 DAA), mature green (MG; 30 DAA), breaker +1 day (BR+1; 32 DAA) and red (R; 36 DAA) tomato fruits. Values are expressed as normalized quantity using a tomato actin gene (Solyc03g078400) as housekeeping. Data are expressed as mean ± SEM of values from

three technical replicates consisting each of pericarp from 15 tomato fruits (n=3). Asterisks represented significant differences compared with the wt determined by t-test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; **** P ≤ 0.0001).

As we demonstrated that silencing of *SlSGT1* results in a reduction of plant size, shorter and thinner internodes, and lower foliar area, we investigated if the silencing of *SlSGT1* results also in alterations of fruit phenotype. To identify changes in fruit phenotype, we first calculated the fruit weight at the growth and ripening stages indicated above. Results of the weight analysis showed that the tomato fruits of mutant lines amiSGT1-31.2 and amiSGT1-61.1 weigh significantly less than wt fruits, with a reduction around 61-65% at 9 DAA (small green, G1), 33-38% at 15 DAA (green, G2), 25-29% at 30 DAA (mature green), 18-25% at 32 DAA (breaker +1 day) and 19-24% at 36 DAA (red) fruits, respectively (Figure 14A-B). Further, we measured the seed production and the analysis revealed a decrease of 42% and 25% in the seed content per fruit in lines amiSGT1-31.2 and amiSGT1-61.1, respectively, when compared with the wt (Figure 14C). Altogether, these results suggest the important role of *SlSGT1* during tomato plant and fruit growth and development since both, vegetative stage and fruits, showed a correlation between lower expression levels of this gene with negative alterations in size.

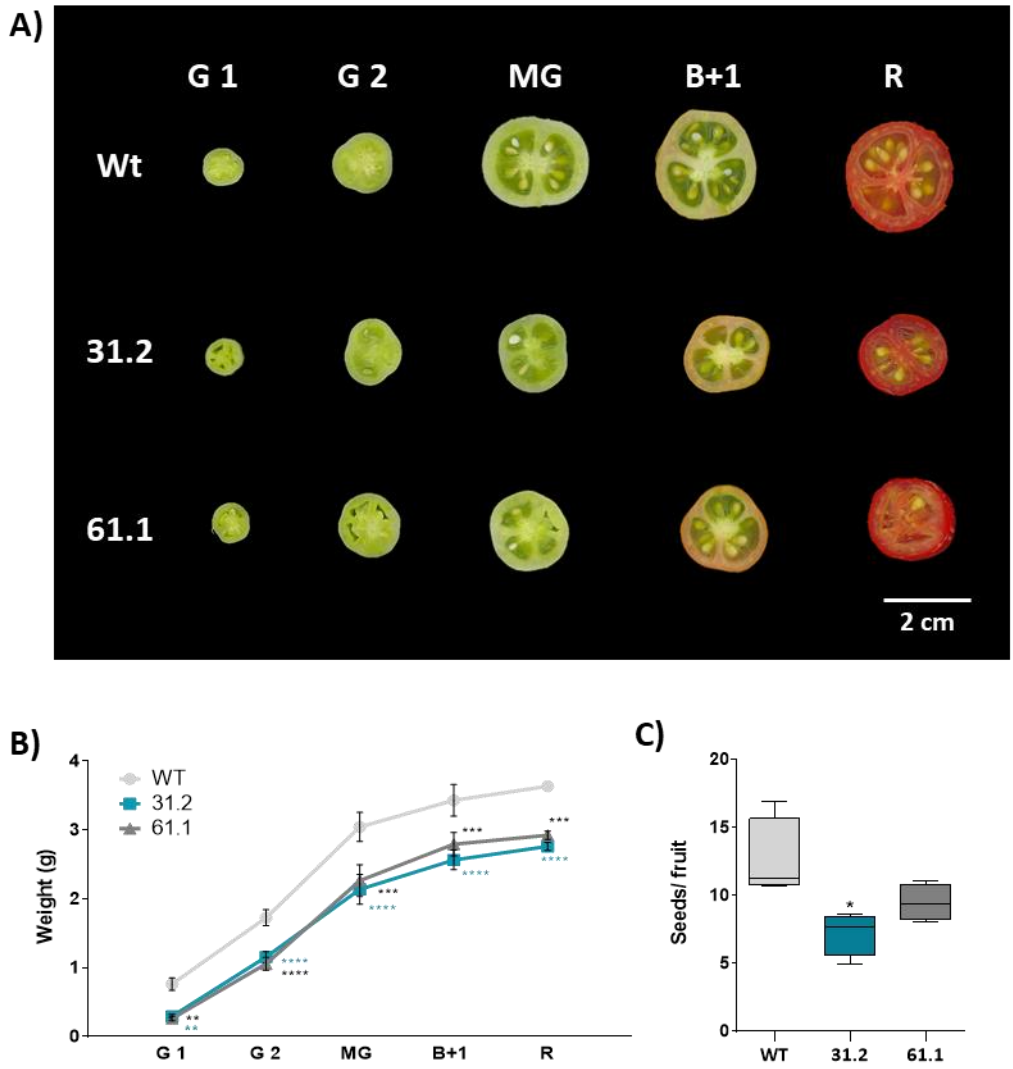

Figure 14. Phenotypic characterization of wt and amiSGT silenced fruits. (A) Representative images of fruits of wt, amiSGT1-31.2 and amiSGT1-61.1 plants at different developmental stages: G1 (green, 9 DAA), G2 (green, 15 DAA), MG (mature green, 30 DAA), B+1 (breaker +1 day, 32 DAA) and R (red, 36 DAA). (B) Fruit weight of wt and amiSGT1 silenced plants at the same developmental stages. Data shows the mean and ± SEM (n=30). (C) Seed content per fruit of the silenced lines and the wt. Data shown are the mean and min and max values. Asterisks represented significant differences in silencing lines compared with wt fruits determined by t-test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001).

3.1.7 Silencing of SlSGT1 results in a reduction of SG levels in tomato fruits.

To gain insight into the metabolic impact of *SlSGT1* silencing in the sterol profile along tomato fruit growth and ripening, extracts from pericarp tissue pooled of wt, amiSGT1-31.2 and amiSGT1-61.1 fruits at the stages previously described were subjected to sterol analysis. Results showed that the total content of SGs in wt fruits gradually decrease along fruit development and ripening, with a 72% reduction from green (0,33 μg/mg DW) to red fruits (0,09 μg/mg DW) (Figure 15A and S. Table 3). Interestingly, silencing of *SlSGT1* in amiSGT1-31.2 and amiSGT-61.1 fruits resulted in a sharp reduction of SGs only at the early stages of development, displaying a reduction of around 50% at green stage and 32% at mature green stage compared with the SG amounts in wt fruits at the same stages. On the contrary, fruits of both mutant lines showed similar SG levels than the wt fruits at both breaker +1 day and red stages (Figure 15A). Overall, changes in the major individual glycosylated sterols profile during fruit development in response to *SlSGT1* silencing are very similar, since their levels are significantly reduced in green and mature green stages whereas at breaker +1 and red stages, they are similar to those in wt fruits (Figure 16A-D and S. Table 4). Only glycosylated stigmasterol displays a slightly different behavior since the levels in the mutant fruits at the four developmental stages are significantly lower than in the wt fruits.

In contrast with the sustained reduction of SG content along growth and ripening, the amounts of ASG in wt fruits progressively decrease from green to breaker +1 day stages and returns to the green fruit levels at the red stage (Figure 15B). The ASG profile throughout development of mutant fruits is very similar to that in wt fruits, although there is an overall reduction of ASG levels at all developmental stages ranging from 12% to 30% compared with wt fruits (Figure 15B), which reflects a general reduction in the contents of all four major sterol species. Interestingly, changes in the profile of each of these species along fruit development shows some differences between mutant and wt fruits (Figure 16E-H). Acylated campesterol glycoside and stigmasterol levels decrease from green to mature green stages and then progressively increase until the red stage, while glycosylated β-sitosterol and cholesterol levels decrease all along fruit development, being this tendency much more pronounced in the case of glycosylated cholesterol, particularly at the very early stages of fruit development.

Taken together, the profiles of SGs and ASGs throughout wt and mutant fruit growth and ripening strongly suggest that SGs are mostly synthesized at the green and

mature green stages whereas an active synthesis of ASGs occurs throughout all stages of fruit development. At the green and mature green stages there is an active cell division and expansion that allows fruits to attain their maximum size. Once the growth phase is completed, fruits enter the ripening phase during which fruit size remains constant (Gillaspy *et al.*, 1993)

Free sterol levels in wt fruits remain fairly constant throughout fruit growth and ripening, with values ranging from 0.025 to 0.045 μg/mg DW (Figure 15C). As previously reported in leaves, the downregulation of *SlSGT1* in lines amiSGT1-31.2 and amiSGT1-61.1 leads to enhanced levels of FS at all stages of fruit development ranging from 45-50% in green fruits, 60-61% in mature green fruits, 68-70% in breaker +1 day and 28-37% in red fruits compared to those in the wt (Figure 15C). As shown in Figure 16I-L, this is due to a marked increase of free cholesterol and β-sitosterol at mature green stage in combination with a general increase of free stigmasterol and campesterol levels at all the developmental stages.

Finally, both the profile and contents of SE are almost identical in wt and fruits of both *SlSGT1* silenced lines, being remarkable in all cases the drastic increase of 650% to 775% observed in mature green fruits compared to green fruits, which is followed by a gradual decrease at breaker +1 day and red stages (Figure 15D). These results indicate that tomato fruits have the capacity to accommodate the excess of free sterols accumulated in *SlSGT1* silenced lines without the need to convert them into their esterified form. The total steryl ester profile reflects perfectly the behavior of esterified cholesterol and β-sitosterol along fruit development and ripening, whereas esterified campesterol and stigmasterol showed extremely low levels, which were undetectable in green and mature green fruits (Figure 16M-P).

As show in Figure 15E, total sterol levels, including free and conjugated sterol fractions, in amiSGT1-31.2 and amiSGT1-61.1 fruits, are only moderately reduced by about of 12-25% along the different developmental stages compared with wt fruits. However, a detailed analysis of each of the four sterol fractions reveals a much more profound effect of *SlSGT1* silencing on the glycosylated sterol fractions, namely SG and ASG, which supports a major role for *SlSGT1* in the glycosylation of sterols, particularly at the earliest stages of fruit development, when important cell division and cell expansion events whose will define the final fruit size take place.

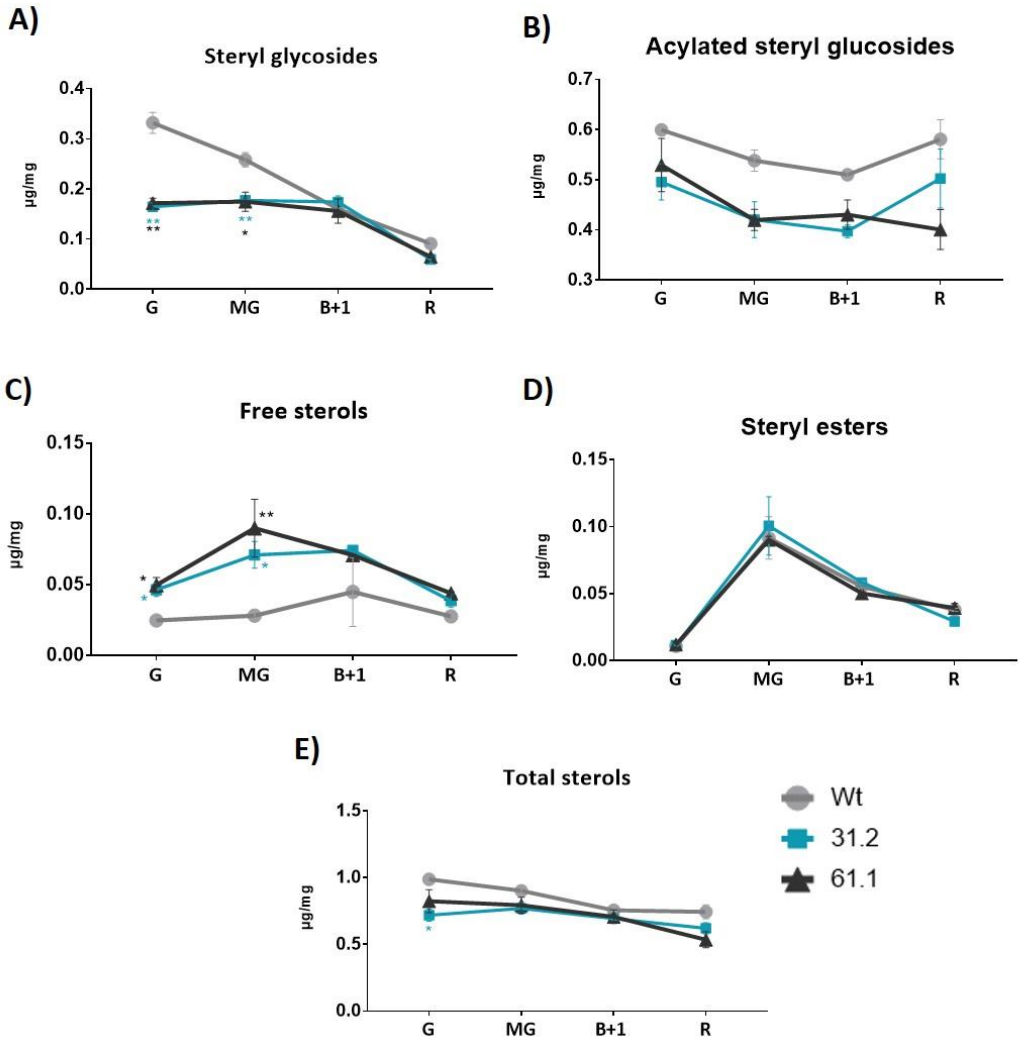

Figure 15. Free and conjugated sterol levels in fruits at different developmental stages of wt and *SlSGT1* silenced lines. (**A**) steryl glucosides, (**B**) acylated steryl glucosides, (**C**) free sterols, (**D**) steryl esters, and (**E**) total sterols in green (G), mature green (MG), breaker +1 (B+1) and red (R) tomato fruits. Data are mean ± SEM from three technical replicates per genotype and fruit developmental stage (n=3). Asterisks represented significant differences in silencing lines compared with wt fruits determined by t-test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001).

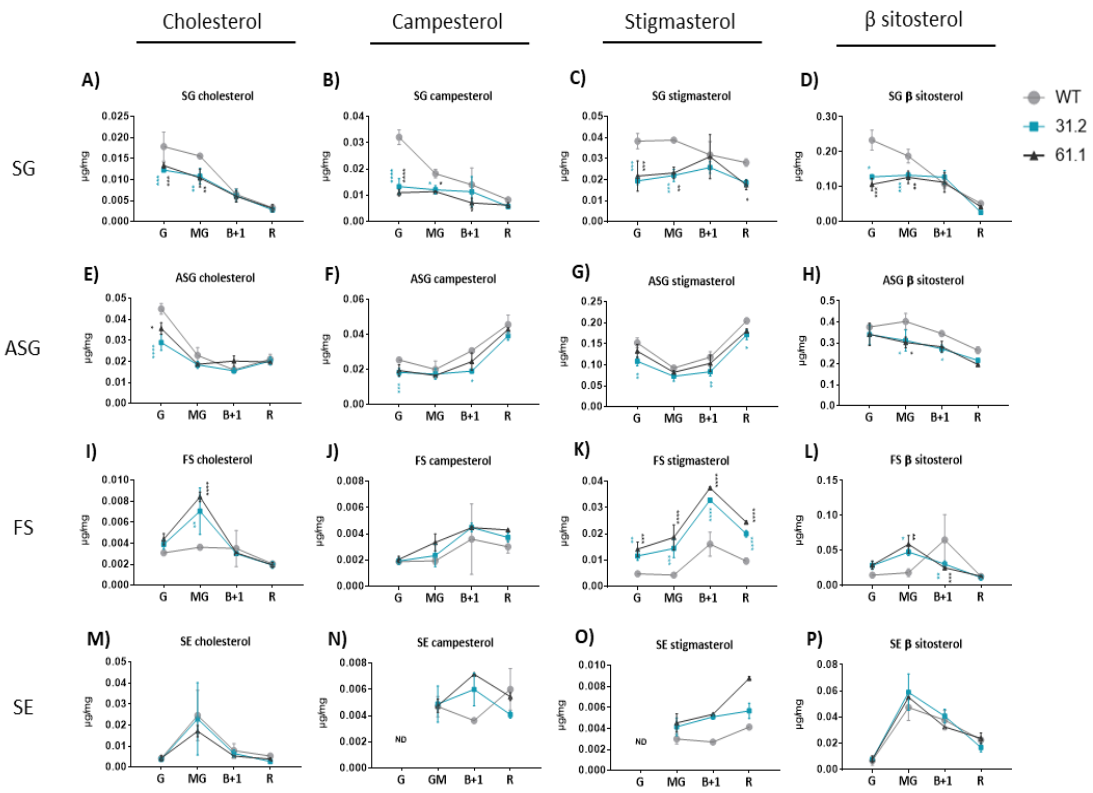

Figure 16. **Major free and conjugated sterol species levels in fruits at different developmental stages of wt and SlSGT1 silenced lines.** (**A-D**) steryl glucosides (SG), (**E-H**) acylated steryl glucosides (ASG), (**I-L**) free sterols (FS), and (**M-P**) steryl esters (SE) in wt, 31.2 and 61.1 tomato fruits at green (G), mature green (MG), breaker +1 (B+1), and red (R) fruits. Data are expressed as mean µg sterol per mg of dry weight ± SEM from three technical replicates per genotype and fruit developmental stage (n=3). Asterisks represented significant differences in silencing lines compared with wt fruits determined by t-test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001).

3.1.8 Transcriptional profiling of fruits silenced for SlSGT1 shows more gens down-regulated than up-regulated.

To further investigate the role of *SlSGT1* and glycosylated sterols during fruit development, we generated whole transcriptome data by RNA-seq using RNA samples from three independent biological replicates of 9 DAA tomato fruits of wt, amiSGT1-31.2 and amiSGT1-61.1 plants. Nine cDNA libraries were constructed and sequenced as previously described (see section 3.1.5) After trimming the obtained raw reads to remove adaptor sequences, empty reads and low-quality sequences, a total of 50,660,982, 52,870,158, and 52,499,562 clean reads were generated for wt biological replicates while a total of 51,363,606, 51,449,142, and 51,499,004 clean reads were generated for amiSGT1-31.2 biological replicates and a total of 51,025,606, 52,043,220, and 52,521,486 clean reads were generated for amiSGT1-61.1 biological replicates. Clean reads were mapped to the *S. lycopersicum* (SL3.0) reference genome with START aligner (version 2.5.2b) in the three genotypes. In addition, more than 96% were unique mapped reads, while the proportion of multiple mapped reads was less than 0.8%. Normalized read counts were obtained from each sample and employed for differential gene expression analysis. The resulting list of DEGs was filtered by $\log_2$ (fold change) of 1.5 or greater or -1.5 or less and a statistical FDR of 0.05 or less. A total of 430 DEGs were identified in fruits of line amiSGT1-31.2 (48 up-regulated and 382 down-regulated) and 633 DEGSs in fruits of line amiSGT1-61.1 (160 up-regulated and 473 down-regulated) (Figure 17A). The number of overlapping DEGs between fruits of both silenced lines was 35 in the set of up-regulated and 353 between the down-regulated (Figure 17B-C). The genes included in the list of overlapping DEGs (S. Table X) were selected as those whose expression was supposed to be altered as a result of *SlSGT1* silencing. Interestingly, this list included several genes coding for proteins which expression has been related with tomato fruit development, such as *7S globulin* (Solyc09g065470) involved in fruit quality (Liu et al., 2016), two members of the *CLV3/ESR* family (Solyc05g053630 and Solyc05g053640) that have been related with fruit organogenesis and maturation (Zhang *et al.*, 2014) nine oleosins (Solyc06g060840, Solyc08g078160, Solyc02g086490, Solyc08g066040, Solyc06g034040, Solyc12g010920, Solyc03g112440, Solyc07g065985 and Solyc06g069260) involved in the formation of oil bodies in seeds and fruits (Frandsen et al., 2001), six non-specific lipid-transfer protein (*nsLTPs*, Solyc01g090350, Solyc01g090360, Solyc02g087910, Solyc10g012110, Solyc10g008205 and Solyc10g012120) associated with fruit development and ripening (D'Agostino *et al.*, 2019), three auxin-responsive GH3 family proteins, including *GH3-12* and *GH3-13* (Solyc00g212260, Solyc10g009610 and

Solyc10g009640), which belong to a family of 24 genes proteins involved in the maintenance of cellular auxin homeostasis (Sravankumar et al., 2018), two B3 domain transcription factors, *FUS3-like* (Solyc02g094460) and *ABI3* (Solyc06g083600 master regulators of seed development (Aziz *et al.*, 2020), two important tomato fruit growth regulators, *SlGRF6* (Solyc02g092070) and *SlGRF10* (Solyc01g091540) (Cao *et al.*, 2016), four members of the late embryogenesis abundant protein family, *SlLEA14* (Solyc07g053360), *SlLEA18* (Solyc09g014750), *SlLEA20* (Solyc09g075210) and *SlLEA17* (Solyc09g008770), an important gene family of proteins that accumulates in response to cellular dehydration that may play a role in tomato development and ripening (Cao and Li, 2015), and finally several proteins involved in cell wall modifications such as expansins *SlEXPB5* (Solyc07g049540), *SlEXPA7* (Solyc03g115300) and *SlEXPA20* (Solyc03g115310), *SldeCWIN1* (Solyc03g121680), one cellulose synthase family protein (Solyc10g038020), two extensins (Solyc04g071070 and Solyc01g006390), a xyloglucan endotransglucosylase/hydrolase (*SlXTH*, Solyc05g005680) and a polygalacturonase QRT3-like protein (Solyc02g068410) (S. Table 4).

Furthermore, the libraries generated in this analysis were used to search for transcript levels of the same genes of the sterol biosynthetic pathway previously analyzed in the RNA-seq expression analysis from leaves (see section 3.1.5). This analysis showed that again the expression of none gene involved in the sterol biosynthetic pathway in 9DAA tomato fruits was affected by the silencing of *SlSGT1.*

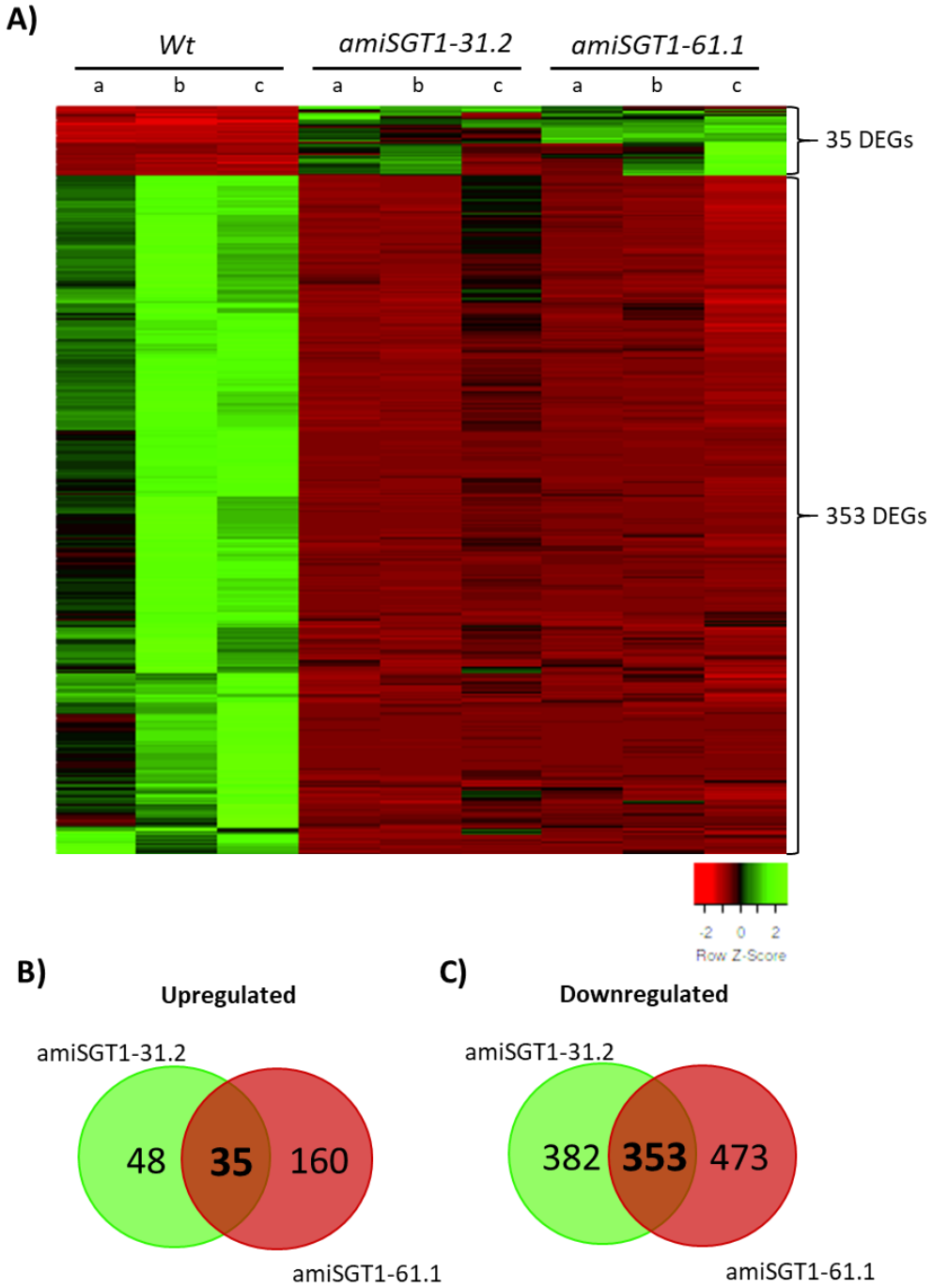

Figure 17. DEGs identified in the amiSGT1 silencing lines. (A) Heatmap representing the normalized expression levels (Z-score) of the identified DEG in the three replicates from 9 DAA fruits from wt, amiSGT1-31.2 and amiSGT1-61.1, and Venn diagrams showing the number of (B) upregulated and (C) downregulated DEGs unique and common between both silenced lines compared with the wt.

From the list of common DEGs between fruits of amiSGT1-31.2 and amiSGT161.1 lines, we selected 10 genes (3 upregulated and 7 downregulated), which considering their biological function might have a role in fruit development (Table 3), and used them to validate the RNA-seq expression data. For this, the transcript levels of these genes were determined using RT-qPCR not only in 9 DAA tomato fruits, but also in fruits harvested at 4 and 15 DAA, to get a time-course overview of their expression during the early stages of tomato fruit growth.

Table 3. Genes selected for RNA-seq validation.

Gene	up/down-regulated	Fold-change 31.2	Fold-change 61.1	Gene ontology
Oleosin (Solyc02g086490)	down	**-6.0**	**-5.4**	Seed oil body biogenesis, lipid storage, seed development, and fruit development.
Oleosin (Solyc03g112440)	down	**-4.0**	**-4.3**	
B3 domain transcription factor (Solyc02g094460)	down	**-5.0**	**-4.1**	Fruit development, and hormone metabolic process.
Expansin (Solyc03g115300)	down	**-4.6**	**-3.2**	Cell wall organization and biogenesis.
Extensin (Solyc04g071070)	up	**2.6**	**2.0**	Cell wall organization.
Xyloglucan endotransglucosylase/ hydrolase 2 (Solyc05g005680)	up	**2.3**	**2.7**	Cell wall organization and biogenesis (GO:0070882)
Pectinesterase (Solyc07g064190)	down	**-4.3**	**-2.7**	Cell wall organization and biogenesis (GO:0070882)
Pentatricopeptide repeat-containing protein family (Solyc08g062580)	up	**10.0**	**10.8**	Protein binding (GO:0045308)
11S Globulin CRU4 (Solyc09g025210)	down	**-13.1**	**-9.6**	Nutrient reservoir activity (GO:0045735)
7S globulin (Solyc09g065470)	down	**-12.2**	**-8.1**	Nutrient reservoir activity (GO:0045735)

The results of the RT-qPCR expression analysis of the ten selected genes demonstrated that the observed changes in their expression in response to the silencing of *SlSGT1* were not restricted to the specific time point (9 DAA) at which the RNA-seq analysis was performed. As can be observed in Figure 18, all these genes,

with the only exception of pectinesterase (Solyc07g064190) (Figure 18G), displayed similar altered expression profiles to those observed at 9 DAA at the earlier (4 DAA) and later (15 DAA) time points examined. Overall, the results of this analysis not only validated the RNA-seq results, but also reinforced the view that changes in the expression levels of these genes are actually related to the observed fruit phenotype, thus representing a first approach to elucidate the underlying molecular mechanisms behind the reduced fruit size induced by *SlSGT1* silencing.

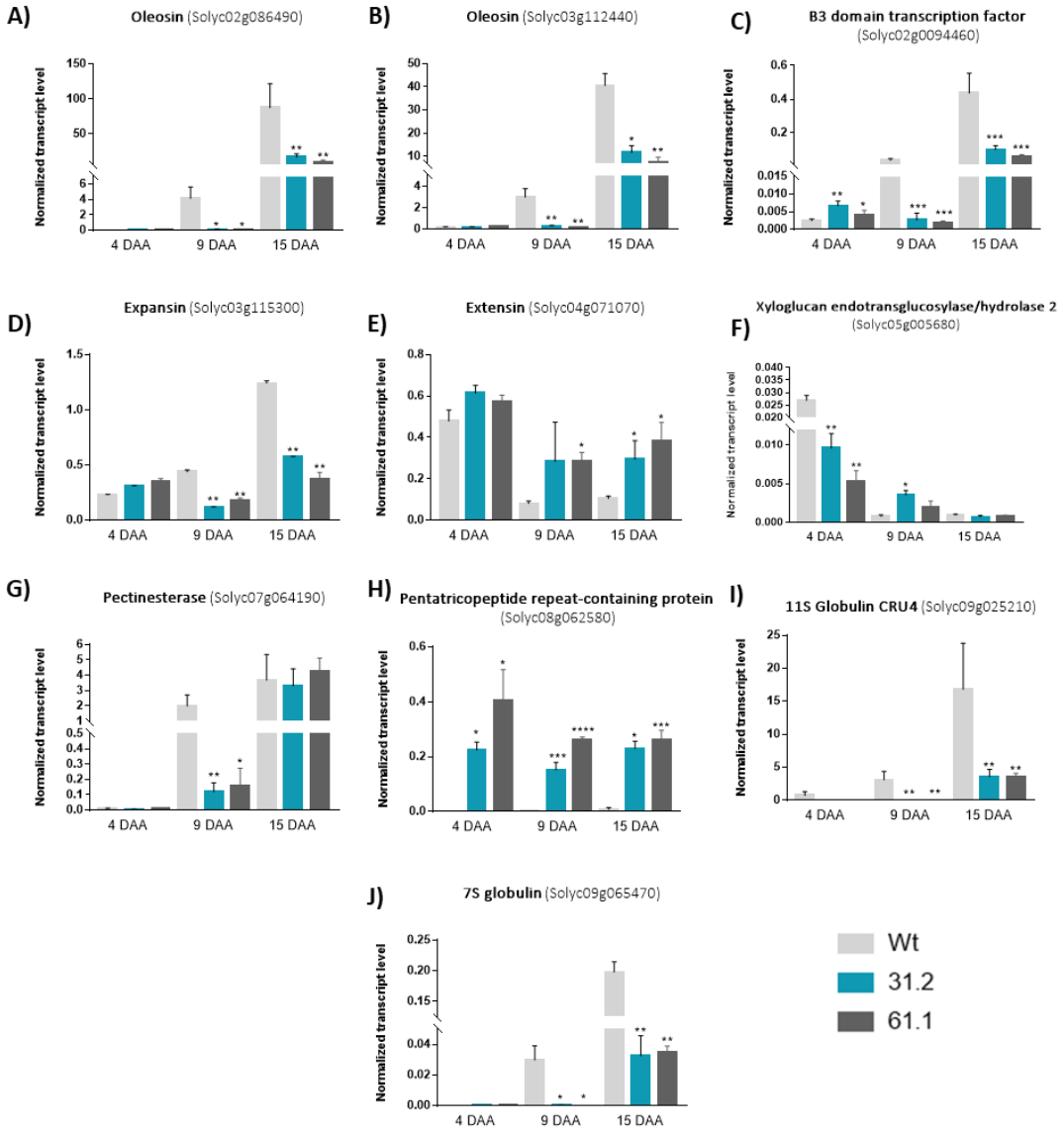

Figure 18. **Time-course expression analysis of selected genes in green fruits at early stages of growth for RNA-seq data validation.** The transcript levels of (**A**) oleosin (Solyc02g086490), (**B**) B3 domain transcript factor (Solyc02g0094460), (**C**) oleosin (Solyc03g112440), (**D**) expansin (Solyc03g115300), (**E**) extensin (Solyc04g071070), (**F**) xyloglucan endotransglucosylase/hydrolase 2 (**G**), pectinesterase (Solyc07g064190), (**H**) pentatricopeptide repeat-containing protein (Solyc08g062580), (**I**)11S Globulin CRU4 (Solyc09g025210) and (**J**) 7S globulin (Solyc09g065470) were determined by RT-qPCR using RNA extracted from green tomato fruits at 4 DAA, 9DAA and 15 DAA. Values are expressed as normalized quantity using tomato CAC (Solyc08g006960) or TIP41 (Solyc10g049850) genes as housekeeping reference genes. Data are expressed as mean ± SEM of values from three biological replicates consisting of 10 fruits from 10 tomato plants with three technical replicates each (n=9). Significant differences between fruits of silenced lines and wt are calculated by t-test and shown by asterisks (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001).

3.1.9 Reduced levels of SGs in tomato leads to reduced tolerance against low temperatures.

Tomato and other plant species which originated from tropical and subtropical regions, are chilling-sensitive plants and its production can be severely impaired by low temperatures (Barrero-Gil *et al.*, 2016; Ré *et al.*, 2017). Thus, it is particularly important to understand the different processes by which tomato plants adapt to low temperatures including those that take place at the plasma membrane since membrane stabilization against freezing damage is crucial in cold acclimatation (Thomashow, 1999). To gain insight into the contribution of glycosylated sterols in plasma membrane integrity at low temperatures, we measured the ion leakage of 12-day old wt and *SlSGT1* silenced tomato seedlings exposed to 0 °C for 3 days. Under normal conditions, the ion leakage of tomato seedlings was not significantly different among wt, amiSlSGT1-31.2 and amiSlSGT1-61.1 plants. After chilling stress, the ion leakage increased on both wt and mutant plants (Figure 19), but this increase was significantly less severe in wt seedlings than in the silenced ones. Thus, while in wt the obtained values were about 22 %, these increased to approximately 27% and 33 % in the amiSlSGT1-31.2 and amiSlSGT1-61.1 plants, respectively (Figure 19).

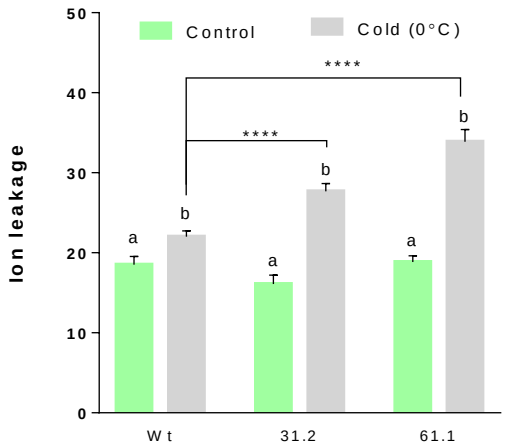

Figure 19. Ion leakage of tomato seedlings after cold stress. Wt, amiSlSGT1-31.2 and amiSlSGT1-61.1 12-day old seedlings wronging *in vitro* were exposed at 0 °C during 3 days. Data are expressed as mean % ion leakage ± SEM from thirty biological replicates (n=30). Letters (a or b) above the SEM bar represented statistical differences between the same genotype exposes or not to cold, while asterisks represent significant differences between wt and silencing lines after cold treatment. In both cases statistical differences were determined by t-test (****P ≤ 0.0001).

Furthermore, we complemented these results with and *in vivo* chilling stress assay. In this case the effect of cold treatment, in acclimated (gradual drop of temperature until chilling temperature) and non-acclimates plants (directly exposed to chilling temperature), was quantified determining the survival percentage after exposition of plants at 4º C during three days followed by four days of recovery at the initial growing conditions (see Material and Methods 7.14.2). For this, the survival rate was classified into three categories according with the damage observed in leaves: (1) dead plants; (2) survivor 1, plants in apparent good condition; and (3) survivor 2, plants with obvious foliar damage (Figure 20A). In the non-acclimated plants, the survival rate of lines amiSlSGT1-31.2 and amiSlSGT1-61.1 was about 80% and 85%, respectively, while all the wt plants survived. Moreover, the survival plants from the silenced lines presented more foliar damage (characterized as survivor 2) than the wt plants (Figure 20B). Regarding acclimated plants, the survival rate was similar in wt and the two *SlSGT1* silencing lines (between 85% and 95%), but the survivor plants looked healthier (characterized as survivor 1) in the wt than in the silenced lines (Figure 20C).

Altogether, these results indicated that amiSGT1 plants are less tolerant to cold stress than wt plants, a phenotype that might be the result of reduced PM integrity, likely as a consequence of their low levels of SG, when these plants are exposed to low temperatures compared to wt plants.

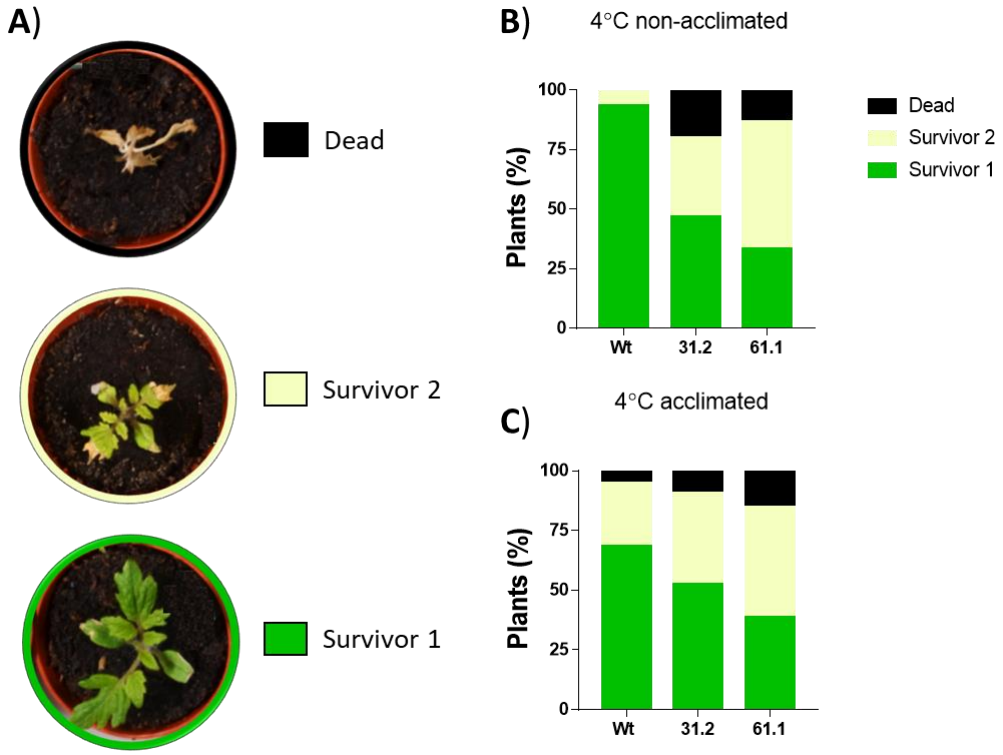

Figure 20. In vivo cold stress response of tomato plants. (**A**) Representative images showing single 19-day plants of each cold-phenotype category: dead (black), survivor 1 (dark green), and survivor 2 (yellow). Survival rates of (**B**) non acclimated and (**C**) acclimated wt, amiSGT1-31.2 and amiSGT1-61.1 plants exposed to 4 °C for 3 days and recovered during 4 days. Data are expressed as percentage of plants classified into each category (n=20).

3.1.10 Reduced levels of SGs in tomato leaves lead to increased tolerance against Botrytis cinerea infection.

The current knowledge about the specific role of glycosylated sterols in plant biotic stress response is scarce, particularly in agronomical-interesting crops, like tomato. To gain some insight about the contribution of these compounds to the plant response against this kind of stress, we evaluated the disease symptoms produced by *B. cinerea*, a common necrotrophic fungal pathogen, in leaves of wt tomato plants and the two *SlSGT1* silenced lines (amiSlSGT1-31.2 and amiSlSGT1-61.1. To this end, the third and fourth leaves of wt and *SlSGT1* silenced plants were inoculated with a *B. cinerea* spore suspension, and the size of the resulting lesions was measured 72h after inoculation. The results of three independent experiments showed a significant reduction (around 39-42%) on the average lesion diameter in leaves of amiSGT1-31.2 and amiSGT1-61.1 plants when compared with the lesion size in wt leaves (Figure

21A-B). These results suggest that the silencing of *SlSGT1* and the concomitant reduction in the levels of SGs results in increased resistance of tomato against *B. cinerea*. These data were further confirmed by the lower amount of genomic fungal DNA, measured as the amount of fungal β-tubulin, detected in infected leaves of the silenced *SlSGT1* plants compared with the wt (72-77% reduction) (Figure 21C), which indicated that *Botrytis* growth in the leaves of amiSGT1 plants is clearly impaired compared to the wt leaves.

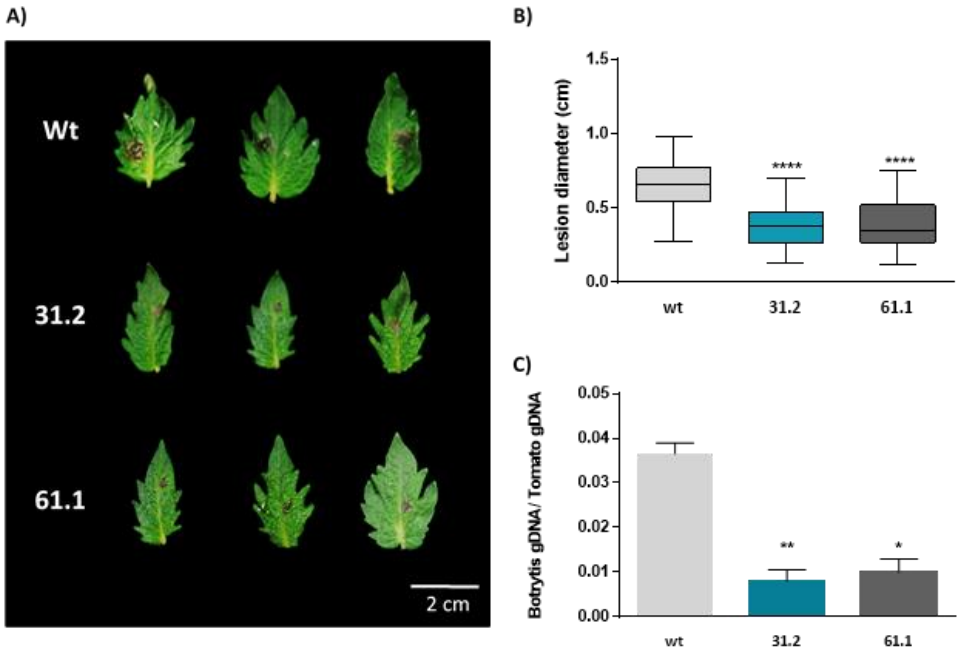

Figure 21. Infection of tomato leaves from wt and SlSGT1 silenced plants with *Botrytis cinerea.* (**A**) Representative images showing lesions in wt, amiSGT1-31.2 and amiSGT1-61.1 tomato leaves 72h post infection**.** (**B**) Average lesion diameter. Data are presented as mean values ± min and max values of 3 independent experiments. (**C**) Relative quantification of *B. cinerea* genomic DNA in leaves at 72h post infection. The ratio *B. cinereal* gDNA/tomato gDNA was calculated from the quantification by qPCR of the *B. cinerea* β-tubulin gene and the tomato actin gene. Asterisks represented significant differences determined by t-test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001).

3.2 CHAPTER II

3.2.1 Obtention of transgenic tomato lines.

Previous results published by our research group showed that *SlSGT4* is barely expressed in different tomato organs (leaves, roots, flowers) and fruit developmental stages, but its expression is remarkably induced when seedlings are exposed to different inducers or stress conditions, suggesting a possible role for this gene in the plant response to stress (Ramirez-Estrada *et al.*, 2017).

In order to start to elucidate the contribution of *SlSGT4* in plant growth and development, and stress response, we generated transgenic tomato lines constitutively overexpressing *SlSGT4*. To this end, the construct containing the *SlSGT4* coding region under the control of the CaMV35S promoter and the gene conferring resistance to kanamycin (*NTPII*) as a marker (*35S:SlSGT4:Tnos*) (Figure 22A), was introduced into tomato plants by *Agrobacterium tumefaciens* mediated transformation of cotyledons. Transgenic plants were selected by resistance to kanamycin and 67 plants were obtained. After 2 weeks of acclimatization in the greenhouse, the presence of the transgene was checked by PCR using two different sets of primers designed to confirm the integrity of the whole transgene. In one of them, the forward and reverse primers correspond to internal sequences on the *CaMV35S* promoter and the *SlSGT4* cDNA, respectively, and should amplify a DNA fragment of 1568 bp (band A, Figure 22B). The second set of primers correspond to internal sequences on the *SlSGT4* cDNA (forward) and the *NOS* terminator (reverse), and a 777 bp amplicon was expected (band B, Figure 22B). From a total of 38 analyzed plants, 30 were PCR-positives and showed amplicons of the expected size using both set of primers (Figure 22A), which demonstrated the presence of the complete *35S:SlSGT4:Tnos* construct in the genome of these plants. Using genomic DNA from those confirmed transgenic lines, the number of copies of *NTPII* gene present in the T-DNA carrying the *35S:SlSGT4:Tnos* transgene was determined by PCR, using the tomato *LAT52* (Solyc10g007270) as endogenous single copy gene. The transgene copy number in each sample was calculated using the equation: *Copy number = 2* $^{(\Delta\Delta Ct)}$. Where, ΔΔCt = ΔCt (reference gene [*LAT52*]) - ΔCt (target gene [*NTPII*]). Values close to 0.5 indicate a single insertion of the transgene. The results of this analysis showed that 12 of the 30 T0 transgenic lines (1, 4, 11, 16, 18, 20, 21, 24, 25, 28, 35 and 37) contained a single transgene copy per genome (Table 4). In all these transgenic lines the transcript levels of *SlSGT4* were higher than in wt plants, albeit overexpression levels were highly variable, with the exception of lines 24 and 28 that

showed transcript levels similar to those of wt (Figure 23). Only 6 of these lines (1, 11, 16, 20, 21, and 35) produced seeds, and were propagated to the next generation (10-15 plants/ line). In order to identify homozygous lines in the T1 generation, the copy number of the transgene was determined by qPCR as described above, but in this case a value of $2^{(\Delta\Delta Ct)}$ close or equal to 1 indicated two copies of the transgene (homozygous). After this analysis, three independent homozygous lines with different *SlSGT4* expression levels were obtained and used for further characterization: 1.8, 11.7 and 35.7 (Figure 23)

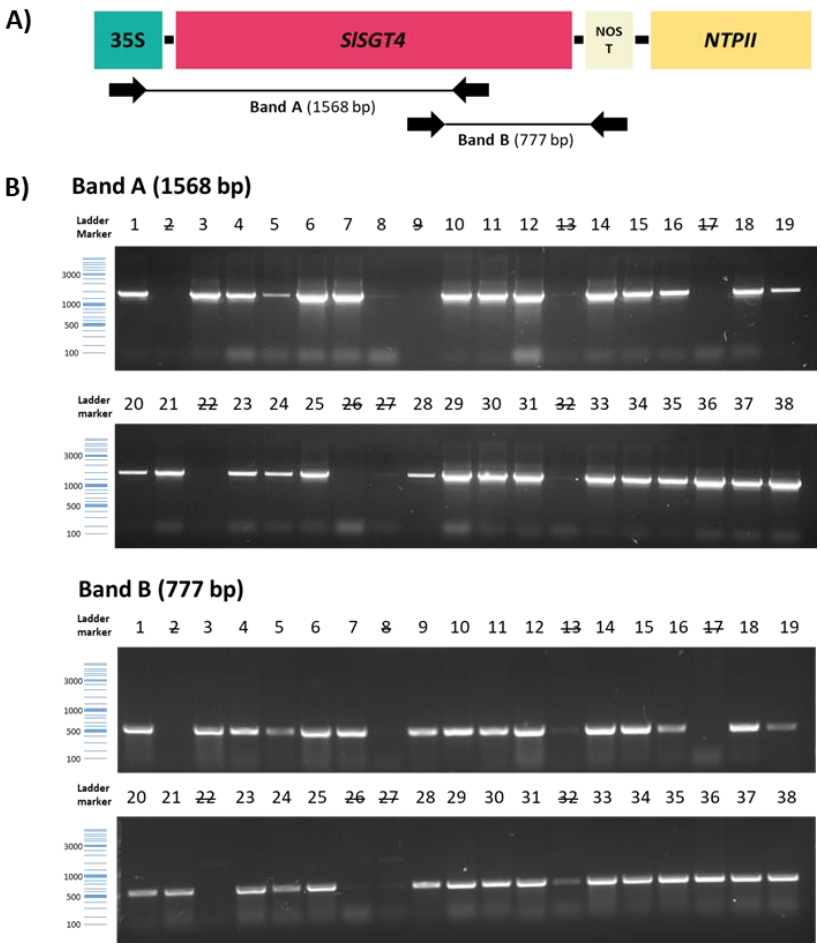

Figure 22. Identification of 35S:SlSGT4:Tnos transgene insertion in transformed tomato plants. (**A**) Schematic representation of the T-DNA in the binary vector used for tomato transformation in order to overexpress constitutively *SlSGT4 using the constitutive promoter CaMV35S (35S), Nos terminator (NOS-T),* and the marker gene for resistance to kanamycin *(NTPII).* Arrows show the position and orientation of the primers used for transgenic lines genotyping, (**B**) PCR genotyping of the transgenic lines harboring *35S:SlSGT4:Tnos*. The presence of the transgene was demonstrated by amplification of fragments of 1568 bp (35S promoter and *SlSGT4* cDNA) and 777 bp (*SlSGT4* cDNA and *nos* terminator) using the primer pairs showed in **A**.

Table 4. Copy number of the *35S:SlSGT4:Tnos* transgene in primary transformants (T0). Selected lines used in this work are shaded. N/A=not analyzed.

Plant ID	$2^{(\Delta\Delta Ct)}$	Number of copies	Seeds
1	0.452	1	15
3	0.968	Multiple	4
4	0.274	1	-
6	2.585	Multiple	6
7	1.778	Multiple	-
10	1.759	Multiple	-
11	0.543	1	100
12	0.797	Multiple	50
14	0.857	Multiple	4
15	1.084	Multiple	4
16	0.280	1	15
18	0.596	1	-
20	0.352	1	5
21	0.138	1	15
23	1.149	Multiple	40
24	0.139	1	-
25	0.423	1	-
28	0.586	1	-
29	1.200	Multiple	-
30	1.206	Multiple	-
31	1.431	Multiple	-
33	3.588	Multiple	-
34	1.035	Multiple	60
35	0.602	1	25
36	1.662	Multiple	-
37	0.261	1	-
38	1.360	Multiple	9
wt	0	0	N/A

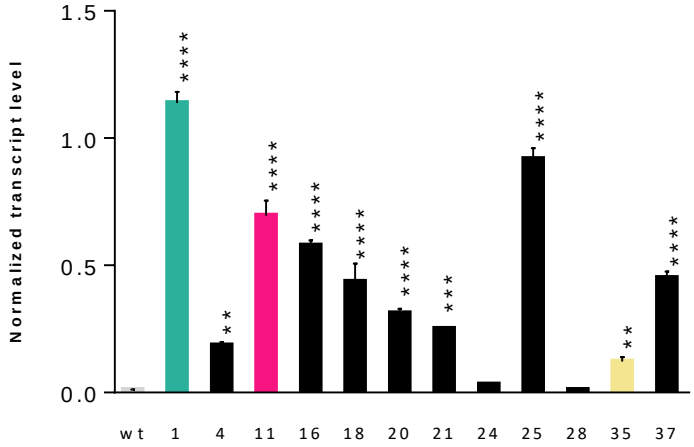

Figure 23. Expression levels of SlSGT4 in T0 single transgene copy lines. Transcript levels of *SlSGT4* in leaves of wt and overexpressing *SlSGT4* plants were determined by RT-qPCR using RNA extracted from leaves of one-month-old plants. Data given for wt and mutant lines are expressed as normalized quantity using the actin gene (Solyc03g078400) as a housekeeping. Values are mean ± SEM from three technical replicates per genotype (n=3). Data were analyzed with t-test (α=0.05). Asterisks show the values that are significantly different compared to the wt (**P ≤ 0.01; ***P ≤ 0.005; ****P ≤ 0.001). Colored bars correspond to selected lines: 1.8 green, 11.7 pink and 37.5 yellow.

3.2.2 Expression analyses of SlSGT4 and other SGT genes in leaves of transgenic 35S::SlSGT4 tomato plants.

The expression of *SlSGT4* was quantified by RT-qPCR in the three selected *35S::SlSGT4* homozygous lines using total RNA extracted from the third and fourth leaves of 1-month-old tomato plants. As expected, the expression of *SlSGT4* was significantly higher in the three transgenic lines than in wt plants (Figure 24). The highest transcript levels were detected in lines 1.8 and 11.7, which presented increases of 136-fold and 77-fold, respectively, compared to the wt, while in line 35.7 the increase was around 13-fold. It is worth to mention that, as reported previously by the research group (Ramírez-Estrada et al. 2017), the expression of *SlSGT4* in wt plants was barely detectable.

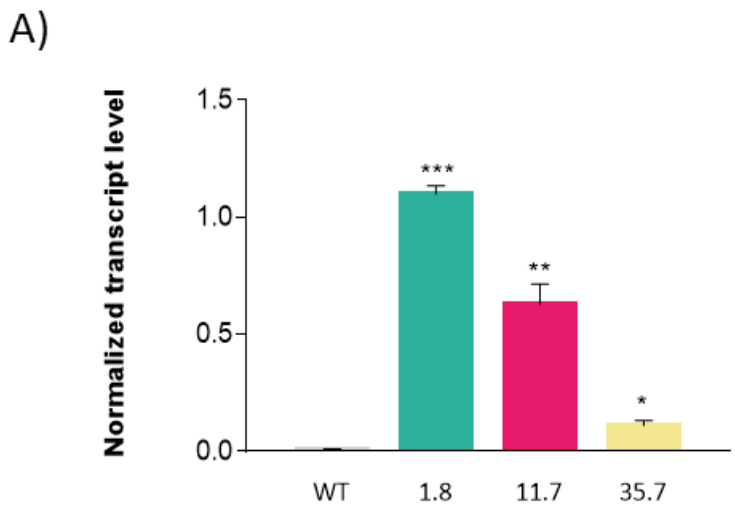

Figure 24. **Expression levels of *SlSGT4* in T1 homozygous single transgene copy lines.** Expression levels of *SlSGT4* in the transgenic and wt plants were determined by RT-qPCR using RNA extracted from leaves of one-month-old plants. Data given for wt and mutant lines are expressed as normalized mRNA quantity using the actin gene (Solyc03g078400) as a housekeeping gene. Values are mean ±SEM from three biological replicates per genotype with three technical replicates each (n=9). Asterisks represent significant differences compared to wt determined by t-test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.005).

In order to investigate if the overexpression of *SlSGT4* resulted in an altered expression of the other tomato *SGT* genes, the transcript levels of the remaining genes of the SGT family (*SlSGT1*, *SlSGT2* and *SlSGT3*) were determined by RT-qPCR using the same RNA samples than for *SlSGT4* transcript quantification.

As shown in Figure 25A, the expression of *SlSGT1* was significantly lower in the three transgenic lines than in wt plants. Concretely, the transcript levels of *SlSGT1* decreased around 36% in transgenic lines 1.8 and 11.7, and about 29% in line 35.7, compared to the wt. Thus, *SlSGT1* downregulation seems to be a consequence of *SlSGT4* overexpression, and not to be due to an insertional effect of the transgene, since it was observed in the three transgenic lines. The expression of the others *SGT* genes (*SlSGT2* and *SlSGT3*), was not significantly affected in any of the transgenic lines compared to wt (Figure 25B-C).

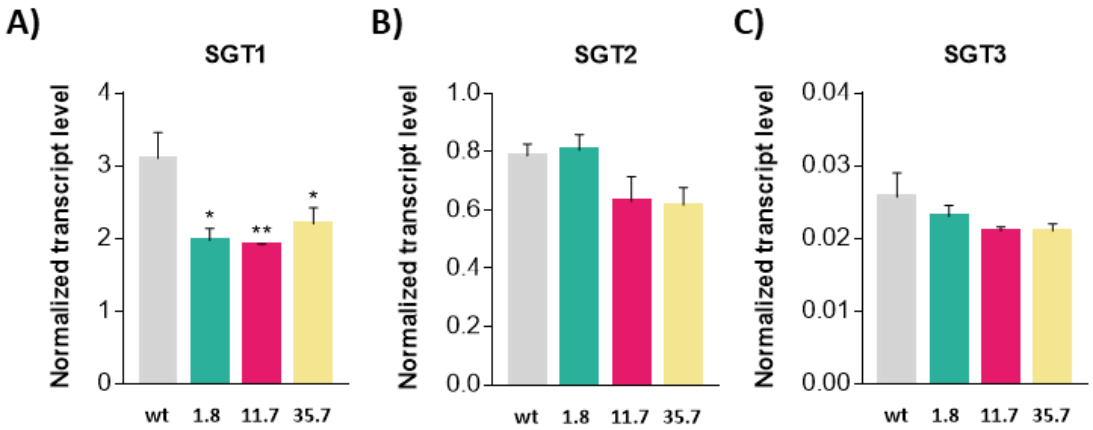

Figure 25. **Expression levels of *SlSGT1-3* in transgenic tomato lines overexpressing *SlSGT4*.** Expression levels of *SlSGT1* (**A**), *SlSGT2* (**B**) and *SlSGT3* (**C**) in the transgenic and wt plants are given as normalized mRNA quantity using the actin gene (Solyc03g078400) as a housekeeping. Values are mean ± SEM from three biological replicates per genotype with three technical replicates each (n=9). Asterisks represent significant differences compared to wt determined by t-test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.005).

3.2.3 Sterol profile in leaves of transgenic lines overexpressing SlSGT4.

In order to investigate if the overexpression of *SlSGT4* results in changes of the sterol profile in tomato leaves, the levels of the different sterol fractions were quantified in extracts obtained from the third and fourth leaves of 1-month-old wt and *SlSGT4* overexpressing plants (1.8, 11.7 and 35.7 lines). Surprisingly, the overexpression of *SlSGT4* did not result in an increase of total glycosylated sterols (SG + ASG). On the contrary, the content of glycosylated sterols in transgenic lines 1.8, 11.7 and 35.7 was 20%, 32% and 40% lower than in wt plants, respectively (Figure 26C and S. Table 8). This effect was mainly due to a decrease in the SGs content, which was around 16%, 50% and 55% lower in lines 1.8, 11.7 and 35.7, respectively, compared to the SGs amounts in the wt plants. As shown in Figure 27A and S. Table 9, the four major sterol species in the SG fraction were reduced at different extent among the *SlSGT4* overexpressing lines, being 11.7 and 35.7 the most affected ones. The levels of stigmasterol and β-sitosterol, the main components of the cell membrane, decrease markedly in these two lines (around 50%-60%) compared to wt plants, and a slighter decrease was observed in line 1.8 (10% stigmasterol and 16% β-sitosterol). However, the levels of campesterol, the precursor of brassinosteroids, were similar in wt and *SlSGT4* overexpressing plants. Finally, the cholesterol content was lower in lines 11.7 and 35.7 than in wt, but remained unchanged in line 1.8.

The total content of ASGs also decreased in the three transgenic lines compared to wt plants, although at lower extent (about 20%) than that of SGs (Figure 26B). In this fraction the low levels of sterols observed in the transgenic lines were mainly due to a significant reduction of campesterol (26%-31% lower than in wt) and stigmasterol (between 25-30%) contents, since the levels of cholesterol and β-sitosterol were similar in wt and transgenic lines (Figure 27B).

Interestingly, overexpression of *SlSGT4* also induces a decrease of the FS levels in the transgenic lines 11.7 and 35.7 (50% approximately compared to wt) (Figure 26D). These alterations were the result of a marked reduction on the content of stigmasterol (around 50%-60%) and β-sitosterol (about 30%-40%) in both lines (Figure 27C). Moreover, free campesterol was not detected neither in line 11.7 nor in line 35.7. However, the content of FS in the overexpressing line 1.8 was similar to that of wt plants. Regarding the total amounts of SEs, no significant differences were observed between any of the three transgenic lines (1.8, 11.7 and 35.7) and the wt plants.

Overall, the decrease in glycosylated and free sterol detected in the *SlSGT4* overexpressing lines resulted in a diminution of the total sterol amount in these lines compared to the wt (Figure 26E). As observed in the different sterol fractions, the reduction in the total sterol content was smaller in line 1.8 (17%) than in lines 11.7 and 35.7 (31% and 36%, respectively).

The reduction of glycosylated sterol levels observed in the transgenic lines could be attributed to the downregulation of *SlSGT1* (Figure 25) induced by the overexpression of *SlSGT4*. Among the genes of the tomato SGT family, *SlSGT1* is the more highly expressed in leaves and other plant organs (see Chapter I). However, since the FS levels were also significantly reduced in the transgenic lines, the possibility that sterol biosynthesis might be regulated at some earlier step of the biosynthetic pathway cannot be discarded.

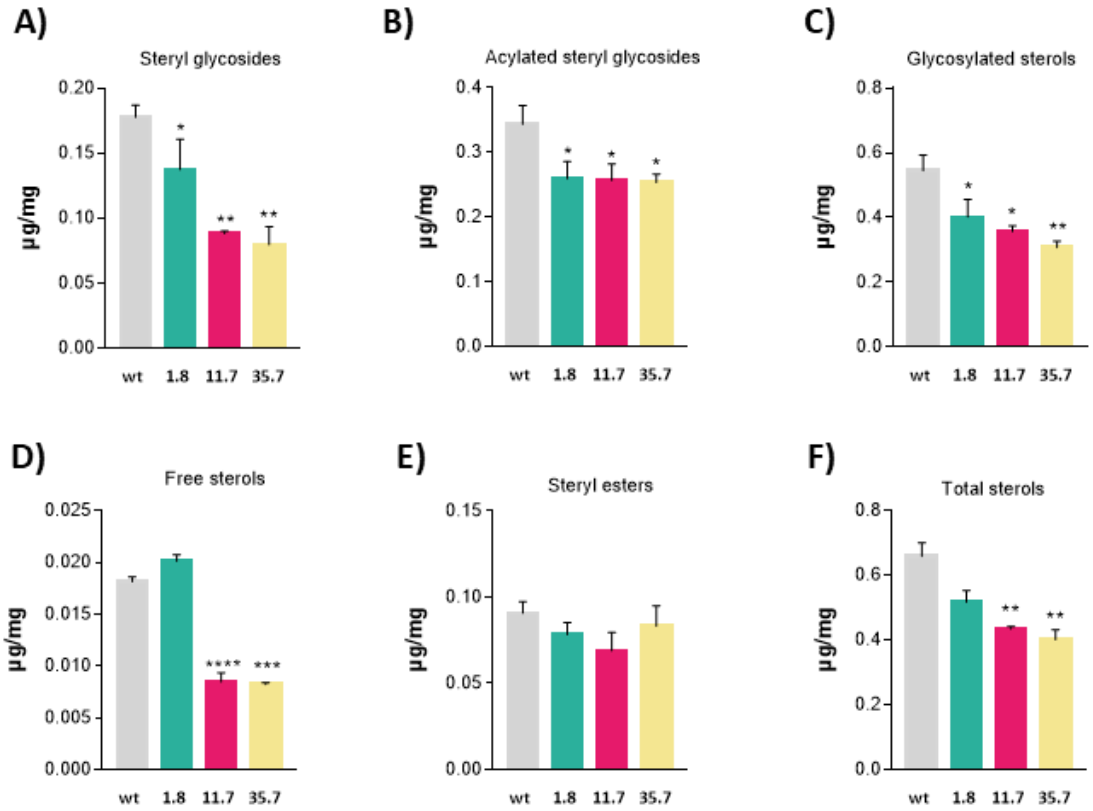

Figure 26. Content of free and conjugated sterol in leaves of tomato wt and *SlSGT4* overexpressing lines. (**A**) Total steryl glucosides, (**B**) acylated steryl glucosides, (**C**) glycosylated sterols (SG + ASG), (**D**) free sterols, (**E**) steryl esters, and (**F**) total sterols (four fractions) in wt and silenced lines amiSGT1-31.2 and amiSGT1-61.1. Total content in each fraction includes cholesterol, campesterol, stigmasterol, and β-sitosterol species. Data are expressed as mean µg sterol per mg of dry weight ± SEM from three biological replicates with three technical replicates each (n=9). Asterisks represent significant differences compared to wt determined by t-test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.005).

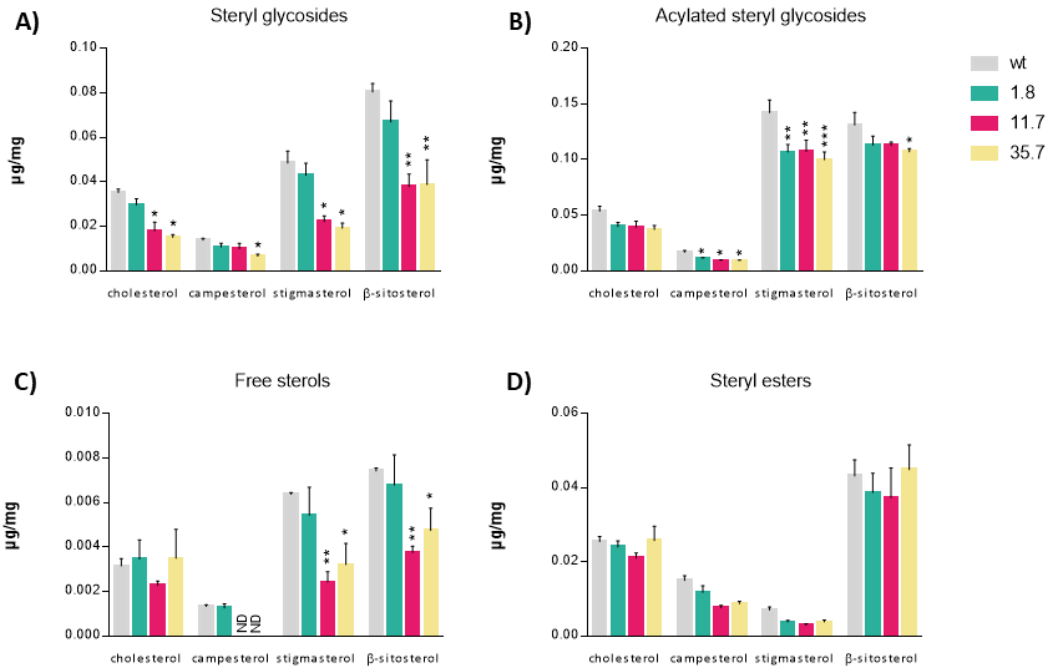

Figure 27. **Content of the main sterol species in leaves of tomato wt and *Sl*SGT4 overexpressing lines.** The total sterol content in the different fractions [(**A**)steryl glycosides, (**B**) acylated steryl glycosides, (**C**) free sterols and (**D**) steryl esters] includes the main sterols: cholesterol, campesterol, stigmasterol and β-sitosterol. Data are expressed as mean µg sterol per mg of dry weight ± SEM from three biological replicates with three technical replicates (n=9). Asterisks represent significant differences compared to the wt determined by t-test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.005; 15 ****P ≤ 0.001).

3.2.4 Phenotype characterization of transgenic lines overexpressing SlSGT4.

As a first approach to explore the role of *SlSGT4* in tomato plant and fruit development, we checked if the overexpression of *SlSGT4* results in some visible phenotype changes in different plant organs and developmental stages.

3.2.4.1 Effect of SlSGT4 overexpression on tomato vegetative development.

At the early stages of vegetative development (one-month-old plants) the height of transgenic lines 11.7 and 35.7 was significantly lower than that of wt plants. Concretely, the plant size was reduced approximately 10% and 14%, respectively, in both overexpressing lines compared to the wt. However, the height of plants from line 1.8, which presents the highest *SlSGT4* expression, was significantly higher than that of wt plants (about 20%) (Figure 29). Altogether, the sterol profile and plant phenotype of lines 11.7 and 35.7 match with the results reported in Chapter I, where we proved the negative impact of a reduction in the levels of SGs to plant size.

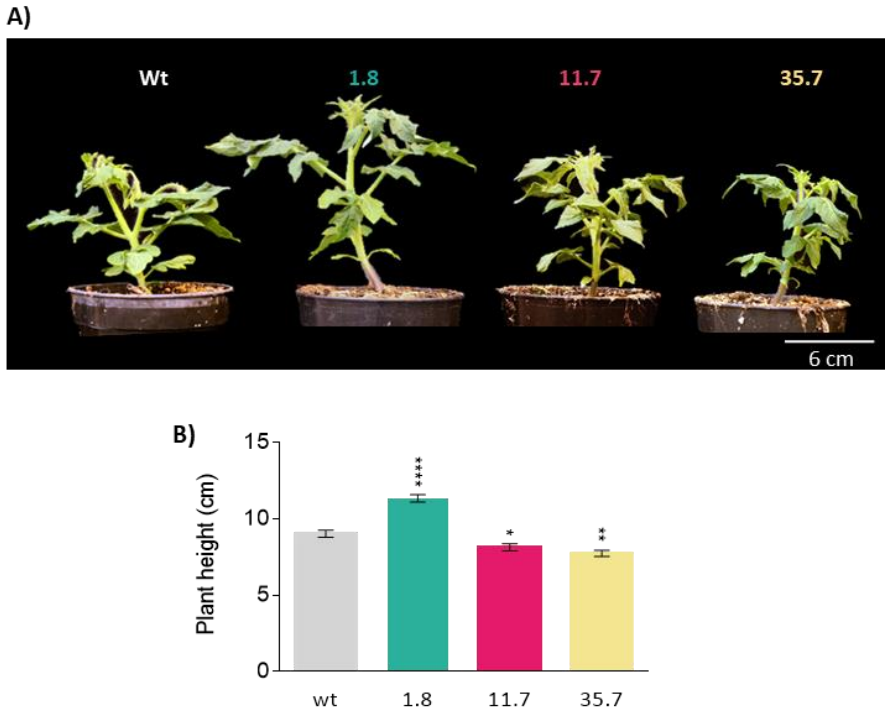

Figure 28. Phenotype of plants overexpressing SlSGT4. (**A**) Representative images of one-month old wt and transgenic plants overexpressing *SlSGT4*. (**B**) Quantitative analysis of plant height. Data are the mean ± SEM from 30 plants per genotype (n=30). Asterisks represent significant differences between transgenic lines and wt determined by t-test (*P ≤ 0.05; **P ≤ 0.01; ****P ≤ 0.0001).

3.2.4.2 Transcript profile of tomato SGT genes in fruits of transgenic 35S::SlSGT4 plants.

The expression degree of *SlSGT4* was also analyzed by RT-qPCR in fruits obtained from the three selected transgenic lines and wt plants at different stages of growth and ripening, including green, mature green, breaker + 1 day and red. As reported previously (Ramirez-Estrada et al. 2017), and can be observed in Figure 13, the expression of *SlSGT4* was barely detectable at any stage of wt tomato fruit development. Nevertheless, as expected, the transcript levels of *SlSGT4* increased on the three transgenic lines, although at different degrees depending on the line and the fruit developmental stage (Figure 29). Thus, the highest differences of *SlSGT4* expression between wt and transgenic lines 1.8, 11.7 and 35.7 were observed at the mature green stage of fruit development. At this stage the transcript levels in the transgenic lines were around 29-fold (line 1.8), 21-fold (line 11.7) and 5-fold (line 35.7) higher than in wt fruits, while these differences were only about 7-fold (line 1.8), 4-fold (line 11.7) and 2-fold (line 35.7) at the breaker stage, which is the stage where the lower overexpression levels were observed.

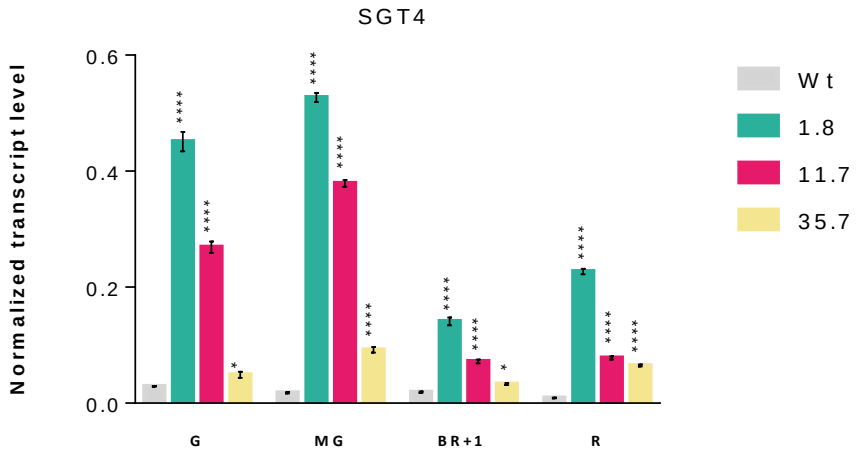

Figure 29. **Overexpression of SlSGT4 in fruits at different developmental and ripening stages**. The transcript levels of *SlSGT4* from wt and overexpressing lines 1.8, 11.7 and 35.7 was determined by RT-qPCR using RNA extracted from green (G,15 DAA), mature green (MG, 30 DAA), breaker +1 day (BR+1, 32 DAA) and red (R, 36 DAA) tomato fruits. Values are expressed as normalized mRNA quantity using a tomato actin gene (Solyc03g078400) as housekeeping. Data are expressed as mean ± SEM from a group consisting of the pericarp from 15 tomato fruits with three technical replicates (n=3). Asterisks represent significant differences between transgenic lines compared to wt determined by t-test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; **** P ≤ 0.0010).

Among the four genes of the tomato SGT family, *SlSGT1* is the most highly expressed in fruits, mainly at the earlier stages of development, but its expression decrease along maturation (Ramirez-Estrada *et al.*, 2017). Moreover, as described in Chapter I, *SlSGT1* plays an important role during fruit development and, as described above, the expression of *SlSGT1* decreased in leaves of the transgenic lines overexpressing *SlSGT4*. Considering all these observations, we decided to also analyze the expression of *SlSGT1* at the different stages of fruit development. Interestingly, the results of this analysis showed that *SlSGT1* mRNA levels in lines 11.7 and 35.3 were significantly higher than in wt during the earlier stages of fruit development. Thus, the expression of *SlSGT1* in fruits from line 11.7 was 16% (green) and 32 % (mature green) higher than in fruits from wt plants at same developmental stages. Similar results were obtained in fruits from line 35.7, where the expression of *SlSGT1* was 16% and 55%, higher in green and mature green, respectively, compared to the wt (Figure 30). Surprisingly, the transcript levels of *SlSGT1* in green and mature green fruits from line 1.8, the one with the higher expression of *SlSGT4*, were depleted markedly (30% and 63%, respectively) as compared to wt fruits at the same developmental stages (Figure 30). As shown in Figure 30, not significant changes in the transcript levels of *SlSGT1* were observed between any of the three transgenic lines and wt fruits at the ripening stages (breaker and red).

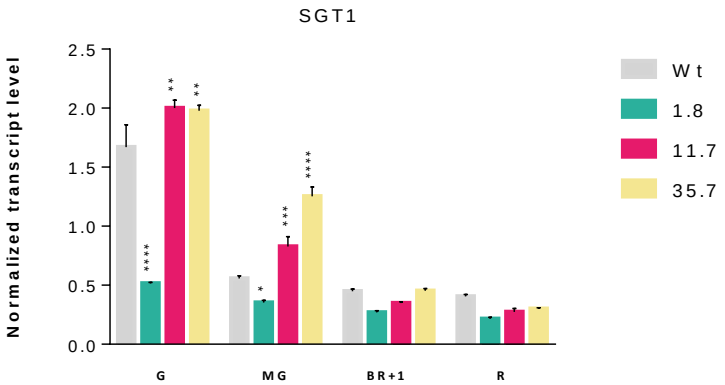

Figure 30. **Effects of the overexpression of *SGT4* in transcript levels of *SlSGT1* in tomato fruits.** The transcript levels of *SlSGT1* from wt and overexpressing lines 1.8, 11.7 and 35.7 was determined by RT-qPCR using RNA extracted from green (G,15 DAA), mature green (MG, 30 DAA), breaker +1 day (BR+1, 32 DAA) and red (R, 36 DAA) tomato fruits. Values are expressed as normalized quantity using a tomato actin gene (Solyc03g078400) as housekeeping. Data are expressed as mean ± SEM from a group consisting of the pericarp from 15 tomato fruits with three technical replicates (n=3). Asterisks represented significant differences between transgenic lines compared to wt determined by t-test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; **** P ≤ 0.0001).

Finally, we analyzed the expression of the remaining two genes of the tomato SGT family (*SlSGT2* and *SlSGT3*). The results showed that the transcript levels of *SlSGT2* were not significantly affected by *SlSGT4* overexpression at the ripening stages (breaker and red) of fruit development, whereas some significant changes of expression were observed in some of the transgenic lines at the earlier stages of development (green and mature green) (Figure 31A). In contrast, the expression of *SlSGT3* was significantly upregulated in almost all the growth and maturation stages of fruits from plants of the three transgenic lines overexpressing *SlSGT4* compared to those of wt plants, but these changes were specially marked in lines 11.7 and 35.7 (Figure 31B).

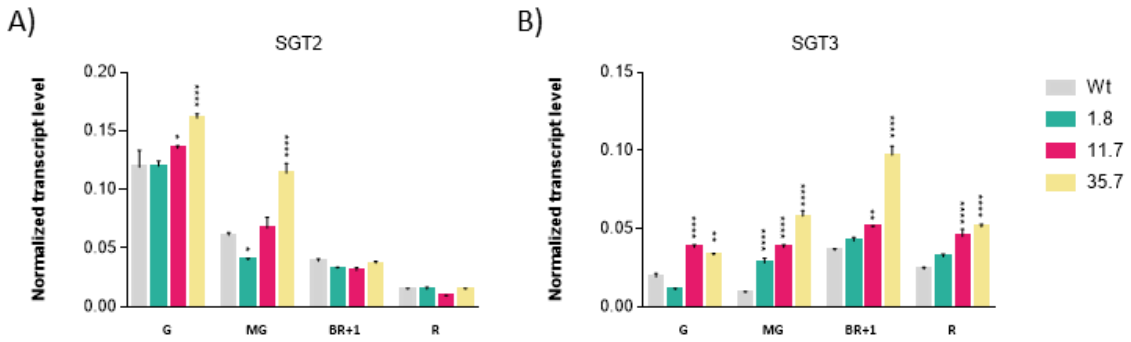

Figure 31. Alteration in the transcript levels of *SlSGT2-3* at different fruit maturation stages of *SlSGT4* overexpression lines. The transcript levels of (**A**) *SlSGT2* and (**B**) *SlSGT3* from wt and overexpressiing lines 1.8, 11.7 and 35.7 was determined by RT-qPCR using RNA extracted from green (G,15 DAA), mature green (MG, 30 DAA), breaker +1 day (BR+1, 32 DAA) and red (R, 36 DAA) tomato fruits. Values are expressed as normalized quantity using a tomato actin gene (Solyc03g078400) as housekeeping. Data are expressed as mean ± SEM from a group consisting of the pericarp from 15 tomato fruits with three technical replicates (n=3). Asterisks represent significant differences between transgenic lines compared to wt determined by t-test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; **** P ≤ 0.001).

Overall, these results showed that in tomato fruits, in contrast to that observed in the vegetative part of the plant (Figure 25, section 3.2.2), the overexpression of *SlSGT4* not only altered the transcript levels of *SlSGT1*, but also those of *SlSGT2* and *SlSGT3* (Figure 31-B).

3.2.4.3 Effect of SlSGT4 overexpression on tomato fruit size.

Fruit development was positively affected in *SlSGT4* overexpressing lines 11.7 and 35.7 (Figure 32A-B). The weight of fruits from these lines was significantly higher (about 30% heavier) than that of wt fruits. However, the weight of fruits from transgenic line 1.8 was similar to that of wt. Furthermore, the fruit seed production was proportional to the fruit weight, with average production levels of about 12 and

15 seeds per fruit in wt and line 1.8 fruits, respectively, which increased to 24 and 22 seeds per fruit in lines 11.7 and 35.7, respectively (Figure 32C). As a result of the phenotypical and transcriptomic differences between tomato fruits from line 1.8 and lines 11.7 and 35.7 we decided not work farther with line 1.8

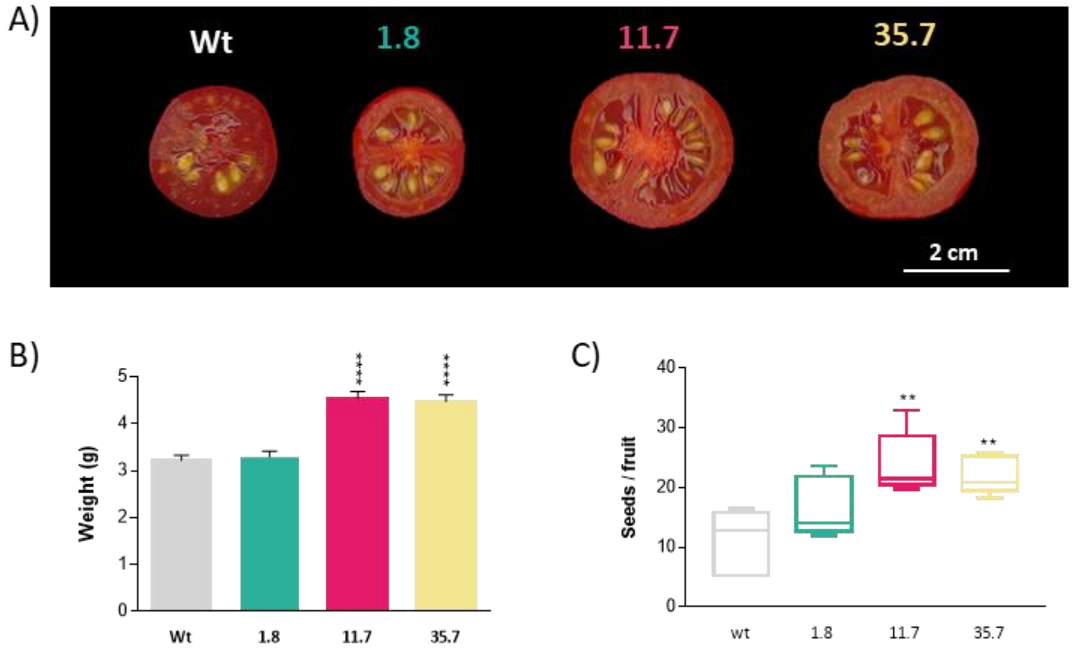

Figure 32. Phenotypic characterization of wt and *SlSGT4* overexpressing fruits. (**A**) Representative images of fruits of wt, and 1.8, 11.7 and 35.7 *SlSGT4* overexpressing lines. (**B**) Fruit weight and (**C**) seed content per fruit of wt and transgenic plants. Data shown are the mean ± SEM (n=150). Seed content was calculated by five pools containing 20 fruits each, and then divided the total of seed between the number of fruits (total seeds/20 fruits) black spots represents the distribution of the values per replicate. Asterisks represent significant differences in overexpressing lines compared to wt fruits determined by t-test (*P ≤ 0.05; **P ≤ 0.01; ****P ≤ 0.001).

3.2.4.4 Overexpression of SlSGT4 affects seed germination and root growth.

To determine if the increased number of seeds detected in fruits from transgenic lines 11.7 and 35.7 might affect the germination process, we next determined the germination rate of seeds collected from plants of these two transgenic lines and wt plants, as a control. Seeds were sown in Petri dishes and seed germination, determined as the radicle emergence, was daily monitored during 13 days. As can be observed in Figure 33, seed germination was remarkably inhibited in both transgenic lines. When compared to wt the germination rate was decreased by 13% and 22% in lines 11.7 and 35.7, respectively (Figure 33). Moreover, this decrease on germination rate was higher one week after seed sowing, being more marked in line 35.7 (47%)

than in line 11.7 (35%). Altogether these results indicate that germination was clearly delayed in both transgenic lines overexpressing *SlSGT4*.

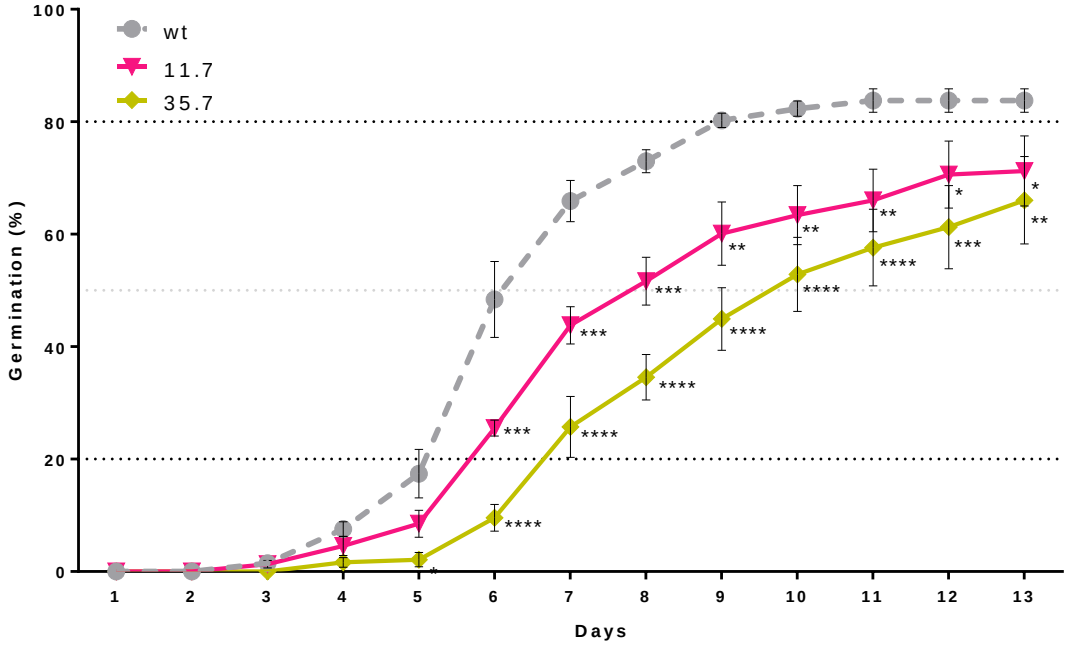

Figure 33. **Seed gemination percentages of wt and *SlSGT4* overexpressing lines**. Data show the mean ± SEM from 3 independent experiments using 50 seeds per line (n=3). Asterisks represent significant differences between transgenic lines compared to wt determined by two-way ANOVA followed by Dunnett test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001).

To determine if the changes observed in seed germination might result in root elongation differences, as reported under different treatments and conditions (Ahammed *et al.*, 2012; Osman Basha *et al.*, 2015), the root length of wt and transgenic seedlings 11.7 and 35.7 grown under hydroponic culture conditions was recorded during two weeks. In both cases the root length was significantly lower than that of wt seedlings. The primary root length of 1-week old seedlings of lines 11.7 and 35.7 displayed a similar reduction of around 62% and 65%, respectively, compared to wt (Figure 34A, C), and these differences were maintained after two weeks of growth, with length reductions of about 54% and 48% in lines 11.7 and 35.7, respectively, compared with the wt (Figure 34B-C).

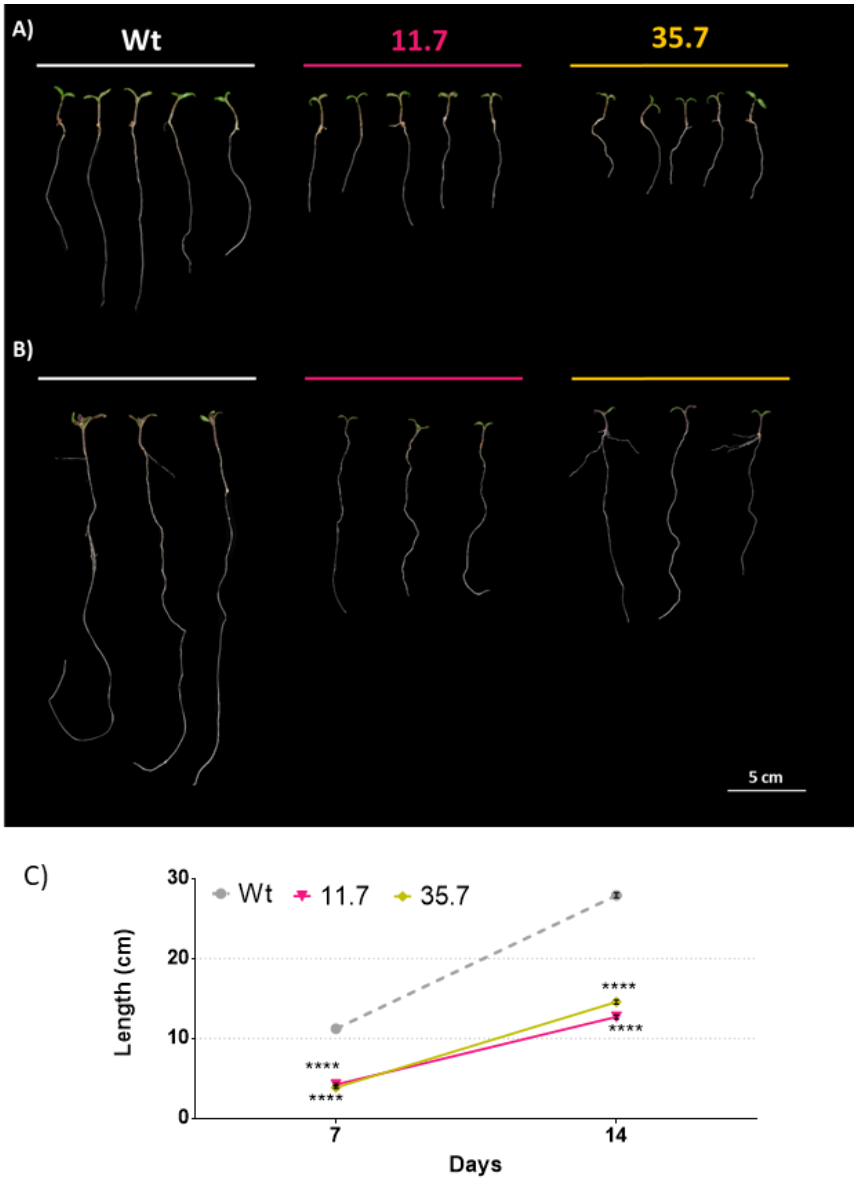

Figure 34. Root length phenotype in *SlSGT4* overexpressing lines. Representative images of seedlings from wt, 11.7 and 35.7 *SlSGT4* overexpressing lines showing root length after (A) one-week and (B) two-weeks of growth under hydroponic conditions. (C) Quantitative root length analysis along the assay. Data show mean values ± SEM from 30 seedlings per genotype (n=30). Asterisks represent significant differences between transgenic lines compared to the wt determined by t-test (****P ≤ 0.001).

4. DISCUSSION

4. DISCUSSION

4.1 CHAPTER I

4.1.1 Silencing of SlSGT1 gene expression in tomato plants.

This doctoral thesis gives continuity to previous work carried out in our research group in which the four tomato sterol glycosyltransferases *SlSGT1-4* were identified, cloned and preliminary characterized (Ramirez-Estrada *et al.*, 2017). The work presented here was aimed to unravel the role of glycosylated sterols on the growth and development of tomato plants and fruits, and also in the response to different abiotic and biotic stresses using transgenic tomato plants with altered transcript levels of *SlSGT1* and *SlSGT4*. The two transgenic lines with reduced expression of *SlSGT1* used in this thesis, amiSGT1-31.2 and amiSGT1-61.1, were previously generated in our research group (Castillo, 2019), each expressing a different artificial microRNA targeting the *SlSGT1* gene. The artificial microRNA technology for silencing of gene expression was chosen over other knock-out technologies such as CRISPR/cas9 for two main reasons: this experimental approach had been successfully used in previous studies carried out in our research group (Manzano *et al.*, 2016), and total inactivation of *SlSGT1*, the most highly expressed in the tomato SGT gene family, might result in drastic alterations in vegetative and/or reproductive stages of tomato which could be detrimental to the obtention of plant material or even result in a lethal phenotype.

The silencing of *SlSGT1* by both artificial microRNAs caused a marked reduction of the transcript levels of this gene in leaves without altering the expression levels of any other gene in the tomato SGT family (Figure 7). Therefore, alterations in biochemical, molecular and morphological phenotypes observed in the silenced lines can be directly attributed to the effect of amiRNA-mediated downregulation of *SlSGT1* expression. The fact that both transgenic lines show identical phenotypic alterations further confirms this view.

In leaves of the amiSGT1 plants, the partial loss of *SlSGT1* function resulted in a reduction around 13-17% in the amounts of glycosylated sterol (SG + ASG) compared with the wt leaves (Figure 8C), which was mainly caused by a marked decrease of around 32-46% in the total amounts of SGs (Figure 8A). The four major sterols species in the SG fraction were reduced, being stigmasterol glycoside the most severely reduced followed by glycosylated β-sitosterol, campesterol and cholesterol (Figure

9A). These results showed that SlSGT1 has indeed the ability to glycosylate the four major tomato sterol species, which is in agreement with previous reports that showed the *in vitro* and *in vivo* ability of SlSGT1 to catalyze the glycosylation of different sterol species (Ramirez-Estrada *et al.*, 2017). Moreover, previous studies have reported the broad substrate specificity of other plant SGTs. For instance, both Arabidopsis SGT isoforms, UGT80A2 and UGT80B1, and SGTL1 and SGTL4 of *W. somnifera* recognize all the major sterol species as substate (DeBolt *et al.*, 2009; Pandey *et al.*, 2015; Stucky *et al.*, 2015). Additionally, *W. somnifera* SGTs beside sterols can glycosylate other structurally related compounds such as the steroidal lactones, also known as whitanolides, to produce SGAs (Pandey *et al.*, 2015; Saema *et al.*, 2015; Singh *et al.*, 2016). These last observations prompted us to investigate if SlSGT1 is also involved in the synthesis of tomato SGAs by measuring the contents of α-tomatine and dehydrotomatine, the predominant SGAs in tomato, in the leaves of *SlSGT1* silenced lines. However, our results showed that SlSGT1 is not involved in the glycosylation of the tomato steroidal lactones, since neither α-tomatine nor dehydrotomatine levels were altered in the silenced plants (Figure 11), which indicates that SlSGT1 only recognize sterols as substrate.

The reduction in the SG levels produced a slight reduction in the total amounts of ASG in the silenced lines, which is mainly due to a reduction in the levels of acylated stigmasterol glycoside. These results might suggest the importance of maintaining high levels of ASG for tomato plant development. It has been proposed that high levels of glycosylated sterols, specifically ASGs, in the PM of tomato cells are needed to protect them against the endogenous high levels of SGAs, toxic defense compounds that causes irreversible leakage of electrolytes in a number of eukaryotic cells (Steel and Drysdale, 1988; Keukens *et al.*, 1995; Blankemeyer *et al.*, 1997).

The reduction in the levels of glycosylated sterols was paralleled by an accumulation of all the four major FS species, particularly stigmasterol (Figure 9C), but it did not significantly alter the total amounts of SE (Figure 8E), considered as the storage form of excess FS (Schaller, 2004; Bouvier-Navé *et al.*, 2010; Silvestro *et al.*, 2013). The analysis of the major sterol species in the SE fraction showed that esterified stigmasterol accumulates in both silenced lines compared with the wt (Figure 9D), but its impact on total SE levels is almost negligible because esterified stigmasterol constitutes less than 10% of the total amount of SE in tomato leaves (S. Table 2). It is remarkable that *SlSGT1* silenced plants transform only a small fraction of the excess of FS into SE, since it has been reported that excess of FS could results in plant toxicity. Wild type Arabidopsis plants treated with sterol precursors accumulate SE without

significant changes in the FS content (Banas *et al.*, 2005; Bouvier-Navé *et al.*, 2010), whereas Arabidopsis *psat1-1* and *psat1-2* mutants defective in the conversion of FS into SE show elevated FS levels which result in toxicity symptoms such as chlorosis, probably caused by the disruptive effects in the plasma membrane sterol homeostasis (Bouvier-Navé *et al.*, 2010). In a recent study, SlPSAT1 and SlASAT1, the tomato orthologues of AtPSAT1 and AtASAT1, have been cloned and functionally characterized (Lara *et al.*, 2018) which proves that tomato has the biochemical machinery to convert the excess of FS into SE. Although in tomato plants FS represent only small fraction of the total sterols, they still have an important structural function in PM alongside glycosylated sterols. This might explain why silenced *SlSGT1* plants accumulate FS instead of storing excess sterols as SE in cytoplasmic lipid droplets, as they might compensate for the decreased levels of glycosylated sterols, which is consistent with the fact that the total amount of sterols (SG + ASG + SE + FS) in the silenced plants did not show significant differences compared to the wt plants (Figure 8F).

The analysis of the major sterol species in the free and conjugated sterols fractions showed that SlSGT1 has some preference for stigmasterol as substrate for glycosylation. In fact, the levels of this particular sterol species and its conjugated derivatives are significantly affected in the four fractions (Figure 9). Different sterol substrate preference, but not strict specificity, has been described in Arabidopsis UGT80A2 and UGT80B1 isoforms. While UGT80A2 seems to account for most of the sitosteryl and stigmasteryl glucosides formation in seeds, UGT80B1 tends to glycosylate preferentially brassicasterol (DeBolt *et al.*, 2009; Stucky *et al.*, 2015). The substrate preference for stigmasterol shown by SlSGT1 and UGT80A2 is in agreement with the phylogeny study comparing SGTs from different plant species performed by Ramirez-Estrada *et al*., (2017) which showed that these two enzymes are closely related.

The reduction in glycosylated sterols induced by the silencing of *SlSGT1* resulted in smaller tomato plants (Figure 10). Previous studies have associated the misregulation of genes involved in biosynthesis of glycosylated sterols and alterations in the glycosylated sterol content with abnormal plant growth. In *W. somnifera,* the silencing of *WsSGTL1* and the concomitant decrease in the glycosylated campesterol, β- sitosterol and stigmasterol amounts negatively affected plant growth (Saema *et al.*, 2015). Further studies showed that downregulation of *WsSGTL1*, *WsSGTL2* and *WsSGTL4* translates into changes in the glycosylated sterol content and a reduction in plant growth manifested as shorter plants and smaller leaves (Singh *et al.*, 2016).

In the Arabidopsis double knock-out mutant *ugt80A2;B1*, the reduced levels of glycosylated sterols in leaves, stem and inflorescences were correlated with multiple morphological phenotypes including defects in embryonic development and significantly lower growth rates (DeBolt *et al.*, 2009). In contrast, transgenic *W. somnifera* plants overexpressing *WsSGTL1* exhibited more vigorous plant growth, characterized by larger plants with higher internode number, increased leaf area, and shoots and roots biomass, which were correlated to an increase in the amounts of glycosylated campesterol, stigmasterol and β-sitosterol (Saema *et al.*, 2015). Our results clearly showed that the reduction in the amounts of glycosylated sterols negatively affects cell division and expansion since reductions in diameter and internode length in the silenced lines (Figure 10C-D) correlates with lower number of cell layers and smaller cell size, which indicates alterations in cell division and cell expansion, respectively (Figure 10E-I). It is well documented that changes in the total sterol content can directly affect cell growth and cell proliferation as a result of possible changes in the physical properties of PM and/or lipid rafts (Piironen *et al.*, 2000; Carland *et al.*, 2002; Schaller, 2004; Laloi *et al.*, 2007; Vriet *et al.*, 2013). However, the results presented in this thesis show that a decrease of a specific type of conjugated sterols, glycosylated sterols, causes similar alterations than those observed when the total sterol content is negatively affected. Thus, we demonstrate the main role of glycosylated sterols in tomato plant growth.

4.1.2 Silencing of SlSGT1 results in a reduction of SG levels in early stages of tomato fruits development and negatively affects fruit size.

The sterol content in tomato fruits was described for the first time by Yamamoto and McKinney (1967). However, it was not until 1988 when Whitaker presented the sterol profile among different development stages of cv. Rutgers tomato fruits. As far as we know, the sterol profile of tomato cv. Micro-Tom plant organs, including fruits at different growth and ripening stages, has not been reported. Consequently, data presented in this thesis represent a major update in the information available regarding tomato fruit sterols profile. Our data are consistent with Whitaker observations that reported important changes in the distribution and content of sterols at early development stages compared to red fruits (Figure 15). As shown in Figure 35, sterol profiles of cv. Rutgers and cv. Micro-Tom are fairly similar at mature green and breaker stages, where glycosylated sterols represent more than 80% of the total sterols, being ASGs the most abundant followed by SGs. Free and SE represent only a minor part of the total content of sterols, although the relative amounts of these fractions are the opposite in these two cultivars. Interestingly, the most

important differences in sterol composition are observed at the red stage. In Micro-Tom, the accumulation of glycosylated sterols reaches up around 95% of the total sterol content, being ASGs, by far, more abundant than SG, which gradually diminish from the mature green stage, whereas similar amounts of ASG and SG are found in cv. Rutgers red fruits. Major differences between red fruits of both cultivars are also observed in FS and SE fractions. In Micro-Tom, FS are poorly represented and SE are almost absent, while in Rutgers both fractions represent near 40% of the total sterol content at this stage.

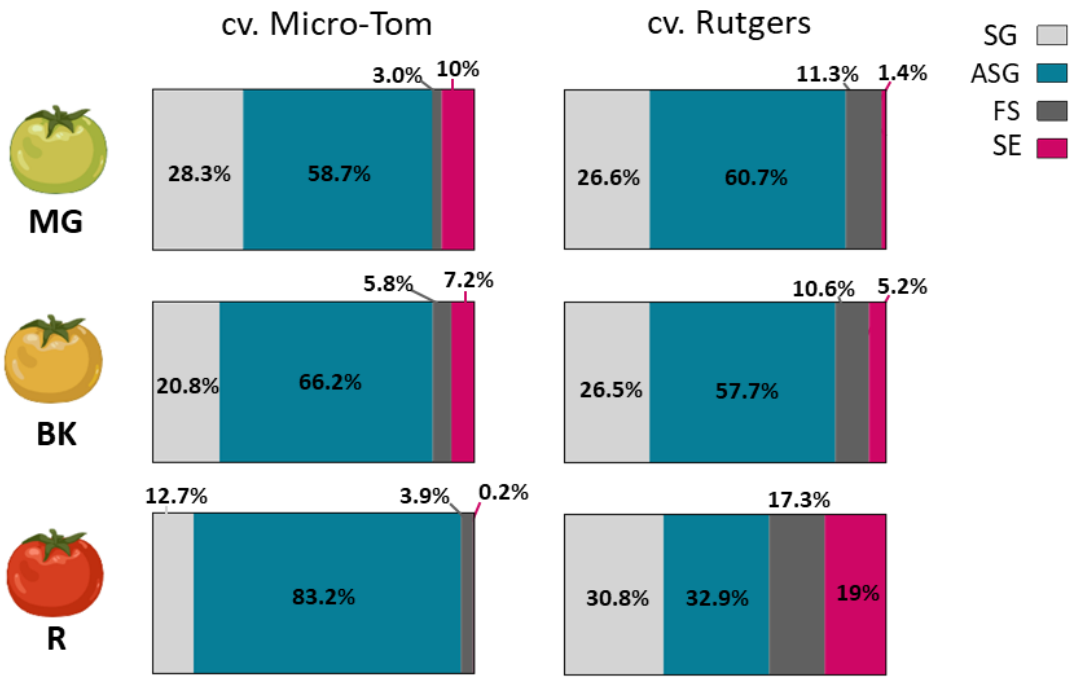

Figure 35. **Comparative analysis of sterol fractions between tomato cv. Micro-Tom and cv Rutgers fruits along development stages.** The percentage of free (FS) and conjugated sterol (SG, ASG and SE) fractions in mature green (MG), breaker (BK) and red (R) cv. Micro-Tom and cv Rutgers fruits is indicated.

The expression analysis of the different *SGT* genes in fruits of amiSGT1-31.2 and amiSGT1-61.1 silenced lines showed the efficiency of the selected artificial micro-RNAs in silencing *SlSGT1* expression also in fruits. Downregulation of *SlSGT1* only produce a moderate upregulation of the expression of *SlSGT2* and *SlSGT3* at mature green and red stages (Figure 13). Interestingly, the sterol analysis of the silenced *SlSGT1* tomato fruits showed a sharp reduction of SGs compared to wt levels only at the early stages of development (i.e. green and mature green) (Figure 15A). The four

major glycosylated sterol species are significantly reduced at these stages (Figure 16A-D). On the contrary, the levels of SG in fruits of silenced plants at breaker +1 and red stages were similar to those in wt fruits at the same stages (Figure 15A). These results suggest that SGs are mostly synthesized at green and mature green stages. As previously mentioned, the ASG levels in wt fruits remain fairly stable along fruit development, and silencing of *SlSGT1* only caused a moderate decrease of these compounds along all fruit developmental stages analyzed (Figure 15B). As in leaves, SGAs accumulate in relatively high amounts in green fruits; thus the high content of ASG might be acting as a PM stabilizer against the chemical damage caused by these compounds (Steel and Drysdale, 1988; Keukens *et al.*, 1995; Blankemeyer *et al.*, 1997). The ASG content of wt and the silenced plants suggest an active synthesis of ASGs throughout all stages of fruit development. The analysis of the major sterol species in this fraction showed that tomato fruits preferentially accumulate acyl cholesterol and β-sitosterol glycosides and the early stages of fruit growth, while acyl campesterol and stigmasterol glycosides tends to accumulate during ripening (Figure 16E-H). Taken together, the expression profile of *SlSGT* genes and the glycosylated sterol profiles in the *SlSGT1* silenced lines (Figure 13 and 15A-B) suggest that SlSGT1 is a major contributor to sterol glycosylation activity in growing tomato fruits and to a much lesser extent, if any, in ripening fruits (breaker +1 and red), where it might be replaced by SlSGT3, since *SlSGT3* transcript levels increase at these stages (Ramirez-Estrada *et al.*, 2017). As in leaves, the reduction of SG in amiSGT1 fruits was paralleled by a marked accumulation of FS particularly at the green and mature green stages where SG levels are reduced (Figure 15C). Contrary to other major free sterol species, accumulation of free stigmasterol was detected along all the developmental stages (Figure 16G) Finally, SE levels were not altered by the partial loss of function of SlSGT1 (Figure 15D). The observed accumulation of SE in fruits at mature green stage of both *SlSGT1* silenced and wt plants is produced by a high accumulation of esterified cholesterol and β-sitosterol (Figure 16M-P). The fast accumulation of these esterified sterols from green to mature green stages matches with tomato seed morphogenesis (Klee and Giovannoni, 2011), which could pave the way for the subsequent mobilization of sterols from fruit tissue into the seeds, since SE and FS are the predominant forms of sterols in tomato seeds (Duperon *et al.*, 1984; Burciaga-Monge, 2020). Altogether, the changes in the sterol amounts, specifically of free and glycosylated sterols, did not result in alterations in the total content of sterols of *SlSGT1* silenced fruits compared to wt fruits (Figure 15E), which as in leaves, is likely to be a mechanism to balance the reduced levels of SG in the PM/lipid rafts.

Similar to the phenotypical alterations observed in tomato plants, downregulation of *SlSGT1* resulted in marked reduction of fruit size and seed yield (Figure 14). The organogenesis of tomato fruits involves multiple events of cell division and expansion which will define the shape and final size of fruits (Bohner and Bangerth, 1988; Tanksley, 2004). Although cell size plays an important role in the final fruit size, in many species, including tomato, differences in fruit size have been attributed to differences in cell number rather than cell size. Tomato fruit development is divided into three well distinguished phases; cell division, cell expansion and ripening. Cell division phase is relatively short and ends about one-two weeks after the anthesis, and is followed by the cell expansion phase which contributes to the growth of the fruit until it reaches the final size. Finally, during the ripening phase, the fruit will face multiple biochemical changes that will define its color, taste and texture, but during this phase the fruit will no longer increase in size (Gillaspy *et al.*, 1993; Bertin *et al.*, 2002; Cheniclet *et al.*, 2005; Musseau *et al.*, 2017). Previous studies have shown the role of sterols in fruit growth. For instance, high mRNA and enzyme activity levels of HMGR, the major rate-limiting enzyme in the sterol biosynthesis pathway, have been reported in early stages of tomato fruit development where cell division and expansion take place, and inhibition of this enzyme during early fruit stages negatively affects fruit size. These results strongly suggested that phytosterols are important elements during cell division and expansion in tomato fruits (Narita and Gruissem, 1989). Moreover, the overexpression of the catalytic domain of melon (*Cucumis melo L. reticulatus*) HMGR (Cm-*HMGR*-CD) in tomato, induced cell division and/or cell extension at early fruit developmental stages, since transgenic fruits showed bigger final size than the non-transgenic fruits (Kobayashi *et al.*, 2003). It is worth noting that alteration in the synthesis of enzymes in early steps in the sterol biosynthesis, as is the case of HMGR, might affect the total content of sterols, and not only a specific sterol fraction (Wang *et al.*, 2012). Our results suggest that negative regulation in the expression of genes involved in the glycosylation of sterols, specifically *SlSGT1*, and subsequent decrease of SG during the tomato fruit growing stages, is enough to induce alterations in fruit size that are reminiscent of those observed when the sterol biosynthesis pathway is inhibited at the level of HMGR and total sterols, most likely including both free and glycosylated pools, are presumably severely depleted. Whether this effect is specifically due to the reduced levels of glycosylated sterols or to changes in the ratio FS/glycosylated sterols remains to be elucidated.

As previously mentioned, the sterol profile and the phenotype observed in the *SlSGT1* silenced fruits strongly suggest that glycosylated sterols have a main role in the

tomato fruit growth. As a first approached to investigate the molecular mechanism underlying the observed fruit phenotype, we performed an RNA-seq analysis of 9 DAA tomato fruits from wt, amiSGT1-31.2 and SGT1-61.1 plants. This particular time-point was chosen because it is considered the edge between the end of cell division phase and the start of cell expansion phase (Gutensohn and Dudareva, 2016; Quinet *et al.*, 2019). The downregulation of *SlSGT1* induced misregulation of 388 genes, of which 91% correspond to downregulated genes (353 DEGs). Thus, silencing of *SlSGT1* and the subsequent decrease in the amounts of glycosylated sterols induced a strong negative regulation of tomato fruit gene expression (Figure 17 and S. table 5). As expected, several genes related with tomato fruit and seed development were strongly downregulated, including *7S globulin* and *11S globulin* genes that encode two of the major storage proteins accumulated during seed development (Kimura *et al.*, 2008; Koziol *et al.*, 2012) and involved in tomato fruit quality (Liu *et al.*, 2016). Downregulation of these genes might be involved in the lower seed production observed in amiSGT1 lines (Figure 14C). Moreover, other genes linked to seed production such as those encoding oleosins, a family of proteins that play a major role in the formation of oil bodies in both seeds and fruits (Frandsen *et al.*, 2001), and the B3 domain transcription factors FUS3-like and ABI3, considered as master regulators of seed development (Aziz *et al.*, 2020), were also downregulated by the silencing of *SlSGT1*. The RNA-seq analysis also showed the downregulation of genes of the *CLV3/ESR* family, which have been related with fruit organogenesis and maturation (Zhang *et al.*, 2014), genes coding for non-specific lipid-transfer proteins (nsLTPs) associated with fruit development and ripening (D'Agostino *et al.*, 2019), auxin-responsive GH3 family genes, including *GH3-12* and *GH3-13*, which belong to a family of 24 genes involved in the maintenance of cellular auxin homeostasis (Sravankumar *et al.*, 2018), genes *SlGRF6* and *SlGRF10* coding for important regulators of tomato fruit (Cao *et al.*, 2016), and genes *SlLEA14*, *SlLEA17*, *SlLEA18* and *SlLEA20* coding for late embryogenesis abundant proteins, an important family of proteins that accumulates in response to cellular dehydration and may play a role in tomato development and ripening (Cao and Li, 2015). The misregulation of all these genes might have important consequences in fruit growth. Finally, multiple genes involved in cell wall modifications such as these coding for expansins proteins (*SlEXPB5*, *SlEXPA7* and *SlEXPA20*), which are highly active elements in cell wall modifications during fruit development and ripening, as well as seed germination (Chen *et al.*, 2001; Nardi *et al.*, 2015). The silencing of *SlSGT1* also induced the misregulation of genes involved in the synthesis or modification of specific cell wall components. For instance, the upregulation of genes encoding CESA and XTH proteins, involved in the

synthesis and modification of cellulose and xyloglucans, respectively (Carroll and Specht, 2011; Tsuchiya *et al.*, 2015), and the downregulation of a gene coding for a pectinesterase, involved in the modification of pectins (Wen *et al.*, 2020), which might suggest alteration in cell wall composition in the silenced *SlSGT1* fruits (S. Table 5). Since, the architecture of plant cell wall is highly dynamic and its correct and punctual re-modulation is key for determining the size and shape of cells, alterations in expression of genes involved in these processes could result in defects in growth and development (De Lorenzo *et al.*, 2019). These results suggest that phenotypical abnormalities in the tomato fruit growth resulting from the silencing of *SlSGT1* involves alterations in the transcript levels of a wide variety of genes including genes involved in hormone response, transcriptional regulation, storage of proteins and lipids, and cell wall biosynthesis and modification. Additionally, we selected ten genes related to different biological processes involved in fruit development (Table 3), and extended the transcriptomic analysis from 4 DAA, a time-point with high activity of cell division, to 15 DAA, a time-point with high activity of cell expansion. This analysis showed that most of the transcriptomic alterations observed are persistent along the cell division phase and/or cell expansion phase (Figure 18), which indicates that the silencing of SlSGT1 and the reduced levels of glycosylated sterols trigger the misregulation of fruit-development-related genes along both phases of fruit growth.

Due to the subcellular location of glycosylated sterols in PM and their enrichment in lipid rafts (Grennan, 2007; Furt *et al.*, 2010; Simon-Plas *et al.*, 2011; Malinsky *et al.*, 2013), changes in their total amount or their relative proportions with respect to FS might affect the fluidity and permeability of the PM, since the sugar moiety of SGs increases their hydrophilic character (Nyström *et al.*, 2012), and the physical properties of lipid rafts. This in turn might affect several lipid-lipid or lipid-protein interactions leading to the alteration of cellular functions involved in plant growth and development (Laloi *et al.*, 2007; Simon-Plas *et al.*, 2011; Cacas *et al.*, 2012). For instance, lipid raft-localization of several proteins involved in the signal transduction of plant hormones including auxin (Boutté and Grebe, 2009; Titapiwatanakun *et al.*, 2009; Yang and Murphy, 2009; Yang *et al.*, 2012) and abscisic acid (Demir *et al.*, 2013) is dependent on the sterol content. Therefore changes in the sterol profile might cause the mislocalization of these proteins, and is frequently accompanied by reduced plant growth (Carland *et al.*, 2002; Men *et al.*, 2008; Pook *et al.*, 2017).

4.1.3 Silencing of SlSGT1 leads to differential response against abiotic and biotic stress.

In nature, plants are exposed to different abiotic and biotic stresses. To cope with these changing environmental conditions, plants have evolved a wide range of molecular mechanisms to perceive the external signals and initiate the optimal defense responses (Fujita *et al.*, 2006). The crosstalk between different stress response pathways results in a more efficient defense response, particularly if common genes and/or compounds are involved in the protective response against more than one type of stress (Atkinson and Urwin, 2012), as seems to be the case of phytosterols. The results of this work showed that tomato plants with reduced levels of glycosylated sterols (mainly SG) (Figure 8), as a result of *SlSGT1* silencing, are more sensitive to cold stress (Figure 19 and 20) but more resistant to the necrotrophic fungus *B. cinerea* (Figure 21) than wt plants. These data, together with the increased levels of FSs detected in the silenced lines, support the view that the ratio of glycosylated versus free forms of sterols might play an important role in regulating different PM-associated processes, like plant adaptation to biotic and abiotic stress conditions (Palta *et al.*, 1993; Uemura and Steponkus, 1994; Moreau *et al.*, 2002; Mishra *et al.*, 2013; Li *et al.*, 2014; Pandey *et al.*, 2014; Tarazona *et al.*, 2015; Takahashi *et al.*, 2016) probably as a consequence of the changes in the properties of the cell membranes and lipid rafts (Moreau *et al.*, 2002; Grosjean *et al.*, 2015; Mamode-Cassim *et al.*, 2019)

The involvement of glycosylated sterols in plant response to abiotic stress, concretely cold, is not unprecedented. An increased sensitivity to cold stress, like that observed in the tomato *SlSGT1* silenced plants (Figure 19 and 20), has also been reported in Arabidopsis and *W. somnifera* plants with reduced expression levels of *SGT* (Singh *et al.*, 2016; Mishra *et al.*, 2017), whereas enhanced tolerance to cold and other abiotic stresses has been associated to overexpression of *SGT*s in Arabidopsis, tobacco, and *W. somnifera* (Mishra *et al.*, 2013; Pandey *et al.*, 2014; Saema *et al.*, 2015). It is worth to mention that, in contrast to other genes of the tomato *SGT* family (*SlSGT2* and *SlSGT4*), the expression of *SlSGT1* is not induced by cold or other abiotic stresses (Ramirez-Estrada *et al.*, 2017). However, the partial silencing of this gene was enough to increase the sensitivity of tomato plants to cold stress (Figure 19 and 20), which supports the preponderant role of this gene in the regulation of SG biosynthesis in tomato and evidences the importance of this conjugated form of sterols in the cold response. The relative proportion of membrane lipids constituents, including sterols, determine the fluidity and the phase transition of the membrane from fluid state to

rigid gel form, a process that allows the cells to mechanically adapt to freezing (Barrero-Sicilia *et al.*, 2017). Our data suggest that SGs may play a relevant role in this process, since the ion leakage, a parameter that reflects the membrane integrity and functionality, was higher in the silenced lines than in the wt plant after exposure of seedlings to low temperature (Figure 19). This is supported by the fact that the levels of the membrane sterol species stigmasterol and β-sitosterol were the more reduced in the glycosylated fraction (Figure 9A). These observations are consistent with changes observed in the relative proportions of glycosylated sterols in the PM of other plant species, such as oat, rye, and potato in association with cold acclimation and freezing tolerance (Palta *et al.*, 1993; Takahashi *et al.*, 2016).

The experimental evidence supporting the involvement of glycosylated sterols in biotic stress responses (Pandey *et al.*, 2014; Singh *et al.*, 2016; Mishra *et al.*, 2017; Castillo *et al.*, 2019) are scarcer than those referring to abiotic stress. Interestingly, most of the published data related with this issue have been obtained using plants from the Solanaceae family such as *N. tabacum* and *W. somnifera*. In both cases, alterations in glycosylated sterols levels are accompanied by changes in the content of glycosylated defense compounds such as whithanolides (SGAs) in *W. somnifera* (Singh *et al.*, 2016) and rutin in tabaco (Pandey *et al.*, 2014), which could be ultimately responsible for the observed responses against pathogens. In contrast to that, the marked reduction on SG levels observed in leaves of the *SlSGT1* silenced lines characterized in this work (Figure 8), was not paralleled by changes in the content of the tomato defense SGAs α-tomatine and dehydrotomatine, whose levels remained similar to those detected in wt plants (Figure 11). In spite of it, silencing of *SlSGT1* increased the tomato resistance against the necrotrophic fungus *B. cinerea* (Figure 21), suggesting than in this Solanaceae species a decrease in SGs could be enough to induce the observed resistance phenotype. These results agree with the enhanced resistance against this fungus reported recently in the Arabidopsis mutant *ugt80A2;B1* (Castillo *et al.*, 2019), which leaves, like those of the tomato silenced *SlSGT1* lines, presented markedly reduced levels of glycosylated sterols (DeBolt *et al.*, 2009). However, the non-glycosylated sterols (FSs + SE) in the Arabidopsis mutant remained at the same level than in wt plants (DeBolt *et al.*, 2009), while in the tomato silenced lines increased clearly as a result of the high content of FE (Figure 8D). Thus, the possibility that the resistance to *B. cinerea* in the tomato *SlSGT1* silenced lines can result from changes in the ratio of free versus glycosylated sterol cannot be discarded. Either way, our data support the view that glycosylated sterols, directly or indirectly, are involved in the plant response to necrotrophic pathogens.

In the Arabidopsis *ugt80A2;B1* mutant the defense response is mediated through signaling pathways involving the stress-hormone JA and some defense specialized metabolites, such as camalexin, the major phytoalexin in *A. thaliana* (Glawischnig, 2007), and indol glucosinolates (Castillo *et al.*, 2019). In the case of tomato further studies, including transcriptomic and metabolomic analyses along the infection process, are necessary to understand the molecular mechanism acting behind the resistance phenotype observed in the silenced *SlSGT1* plants. However, the upregulation of several genes related to stress responses (Table 1) observed in the RNA-seq transcriptomic analysis of non-infected leaves from the amiSGT1-31.2 line might help to understand, at least in part, the resistance (Table 1).

Between the upregulated genes we found a set of genes involved in the phenylpropanoid and flavonoid biosynthetic pathways (Table 1), like two genes encoding different phenylalanine ammonia-lyase isozymes (PAL5 and PAL7) (Table 1). This is a key enzyme that catalyzes the first committed step in the phenylpropanoid biosynthetic pathway (Jones, 1984), and has been reported to be important to many plant defense responses. PAL5 is the main isoform induced in response to biotic stresses in tomato (Chang *et al.*, 2008), while the expression of *PAL7* is upregulated in tomato plants infected with *B. cinerea*, and its silencing in the ABA-deficient tomato mutant *sitiens* increased the susceptibility to *B. cinerea* (Seifi *et al.*, 2013). Enhanced expression of *PAL* has also been reported in Arabidopsis plants overexpressing *WsSGTL1,* which presented increased tolerance to the necrotrophic fungus *Alternaria brassicicola* (Mishra *et al.*, 2017). Other gene which expression was upregulated by *SlSGT1* silencing encodes a 4-coumarate:CoA ligase-like protein (Table 1). The 4-coumarate:CoA ligase (4CL) catalyzes the formation of coumaroyl-coenzyme A, a central intermediate in the biosynthesis of a myriad of phenylpropanoids compounds (Vogt, 2010). Flavonoids are a group of plant specialized metabolites with a vast array of biological functions, including a role in stress protection (Winkel-Shirley, 2002; Schenke *et al.*, 2019), which biosynthetic pathway lies downstream of the phenylpropanoid one, and is initiated by the key enzyme chalcone synthase (CHS) (Holton and Cornish, 1995). A gene encoding this enzyme, and other encoding a member of the dihydroflavonol 4-reductase family (DRF), a protein involved in the synthesis of anthocyanins (Lepiniec *et al.*, 2006), were also upregulated in the *SlSGT1* silenced line (Tabla 1). Moreover, the expression of some genes encoding defensin-like proteins was also incremented in leaves of the amiSGT131.2 line compared to wt (Table 1). These proteins constitute a group or antimicrobial peptides that are part of the plant innate immune system against pathogens, whose overexpression in tomato

has been reported to enhance the foliar resistance to *B. cinerea* (Stotz *et al.*, 2009). These results support the hypothesis that changes in the relative proportions of sterols may be perceived by plants as a stress signal that activates different defensive responses in a sterol profile-dependent manner (Wang *et al.*, 2012; Singh *et al.*, 2015; Manzano *et al.*, 2016).

Altogether, these results suggest that SGs play important, but opposite, roles in the tomato response against biotic and abiotic stresses. Although the mechanisms that trigger these responses are still far to be known, some hypothesis could be proposed. It is known that SGs are enriched in the PM lipid rafts, which control dynamic protein interactions in a specific sterol-lipid environment (Zauber *et al.*, 2014), and the biological function of these microdomains has been linked to signaling and transport (Shahollari *et al.*, 2004; Kierszniowska *et al.*, 2009). Thus, it might be hypothesized that an altered composition of glycosylated sterols in the membrane rafts might affect their structure and function, resulting in an indirect differential modulation of some signaling pathways, such as those involved in the stress responses studied in this work.

Overall, the results of this work show that in tomato a reduction in the levels of glycosylated sterols, mainly SG, results in detrimental effects in plant and fruit development, as well as in differential responses to biotic and abiotic stress. Further studies aimed to dissect the transduction pathways involved in these processes will provide more insights about the mechanism of action by which glycosylated sterols may modulate these plant physiological events.

4.2 CHAPTER II

4.2.1 Overexpression of SlSGT4 affects tomato plant and fruit development.

Of the four tomato genes encoding SGTs, *SlSGT4* is barely expressed in different tomato organs and throughout fruit developmental stages (Ramirez-Estrada *et al.*, 2017). Thus, the constitutive overexpression of *SlSGT4* could be a good strategy to start investigating the role of the SlSGT4 isozyme in tomato growth and development. To this end, we obtained 3 homozygous transgenic lines with expression levels of *SlSGT4* significantly higher than in wt plants, being line 1.8 the one with highest transgene expression, followed by line 11.7 and finally line 35.7 (Figure 24).

Surprisingly, the levels glycosylated sterols decreased significantly in the leaves of the three transgenic lines compared to wt plants (Figure 26C), and this decrease was observed in both the SG and ASG sterol fractions, being SG the most affected (Figure 26A-B). This unexpected reduction of the glycosylated sterol levels could be attributed to the significant *SlSGT1* downregulation observed in the three transgenic lines (Figure 25A), since the expression levels of *SlSGT2* and *SlSGT3* were not affected by overexpression of *SlSGT4* (Figure 25B-C). As mentioned before, *SlSGT1* is the most highly expressed gene of the tomato *SGT* gene family (Ramírez-Estrada et al., 1997) and, as observed in the characterization of tomato *SlSGT1* silenced lines, it plays a key role in the regulation of glycosylated sterol levels in leaves and fruits (Figures 8 and 15). However, in contrast to the *SlSGT1* silenced lines, the levels of FS in the *SlSGT4* overexpressing lines were not increased, but significantly reduced compared to wt plants (Figure 26D). This marked reduction in different sterol fractions suggest a possible downregulation of some genes coding for enzymes that catalyze early steps of the sterol biosynthesis pathway in the plants overexpressing *SlSGT4*. It has been previously reported that downregulation of *W. somnifera SGT*s induces the expression of genes involved in the early steps of the isoprenoid biosynthesis pathway such as *HMGR* and *FPS*, which in turn leads to enhanced accumulation of sterols (Singh *et al.*, 2016). Altogether these results support the idea that altered transcript levels of *SlSGT4* can modulate the expression of genes upstream the sterol pathway whose expression might affect different fractions of sterols profile.

The overexpression of *SlSGT4* resulted in a negative effect in the plant size in 11.7 and 35.7 transgenic lines (Figure 28) which is comparable to the pleiotropic phenotype induced by the reduction of glycosylated sterols contents in *SlSGT1* silenced lines (Figure 10). Intriguingly, neither height nor total sterol levels in 1.8 plants were

affected, which suggests that the phenotype of 11.7 and 35.7 plants is likely caused by the drastic reduction in the total amount of sterols (SG + ASG + FS + SE). It is well documented that mutant plants defective in late steps of the sterol pathway whose total sterol amounts are reduced show growth defects (Jang *et al.*, 2000; Schaeffer *et al.*, 2001; Carland *et al.*, 2002; Schaller, 2004; Vriet *et al.*, 2013). In Arabidopsis, suppression of *SMT2* resulted in pleiotropic effects on development such as reduced growth caused by alterations in sterol composition (Schaeffer *et al.*, 2001). Additionally, the Arabidopsis *fackel* mutant displays several development defects including dwarfism as result of abnormalities in cell division attributed to a negatively altered composition of sterols (Jang *et al.*, 2000).

In contrast to the phenotypic alterations on vegetative development, plants from transgenic lines 11.7 and 35.7 produced fruits significantly bigger than those from wt plants, while the size of fruits from plants of line 1.8 was similar to that of wt (Figure 32). As expected, in the three transgenic lines the expression of *SlSGT4* increased significantly in all the fruit developmental stages (from green to red) (Figure 29). But, contrary to that observed in the vegetative tissues, the expression of *SlSGT1* only at decreased at green and mature green stages in line 1.8, while this gene was significantly upregulated in green and mature green fruits of lines 11.7 and 35.7, when cell division and expansion takes place (Figure 30). A similar pattern of expression, although at lower level, was observed in the *SlSGT2* expression, while the transcript levels of *SlSGT3* in these two transgenic lines increased significantly during all the fruit developmental stages, but this was not observed in line 1.8 (Figure 31). Thus, although sterol levels in fruits of these transgenic plants remain to be quantified, we can hypothesize that the observed increase in fruit size in transgenic lines 11.7 and 35.7 might be due to an increase in the content of glycosylated sterols. To date the effects of altered sterol composition in tomato fruit development have been poorly studied (Narita and Gruissem, 1989; Kobayashi *et al.*, 2003). However, our hypothesis is supported by the results presented in Chapter I, since in *SlSGT1* silenced lines, with reduced levels of glycosylated sterols, tomato fruits are smaller that wt fruits (Figure 14, section 3.1.6). The differential expression response of the *SlSGT* genes in leaves and fruits of plants overexpressing *SlSGT4* strongly suggest that different regulation mechanisms controlling glycosylated sterol levels operate in these two organs.

Formation of seed completes the reproductive cycle of many plants(Buchanan *et al.*, 2015). In tomato, it has been reported that increased fruit weight, as that observed in the *SlSGT4* overexpressing lines 11.7 and 35.7, is commonly accompanied by an increment on seed production (Imanishi and Hiura, 1975; Prudent *et al.*, 2009).

According with this, the number of seeds in fruits from both *SlSGT4* overexpressing lines was significantly higher than in those from wt (Figure 32C). Since seeds are a plant storage organ, during their development they compete for the assimilates accumulated in the fruit sink organ. Thus, the bigger are the fruits, the greater is the amount of assimilates available for seed production (Nitsch, 1970; Bertin *et al.*, 1998; Prudent *et al.*, 2009). Overall, our results suggest that changes in sterol content, mainly glycosylated sterols, play an important role in the development of tomato fruits, specifically at the fruit growing stages. This in turn results in changes of the seed yield, which is lower in smaller fruits, as those produced by the *SlSGT1* silenced lines (Figure 14, section 3.1.6), but higher in the bigger fruits produced by *SlSGT4* overexpressing lines (Figure 32C).

A proper seed reserve content has been correlated to the rate germination (Sun *et al.*, 2015; Shrestha *et al.*, 2016; Zhao *et al.*, 2018). This physiological process involves the breakdown of storage reserves accumulated in the endosperm, like carbohydrates, lipids and proteins, into soluble molecules that are mobilized through the embryo to allow seedling development (Bewley and Black, 1994; Pritchard *et al.*, 2002). The results of this work show that although tomatoes from lines 11.7 and 35.7 produced more seeds than those from wt plants, the germination rate was significantly reduced in both transgenic lines (Figure 33), suggesting that the reserve content in the transgenic seeds and/or its mobilization during germination is negatively affected. The role of sterols during germination still remains controversial. Mobilization of these metabolites during seed germination has been reported in several plant species including *Medicago sativa, Sorghum bicolor, Zea mays, Cucurbita maxima, Brassica napus, Corylus avellana* and tomato (Bush and Grunwald, 1972; Shewry and Stobart, 1974; Duperon *et al.*, 1984; Garg and Nes, 1985; Heupel *et al.*, 1986; Huang and Grunwald, 1988; Guo *et al.*, 1995; Zhang *et al.*, 2007). The most abundant sterol fractions in tomato seeds are SE and FS, while the levels of glycosylated sterols are relatively low. However, during germination the glycosylated sterols became predominant (Duperon *et al.*, 1984). On the other hand, the seeds of a tomato *psat1* knock-out mutant, generated in our laboratory by CRISPR- Cas9, showed altered levels of free and conjugated sterols (increased levels of FS and SG, and reduced levels SE) that resulted in a reduced germination rate (Burciaga, 2020). Altogether these results suggest that mobilization of sterols during tomato seeds germination may be affected by changes in their sterol content or in the ratio of the different conjugated fractions, mainly that of glycosylated sterols, which might play an important role during this physiological process. However, the possibility that

altered levels of sterols, including glycosylated sterols, might affect directly the germination signaling pathway cannot be discarded, since during plant growth and development phytosterols can act as signaling molecules interacting with different hormone pathways (Vriese *et al.*, 2019), including gibberellins (He *et al.*, 2003), which have been reported as essential factors for tomato seed germination (Groot and Karssen, 1987; Bassel *et al.*, 2004). The plant material generated in this thesis could be a useful tool for further studies aimed to unravel the specific role of glycosylated sterols during tomato seed germination.

It has been reported that delayed tomato seed germination might affect root elongation (Kummerová and Kmentová, 2004; Ahammed *et al.*, 2012; Basha *et al.*, 2015). Thus, we decide to determinate if this was the case in the transgenic lines overexpressing *SlSGT4*. We observed that, as the germination rate, root elongation was negatively affected in transgenic lines 11.7 and 35.7, since root length was drastically reduced when compared with wt roots (Figure 34). The involvement of sterols in root development has been previously reported in the Arabidopsis sterol deficient mutants *hydra1* and *hydra2/fackel* (Topping *et al.*, 1997; Schrick *et al.*, 2000), and *smt1 (*Diener et al., 2000; Willemsen et al., 2003*),* which showed a severe reduction in root growth, attributed to a direct effect of sterols on the auxin and ethylene signaling pathways. Alterations in the levels of glycosylated sterols have also been related with different root phenotypes. Thus, accumulation of glycosylated sterols by overexpression of *SGT1* in *W. somnifera* induces a drastic increase of the root biomass (Saema *et al.*, 2015). Conversely, the Arabidopsis mutant *ugt80B1,* exhibits shorter roots than wt and this phenotype is more marked in the double mutant *ugt80A2;B1*, which contains very low levels glycosylated sterols (DeBolt *et al.*, 2009). Since the effect of *SlSGT4* overexpression on the expression of the other genes of the *SlSGT* family seems to be different depending on the plant organ (Figures 25, 30 and 31). Quantification of sterols in the different fractions are necessary to understand how these compounds, mainly the glycosylated forms, are affecting root development in the *SlSGT4* overexpressing lines.

Overall, the results of this work suggest that in tomato overexpression of *SlSGT4* affects plant and fruit growth, seed production and germination, and root elongation. However, further studies directed to determine the sterol profile during these physiological processes are necessary to correlate the observed phenotypes with specific changes on the different sterol fractions and to determine the specific role of glycosylated sterols. Either way, these preliminary results set the basis for new

research aimed to understand the molecular mechanisms through which sterols might regulates the mentioned physiological processes.

5. CONCLUSIONS

5. CONCLUSIONS

1. The *SlSGT1* gene encodes isozyme SlSGT1, which plays a major role in the biosynthesis of tomato SGs, since the downregulation of this gene by artificial miRNA-mediated gene silencing caused a marked reduction of glycosylated sterols, specifically SGs, in leaves and fruits.

2. *SlSGT1* silencing does not significantly alter the expression levels of the remaining members of the tomato *SlSGT* gene family (*SlSGT2-4*), which indicates that the reduced activity of SlSGT1 cannot be compensated by increasing the expression of any other SlSGT isozyme.

3. Downregulation of *SlSGT1* gene expression leads to decreased levels of the four major glycosylated sterol species in both leaves and fruits, but stigmasterol seems to be the preferred substrate of SlSGT1 for sterol glycosylation.

4. SlSGT1 is not involved in the glycosylation of tomatidine and dehydrotomatidine, the aglycones of the main tomato defense steroidal glycoalkaloids α-tomatine and dehydrotomatine.

5. *SlSGT1* plays an important role during tomato plant and fruit growth and development since the reduced levels of glycosylated sterols in amiSGT1 mutant plants have a negative effect on the size of both plants and fruits, likely affecting cell division and expansion, and on fruit seed production, which highlights the importance of maintaining normal amounts of this type of sterols for a right tomato plant growth and development.

6. The profile of glycosylated sterol levels throughout fruit development in wt and *SlSGT1* silenced plants strongly suggest that SGs are mostly synthesized at the green and mature green stages, where an active cell division and expansion that allows fruits to attain their maximum size occurs, whereas an active synthesis of ASGs seemingly occurs throughout all stages of fruit growth and ripening.

7. The reduction on glycosylated sterol content in *SlSGT1* silenced plants is paralleled by the accumulation of FS, most likely as a consequence of the

reduced ability of mutant plants to glycosylate sterols. The excess of FS in leaves and fruits can be accommodated without converting them into their esterified forms, as there is only a slight increase in the content of SE, but cannot replace the biological function of their glycosylated forms, since their accumulation cannot reverse the phenotypes observed in the silenced *SlSGT1* plants.

8. Whole genome expression analysis by RNA-seq shows that silencing of *SlSGT1* expression and the concomitant reduction of fruit SG levels induce a strong misregulation, mainly downregulation, of different sets of genes involved in hormone response, transcriptional regulation, storage of proteins and lipids, and cell wall biosynthesis and modification that are consistent with the reduced fruit size and seed yield phenotypes.

9. Glycosylated sterols play important but differential roles in tomato response to biotic and abiotic stress, since *SlSGT1* silencing increases the resistance against *B. cinerea* infection, but reduces the tolerance to stress induced by cold exposition.

10. Contrary to expectations, the leaves of transgenic tomato plants constitutively expressing the *SlSGT4* gene show lower levels of glycosylated sterols than wild type plants, which might be due to the concomitant reduction of *SlSGT1* transcript levels detected in these mutant plants.

11. Overexpression of *SlGT4* affects tomato plant and fruit growth and development, as well as the seed number and germination rate.

6. MATERIALS AND METHODS

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6.1 Plant material.

Tomato plants used in all the assays presented in this work were *Solanum lycopersicum* cultivar Micro-Tom

6.1.1 Tomato cv. Micro-Tom cultivation in soil.

Seeds were sown 5 mm deep in peat (Kalsmann TS2) previously moistened in small pots (5 cm x 5 cm x 6.5 cm), placed in plastic trays filled with water up to 1 cm high, and covered with plastic film to maintain 100% relative humidity (RH). Trays containing the pots were placed in the greenhouse under long day conditions (16h light / 8h dark) at 26-28°C (day) and 22-24°C (night). Once cotyledons appeared, approximately one week after planting seeds, the cover was drilled for air circulation and to start acclimation to greenhouse conditions. One week later, the cover was completely removed (RH of 60-70%). Once cotyledons were fully extended and the first true leaves appeared (3 weeks after planting), seedlings were transferred to larger pots (13 cm diameter x 10 cm height) and the substrate was replaced by a mixture of peat (Klasmann TS2), perlite and vermiculite (3:1:1). Plants were kept in the greenhouse under the same conditions until they completed their life cycle. The seeds were extracted from ripe red fruits, rinsed thoroughly with plenty of distilled water in a strainer, and placed on filter paper sheets for 3 to 4 days until complete dryness. Dry seeds were then collected in paper envelopes that were kept at room temperature.

6.1.2 Tomato cv Micro-Tom in vitro culture.

Seed for *in vitro* experiments were rinsed by immersion in sterile water for 15 min with constant shaking, and surface-sterilized by treatment with an antifungal solution of 0.3% (w/v) Captan50 (Bayer) for 5 min, followed by three 5 min washes in sterile water. Then, seeds were disinfected for 15 min with a solution of 40% (v/v) sodium hypochlorite, and washed again with sterile water. Sterilized seeds were sown on petri dishes containing semi solid (0.4% agar) MSO medium (0.5% Murashige and Skoog (MS) basal salts (pH 5.8) supplemented with Gamborg B5 vitamins (inositol 100 mg/L, nicotinic acid 1 mg/L, piridoxine 1 mg/L and thiamine 1 mg/L), and sucrose 3% [w/v]). Petri dishes were transferred to a growth chamber set for long day conditions (16h light/8h darkness) at an irradiance of 150 μmol^{-2} and 24°C.

6.1.3 Tomato cv Micro-Tom cultivation on hydroponic culture.

Tomato seeds were directly sown on 5 cm x 5 cm x 6 cm pots containing 100% vermiculite as plant support. Pots were place on trays flooded with 3 cm high water and then covered with a plastic film for 100% RH until seed germination in a growth chamber set for long day conditions. Once cotyledons were fully extended (around 1 week), the plastic fill was perforated for air circulation. Two days later, the cover was totally removed and water level was maintained at 3 cm.

6.2 Bacterial strains.

Escherichia coli TOP10. F– mcrA Δ (mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 recA1 araD139 Δ (ara-leu) 7697 galU galK λ– rpsL (StrR) endA1 nupG.

Agrobacterium tumefaciens GV3101::pMP90. Rifampicin and gentamicin resistant strain carrying the Ti plasmid pMP90 (pTiC58DT-DNA), used in the generation of transgenic tomato lines.

6.3 Culture media.

LB (Luria-Bertani): 10 g/L NaCl, 5 g/L yeast extract and 10 g/L tryptone at pH 7.4. Solid medium was prepared by adding 15 g/L of bacteriological agar.

YEB (Yeast Extract Broth): 1 g/L yeast extract, 5 g/L meat extract, 5 g/L peptone, 5 g/L sucrose and 0.5 g/L $MgSO_4 \cdot 7H_2O$ at 7.2 pH. Solid medium was prepared by adding 15 g/L of bacteriological agar.

Chemically competent TOP10 *E coli* cells were used for all the subcloning steps. Transformants were selected on LB plates supplemented with gentamycin (Table 5). *A. tumefaciens* GV3101 was used for tomato cotyledon transformation. Binary plasmids were introduced into this strain by thermal shock (see, section 6.7) Positive transformants were selected on YEB plates supplemented with kanamycin, rifampicin and gentamycin at the indicated working concentrations (Table 6). In all cases, the presence of the recombinant plasmid in the antibiotic-resistant bacterial colonies was checked by colony PCR.

Table 5. Antibiotics used for selection of bacterial cultures.

Antibiotic	Stock concentration	Working concentration
Kanamycin	25 mg/mL	25 µg/mL
Rifampicin	50 mg/mL	100 µg/mL
Gentamycin	100 mg/mL	25 µg/mL

6.4 Escherichia coli transformation by thermal shock.

Plasmid DNA (5-10 ng) or the product from the ligation or Gateway recombination reactions (5-10 µL) were added to 100 µL of competent *E. coli* cells, which were maintained on ice for 5 min, then incubated at 42°C for 30 seg and finally 2 min on ice. After adding 300 µL of LB medium, the mix was incubated at 37°C for one hour to allow cells recovery. Aliquots of 100 µL were plated onto solid LB medium supplemented with the appropriate antibiotics and incubated overnight at 37°C. Transformed colonies were identified by colony PCR.

6.5 Analysis of recombinant colonies by PCR.

Ten colonies from the transformation events were randomly selected from the corresponding bacterial plate culture and transferred with a sterile pipette tip to a new plate with the same medium as the original in order to replicate the colony and preserve it. The bottom of a PCR tube was rubbed with the same tip in order to transfer the remaining bacterial cells to the tube, whose genetic material would serve as a template in the PCR reaction. A reaction mixture containing 1 µL of each primer (10 mM stock solution), 5 µL of Green Taq Master Mix (Promega), and 3 µL of mQ water were added to the PCR tube to have a final volume of 10 µL. The PCR conditions were: 5 min of initial denaturation at 94°C followed by 30 to 32 cycles consisting of denaturation at 94°C for 30 sec, annealing at a temperature 3°C lower than the lowest Tm of the primers for 30 sec, 1 min/kb of extension at 72°C, and a final extension step at 72°C for 5 min. The resulting amplification products were analyzed by agarose gel electrophoresis.

6.6 Miniprep for plasmid DNA extraction.

Five mL of LB medium with the appropriate selection antibiotics (10 mL for low copy number vectors) were inoculated with an isolated colony containing the plasmid of interest and incubated at 37°C overnight with constant shaking at 180 rpm. Cells were harvested by centrifugation at 14,000 rpm for 30 sec. and plasmid DNA was purified using the Macherey-Nagel Nucleospin plasmid kit following the manufacturer's instructions.

6.7 Agrobacterium transformation by thermal shock.

Plasmid DNA (5-10 µL) was added to 100 µL of competent *A. tumefaciens* cells, which were maintained on ice for 5 min, then 5 min in liquid nitrogen and finally 5 min at 37°C. After, the bacterial cells were diluted in 1 mL YEB-medium and shaken at 120 rpm and 28°C during 2-4 h. Aliquots of 200 µL were plated on YEB containing appropriate antibiotics and incubated for 2 days at 28°C. Transformed colonies were identified by colony PCR as described above.

6.8 Generation of transgenic tomato lines overexpressing SlSGT4.

The open reading frame of *SlSGT4* was amplified by PCR as previously described in Ramírez-Estrada et al. (2017), and the amplification product was cloned into pENTR/pDONOR207 vector using Gateway technology (Invitrogen). The insert in pENTR-*SlSGT4* was sequenced to exclude the presence of amplification mutations, and subcloned into the binary vector pGWB414 by recombination. The coding sequence was flanked by the CaMV35S promoter and the *Nos* terminator sequence. The selection marker gene in the binary vector was *NPTII* that provides resistance to kanamycin.

Transgenic tomato lines were obtained by cotyledon transformation method (Wittmann *et al*., 2015) with some modifications. Seeds were sterilized as described previously (section 6.1.2). Cotyledons from 14 days old seedlings growing *In vitro* at 24°C were longitudinally cut into two pieces. Apexes and base were discarded and around 20 explants were placed adaxial surface down in Petri dishes with conditioning medium (0.43% MS, 3% sucrose, 100 mg/L inositol, 10 mg/L, 1 mg/L nicotinic acid, 1mg/L pyridoxine, and 0.8% agar. BAP [0.1 mg/L] and NAA [1 mg/L] were added after autoclaving) and incubated in darkness for 48h at 22°C.

A fresh overnight culture of *A. tumefaciens* GV3101 strain transformed with *35S:SlSGT4:Tnos* grown in LB medium at 28°C was centrifuged at 5,000 rpm for 15 min, and the pellet suspended in 10 mM $MgSO_4$ supplemented with 200 µM acetosyringone, to an OD_{595} of 1,0. Two droplets of the Agrobacterium solution were applied per cotyledon and they were co-cultivated for 48 h in the dark at 22°C. Cotyledons were then placed abaxial surface down on a shooting selection medium 1 (SM1) (0.43% MS, 3% sucrose, 100 mg/L inositol, 10 mg/L thiamine, 1 mg/L nicotinic acid, 1 mg/L piridoxine and 0.8% agar at pH 5.8; zeatin [1 mg/L], timentin [250 mg/L] and kanamycin [35 mg/L] were added after autoclaving) in small crystal jars. After 15-20 days, cotyledons were transferred to selection medium 2 (SM2) (the same recipe as SM1 but supplemented with 50 mg/L kanamycin). One-month later, kanamycin was increased to a concentration of 100 mg/L for selection medium 3 (SM3). Finally,

shoots generated from callus were transferred to rooting medium (0.43% MS, 3% sucrose, 100 mg/L inositol, 10 mg/L, 1 mg/L nicotinic acid, 1mg/L pyridoxine and 0.8% agar. IAA [0.1 mg/L], vancomycin [500 mg/L] and kanamycin [20 mg/L] were added after autoclaving). After roots were developed, plantlets were transferred to soil and cultivated in plastic incubators for acclimation.

6.9 Acclimatization of tomato plants from in vitro culture.

The seedlings were removed from the medium and the root was washed with running water to remove medium and callus residues. Seedlings were immediately sown in moistened substrate (Klasmann TS2) contained in small pots, which were placed in plastic incubators (mini-greenhouse cabin) to maintain humidity under long day conditions (16h light/8h dark) at 26-28°C (light) and 22-24°C (dark). After one week, one window of the incubator was opened and a second one was opened a week later. Finally, further one more week the lid was partially lifted to allow gradual homogenization of temperature and humidity conditions between the interior of the incubator and the greenhouse. One week later, the cover was completely removed and plants were transferred to larger pots as described above.

6.10 Genotyping of transgenic tomato plants.

The genomic DNA used for genotyping transgenic tomato lines was obtained from leaf tissue using the cetyltrimethyl ammonium bromide (CTAB) method. Fresh material was frozen in liquid nitrogen, grinded to obtain a fine powder, and mixed with 600 µL of ice-cold extraction buffer (50 mM Tris-HCl, pH 8.0 and 20 mM EDTA). After adding 80 µL of SDS 10% (w/v), samples were vortexed and incubated at room temperature for 15 min. Then, 180 µL of 3M NaAc, pH 5.2, were added to the mix and incubated on ice for 30 min. Samples were centrifuged at 10,000 rpm for 15 min and the supernatant was transferred to a new tube. The same volume of isopropanol was added and the mixture incubated on ice for 30 min. Samples were centrifuged at 10,000 rpm for 10 min and the pellet was suspended in 300 µL 10 mM Tris-HCl, pH 8.0. After that, samples were mixed with 300 µL CTAB (2% CTAB [w/v], 2M NaCl, 0.2 M Tris-HCl, pH 8.0, and 0.05 M EDTA) and incubated at 65°C for 15 min. Finally, 600 µL of chloroform were added, mixed, and centrifuged for 5 min at maximum speed. The aqueous phase was recovered, mixed with the same volume of isopropanol, and incubated at -20°C for 2 hours. Samples were centrifuged at maximum speed for 10 min and the dried DNA pellet suspended with 50 µL of water. Genomic DNA integrity was checked by PCR amplification of a fragment of the actin gene (*SlACT*, Solyc03g078400) (Table 6). DNA samples were stored frozen at -80°C until use.

Table 6. **Primers used to check DNA integrity.**

Name	Sequence	Gene
Actin.fw	CCTTCCACATGCCATTCTCC	Actin (Solyc03g078400)
Actin.rv	CCACGCTCGGTCAGGATCT	

The presence and integrity of the whole *35S:SlSGT4:Tnos* transgene was assessed by PCR using two different sets of primers: (a) 35S promoter forward primer and internal *SlSGT4* reverse primer, and (b) internal *SlSGT4* forward primer and reverse Tnos terminator primer (Table 7). The transgene copy number in the different plant generations was determined by qPCR as described in (Yang *et al.*, 2005), using the tomato *LAT52* gene (Solyc10g007270) as an endogenous single copy gene and *NPTII* as target gene. Transgene copy number in each sample was calculated using the equation proposed by Ingham et al., (2001):

$$\text{Copy number} = 2^{(\Delta\Delta Ct)}$$

Where, ΔΔCt = ΔCt (reference gene [*LAT52*])- ΔCt (target gene [*NTPII*])

For instance, if a plant contains a single copy of the transgene (heterozygous), the $2^{(\Delta\Delta Ct)}$ must be equal or close to 0.5. Thus, in the next generation the corresponding homozygous plants for the transgene will display $2^{(\Delta\Delta Ct)}$ values equal or close to 1.0.

Once transgenic plants with a single copy of the transgene in the T0 generation were identified, they were kept in the greenhouse until red ripe fruits could be harvested. The seeds collected from these fruits were sown and grown in soil as described in section 6.1.1 to obtain the next plant generation. Three generations were analyzed to assure transgene homozygosity in segregating plants.

Table 7. **Primers used for genotyping and transgene copy number quantification in transgenic tomato plants.**

Name	Target sequence	Sequence
qPCR.35S.fw	*CaMV35S* promoter	CTATCCTTCGCAAGACCCTTC
SGT4 qpcr fw	*SlSGT4*	GGGATTCGGGATGTGGTAAA
SGT4 qpcr rev		AAAGGCACATCTGAATGGAAAC
Tnos rev	*Nos* terminator	TGGGTACCCGATCTAGTAACATAG
Lat1	*LAT52*	AGACCACGAGAACGATATTTGC
Lat2.1		GCCTTTTCATATCCAGACACAC
Ntp 1-5	*NPTII*	GACAGGTCGGTCTTGACAAAAAG
Ntp 1-3		GAACAAGATGGATTGCACGC

6.11 Gene expression analysis by RNA-Seq.

For RNA-seq expression analysis of leaves, amiSGT1-31.2 and wt plants were grown under greenhouse conditions as described above (section 6.1.1). Three biological replicates, each consisting of pooled third and fourth leaves from 10 one-month-old tomato plants, were prepared for RNA extraction. For RNA-seq of fruits, newly opened flowers of amiSGT1-31.2, amiSGT1-61.1 and wt plants were labeled and fruits were collected 9 days after anthesis (DAA). Three independent pools consisting of 10 whole fruits per replicate were collected for each genotype. Samples were frozen and ground in liquid nitrogen to obtain a fine powder. Total RNA was isolated from 100 mg of each sample using the Maxwell RSC Plant RNA Kit with the Maxwell RSC Instruments (Promega) according to manufacturer's instructions (see section 6.12). Total RNA concentration was measured using Qubit® 2.0 Fluorometer (Life Technologies), and integrity was assessed using the RNA Nano 6000 Assay Kit with a Bioanalyzer 2100 system (Agilent Technologies). RNA sequencing and data analysis was performed by Sequentia Biotech SL (Barcelona, Spain). A total of 3 µg of RNA per sample was used as the input material for the RNA sample preparations. Libraries were prepared according to Illumina protocols and sequenced using an Illumina HiSeq 2500 machine to perform 2 x 75 bp paired-end sequencing. The quality of the reads obtained by HiSeq 2500 sequencing was checked with FastQC software (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). The reads were aligned against the *S. lycopersicum* (SL3.0) reference genome with STAR aligner (version 2.5.2b). FeatureCounts (version 1.5.1) was used to calculated gene expression values as raw fragments counts (annotation version ITAG 3.2) Normalization was applied to the raw fragment counts by using the Trimmed Mean of M-values (TMM) and Fragments per Kilobase Million (FPKM) normalization. All the statistical analyses were

performed with R with the packages HTSFilter, edgeR and NOISeq. No expressed genes and the ones showing high variability were removed. The HTSFilter package was chosen for this scope, which implements a filtering procedure for replicated transcriptome sequencing data based on a Jaccard similarity index.

6.12 Gene expression analysis by RT-qPCR.

Total RNA was isolated from around 100 mg of ground frozen tissue (tomato leaves, roots or fruits). RNA was quantified by NanoDrop and its quality was assessed by electrophoresis on 0.8% agarose gels. RNA samples were stored frozen at -80 ºC until use. The cDNA synthesis reaction by reverse transcription (RT) was performed from 1 µg of total RNA from each sample using the NZY First-Stand cDNA Synthesis kit (NZYtech) according to the manufacturer's instructions. The reaction mixture, consisting of 10 µL of NZYRT 2X Master Mix (includes oligo (dT) 18, random hexamers, $MgCl_2$, and dNTPs), 2 µL of NZYRT Enzyme Mix (includes NZY Reverse transcriptase and NZY Ribonuclease Inhibitor), 1 µg of total RNA and DEPC-treated water up to a final volume of 20 µL, was incubated in a thermocycler at 25°C for 10 min, followed by 30 min at 50°C. The reaction was stopped by heating the mixture at 95°C for 5 min. and then cooled on ice. Finally, 1 µL of NZY RNase H (*E. coli*) was added to the mixture and incubated at 37°C for 20 min. The integrity of the cDNA was verified by the amplification of a fragment of the actin gene (*SlACT*) using primers shown in Table 6.

Quantitative PCR analysis was performed with a LigthCycler 480 Real Time PCR System (Roche) in a total volume of 10 µL containing 5 µL LigthCycler 480 SYBER Green I Master Mix (Roche), 0.25 µM of each specific forward and reverse primers (Table 8), 2.5 µL water, and 2 µL of ten-fold diluted cDNA sample. The LigthCycler experimental run protocol used was: 95°C for 10 seg, 60°C for 30 seg, and a final cooling step at 4°C, the raw PCR data from the LigthCycler software 1.5.0 was used in the analysis. Normalized transcript abundances were calculated as follows: $\Delta Ct = Ct\ target - Ct\ reference.$

The fold-change value was calculated using the $2^{-\Delta Ct}$ expression (Livak and Schmittgen, 2001) using tomato actin gene as an endogenous reference gene.

Table 8. **Primers used for RT-qPCR**

Name	Sequence	Gene
SGT1qpcr for	TAGGCCCTCAACCCATAGAA	*SlSGT1* (Solyc06g007980)
SGT1qpcr rev	TCAAATGGACCGCTCTTATC	
SGT2qpcr for	CCTCGATCTTGCATCTGGTTAT	*SlSGT2* (Solyc09g061860)
SGT2qpcr rv	GGCTCTTGAACAGGAAGACTAC	
SGT3qpcr for	CATGTTGTGCCAAAGCCTAAA	*SlSGT3* (Solyc04g071540)
SGT3qpcr rev	CTTCAGGAGGTTGGTAGTTTCTC	
SGT4qpcr for	GGGATTCGGGATGTGGTAAA	*SlSGT4* (Solyc04g051150)
SGT4qpcr rev	AAAGGCACATCTGAATGGAAAC	
qPCR.Actin fw	CCTTCCACATGCCATTCTCC	*SlACT* (Solyc03g078400)
qPCR.Actin rv	CCACGCTCGGTCAGGATCT	
Solyc02g086490_Fwd	TGTACCTGCTACGCTTACTATTG	Oleosin (Solyc02g086490)
Solyc02g086490_Rev	CTCTCTGGGACCATCGATTTAC	
Solyc02g094460_Fwd	GAGACTACTTTGCACTCCACTAC	B3-domain-transcription factor (Solyc02g094460)
Solyc02g094460_Rev	CCGCTTCATAACCTCGGAATAC	
Solyc03g112440_Fwd	GGCTGTCACCGGATTTTTGAC	Oleosin (Solyc03g112440)
Solyc03g112440_Rev	CCATCTGCTCTCCACCTGTT	
Solyc03g115300_Fwd	GGAGGTGTTGGAGATGTTGT	Expansin (Solyc03g115300)
Solyc03g115300_Rev	GTCCTCCCAACTGCACATTA	
Solyc04g071070_Fwd	CCACCACCAGTGCATGTCTA	Extension (Solyc04g071070)
Solyc04g071070_Rev	TGTATACCGGGGTGTGTGGA	
Solyc05g005680_Fwd	GTGTTCACACTGGAAGAGAAGA	Xyloglucan endotransglucosylase/ hydrolase 2 (Solyc05g005680)
Solyc05g005680_Rev	CTGGTACATTGTCCACGAGAAAT	
Solyc07g064190_Fwd	ACTTCGAGTTGGAGCTGATATG	Pectinesterase (Solyc07g064190)
Solyc07g064190_Rev	TGTCACGTAGGAGTCTCGATAG	
Solyc08g062580_Fwd	GAGGGTGTGCAAAGATTGTC	Pentatricopeptide repeat-containing protein family (Solyc08g062580)
Solyc08g062580_Rev	CTGCAAGAGCACAAACCTTCTT	
Solyc09g025210_Fwd	GCAGTGCTAGATGACAGAGTAAG	11S Globulin CRU4 (Solyc09g025210)
Solyc09g025210_Rev	GAACACCACCCACTCAAAGA	
Solyc09g065470_Fwd	TCGGCGTAACTTCCACAAA	7S globulin (Solyc09g065470)
Solyc09g065470_Rev	CCCTCTCCAGTTGACTCATTAC	

6.13 Sterol analysis.

All plant tissue samples were collected, frozen in liquid nitrogen, ground to a fine powder and lyophilized prior to use. When necessary, grinded samples prior to lyophilization can be stored until further use at -80°C. Around 20-25 mg of the lyophilized tissue was placed in a pyrex glass tube (10 x 15 mm) and 200 µL of a mix of internal standards containing 5 µg of cholestanol (FS), 5 µg of palmitoyl-cholestanol

(SE), 5 µg of cholestanyl-β-D-glucoside (SG) and 5 µg of palmitoyl-β-D- glucosyl-cholestanol (ASG) dissolved in dichloromethane-methanol 2:1 was added. Sterols were then extracted with 3 mL of a dichloromethane-methanol solution (2:1). Samples were vigorously homogenized and sonicated for 10 min at room temperature (25-28°C). Then, 1.5 mL of 0.9% (w/v) NaCl were added to facilitate further phase separation. The organic phase was recovered by centrifugation at 5,000 rpm for 5 min in a JA-20 rotor (Beckman Coulter) and filtered through a Pasteur pipette with a glass wool filter. The remaining aqueous phase was re-extracted with 3 mL of the dichloromethane-methanol solution (2:1) and the two organic extracts were mixed together and evaporated to dryness using a SpeedVac® Concentrator (Savant). The dried residue was dissolved in 150 µl of chloroform and the four sterol fractions were separated by thin-layer chromatography (TLC) using precoated silica gel PLC 60 F254 plates (20 cm x 20 cm) (Merck) and a dichloromethane-methanol-acetic acid (92:8:2) mix as a mobile phase. A mix of the respective standards was also applied onto the TLC plates as markers. For fraction visualization, plates were sprayed with a 0.01% primuline (Sigma-Aldrich) solution and detected with a UV lamp. All fractions were separately scraped from the silica plates and placed in a glass tube. For the acidic hydrolysis of SG and ASG, 1.5 mL of a 0.5 N HCl methanolic solution was added to the silica powder, while for the basic hydrolysis of SE, 1.5 mL of 7.5% (w/v) KOH methanolic solution was used. After incubation at 85°C for 2 h, the reaction was stopped with 1.5 mL of 0.9% (w/v) NaCl, and the released FS moieties were extracted twice with 3 mL of n-hexane. The mixture was centrifuged at 5,000 rpm for 5 min and the hexanic phases were collected in a new tube, mixed and evaporated to dryness. Sterol samples were resuspended in 50µl of tetrahydrofuran (THF) and derivatized by adding 50 µl of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Regis technologies). The mix was incubated 20 min at 60°C, evaporated to dryness, and the residue dissolved in 50 µl of isooctane and transferred to 1.5 mL amber glass vials (VWR) with 0.1 mL glass insert (VWR). Samples were then analyzed by GC-MS using an Agilent 7890A gas chromatograph equipped with a Sapiens-X5ms capillary column (30 m x 0.25 mm x 0.25µm) (Teknokroma) coupled with a 5975C mass spectrometer (Agilent). The oven was programmed as follows: injection temperature of 70°C with a temperature increase from 70°C to 255°C at 20°C per min during 40 min, 255°C to 300°C at 3°C per min during 5 min. Total time of the program was 69.75 min. Helium was used as carrier at a pressure of 8,8085 psi. Using a 10 µL syringe, 1 µL of sample was injected in Split mode (5:1) at 270°C for all sterol fractions (FS, SE, SG and ASG). The mass spectrometer was operated in scan mode with an electron impact source operated at 70 eV. The ion source temperature was 230°C and 280°C at the interface.

Peaks were identified in the chromatographic profile based on the relative retention times and the comparison of the mass spectra of the different peaks whit those of known standard and/or those available in the database incorporated in the program software. The content of each sterol was calculated by integrating the area of the peaks of interest using the MSD Productivity ChemStation (Agilent) program, which was normalized to the peak area of the internal standard 5α-cholestanol. The concentration of the different sterols was expressed in µg of sterols per mg of dry weight of tissue, according to the formula:

$$\frac{\text{µg of sterol}}{\text{mg of dry weight}} = \frac{\text{Sterol peak area}}{\text{Standard peak area}} \times \frac{\text{Concentration of standard (µg)}}{\text{Sample weight (mg)}}$$

6.14 Botrytis cinerea bioassays

Three-week-old tomato plants grown under greenhouse conditions, as previously described (6.1.1), were used for *Botrytis cinerea* bioassay. The necrotrophic fungus used for tomato plant infections was *B cinerea* CECT2100 (Spanish collection of type cultures, Universidad de Valencia, 46100 Burjassot, Spain). It was cultured in oatmeal agar (Difco) at 24°C. *B. cinerea* spores were collected from 15-20 days old cultures with sterile water, filtered through a Pasteur pipette with a glass wool filter, quantified with a hemacytometer and adjusted to a spore concentration of 1.8×10^7 mL^{-1}. Conidial collected from 15-20 days old oatmeal agar were maintained in Gambor's B5 medium (Duchefa, Haarlem, The Netherlands) supplemented with 10 mM sucrose and 10 mM KH_2PO_4 for 2 h in the dark with no shaking (Flors *et al.*, 2007).

The third and fourth leaves of 3-weeks-old plants at 100% RH were inoculated by applying two 6 µL droplets of a suspension of *B. cinerea* spores (1.8×10^7 mL^{-1}) per leaf. Plants inoculated with Gambor´s B5 medium supplemented with 10 mM sucrose and 10 mM KH_2PO_4 were used as non-infected (mock) control. Disease symptoms were assessed 72h after inoculation by (1) measuring the average lesion diameter in 15 plants per genotype, and (2) quantitative analysis of the amount of *B. cinerea* DNA in the infected leaves. For genomic DNA extraction, the leaf necrotic areas were cut into discs of 14 mm diameter, which were immediately frozen in liquid nitrogen, ground to a fine powder and extracted using the CTAB method (as described in section 7.10). Samples were stored frozen at -80°C until use. DNA quantifications were conducted by qPCR using 100 ng of total gDNA as a template and specific primer pairs for *B. cinerea* β-tubulin gene (GenBank: KC620303) and the tomato actin (*SlACT*,

Solyc11g005330.1), which was used as reference endogenous gene (Table 9) (Ueda *et al.*, 2018).

Table 9. **Primers used for *B. cinerea* gDNA quantification.**

Name	Sequence	Gene
Inf.Actin FW	CCAGGTATTGCTGATAGAATGAG	*SlACT*, Solyc11g005330
Inf.Actin RV	GAGCCTCCAATCCAGACAC	
Inf.β-tubulin fw	GTTACTTGACATGCTCTGCCATT	*B. cinerea* β-tubulin DNA (accession no. KC620303)
Inf.β-tubulin rv	CACGGCTACAGAAAGTTAGTTTCTACAA	

6.15 Cold stress assay.

6.15.1 In vitro cold stress assay.

Tomato seeds were surface-sterilized as described in section 6.1.2, sown on Petri dishes (150 mm x 20 mm) containing MS medium and maintained at 24°C under long day photoperiod conditions until full expanded cotyledons were developed (7 days). Next, 10-15 seedlings were transferred to new plates (150 mm x 25 mm) containing fresh MS medium (6 plates/genotype) and maintained during 5 days at the same growing conditions. After this time, 3 plates per genotype were chilled at 0°C in continuous light (100 mmol m^{-2} s^{-1}) for three days and returned to previous growing conditions during one day for recovery, while the remaining 3 plates were maintained in the same growing conditions and used as a control. Samples were collected from non-chilled and chilled plants to determine cold tolerance by electrolyte leakage. For this, seedlings were carefully pulled out of the agar and rinsed with deionized water to discard remaining agar. One seedling was subsequently placed in a glass tube (16 mm diameter) containing 7 mL of deionized water and shaken at 320 rpm for 1 h. After, the initial conductivity (C_0) was measured using a Basic 30 conductivity meter (Crison Instruments). Next, tubes were boiled for 30 min and shaken again for 1 h, as above, before measuring the final conductivity (C_F). The relative membrane damage was represented as a percentage of the total leakage for boiled samples (% ion leakage), and was determined by the equation:

$$\textit{Ion leakage (\%)} = \left(\frac{C0}{CF}\right) X\ 100$$

6.15.2 In vivo cold stress assay.

Tomato seeds were directly sown on pots containing 100 % peat (Klasman T2) and cultivated as described in 6.1.1. For cold stress assay, 12-days-old plants were separated into two groups before chilling treatment: non-acclimated and acclimated.

Non-acclimated plants were chilled at 4°C for 3 days in the dark. For the acclimation treatment, plants were transferred to a growth chamber set for a gradual drop in temperature of 15°C for 3 days, 12°C for 3 days, followed by 10°C for 3 days with a 12-hour photoperiod, before chilling at 4°C for 3 days in the dark. After the chilling treatments, both sets of plants were returned to the initial growing conditions and allowed to recover for four days. Cold sensibility was expressed as percentage of plant cold-damage phenotype, considering 3 possibilities: plants dead, plants that survive but present damage symptoms in the leaves (survivor 2) and plants that survive without damage symptoms (survivor 1).

6.16 Tomato seed germination assay.

Germination assays were carried out by sowing 50 seeds, sterilized as previously described (section 6.1.2), in Petri dishes (150 mm diameter and 25 mm high) containing semi solid MSO medium (0.5% Murashige and Skoog basal salts supplemented with Gamborg B5 vitamins and sucrose 3% (w/v), adjusted to pH 5.8 and solidified with 0.4% agar). Seed germination, defined as the radicle emergence through the seed coat, was monitored daily during 13 days.

6.17 Histological analysis.

The fifth internode of one-month-old plants was carefully cut and placed in fixing solution (FAA; 10% formaldehyde (37%), 5% glacial acetic acid, 50% absolute ethanol and 35% water) for 1 h on ice under vacuum to favor tissue penetration of the solution. Vacuum was cut and restored every 20 min. Then, FAA solution was replaced by fresh FAA solution and samples stored at 4ºC for 24 h. After, FAA solution was discarded, replaced with 10% ethanol and kept under gentle shaking at 4°C for 1 h. Ethanol concentration was progressively increased to 30%, 50%, 70%, and 95 % (1 h at 4°C with gentle shaking at each concentration), and finally 95% ethanol solution was refreshed and samples stored overnight at 4°C. Then, 95% ethanol solution was discarded and samples were transferred to absolute ethanol which was refreshed every 30 min over the next 2 hours. Samples were transferred to a histoclear/absolute ethanol solution (1:2 v/v) and stored at 4°C with gentle shaking for 1 h, then were sequentially incubated at 4ºC with shaking for 1 h in 1:1 and 2:1 histoclear/absolute ethanol and finally in 100% histoclear. Then, samples were transferred to a solution 50% histoclear and 50% Paraplast® (Merck) and incubated at 60ºC for 1 h. Samples were subsequently transferred to 100% paraplast and incubated overnight at 60°C. Finally, samples were embedded in paraffin, transversally cut into 8 µm slices, and placed on microscope slides. Paraplast was removed by successive immersions in 100% histoclear (Natural Diagnostics), absolute ethanol, 95% ethanol, 80% ethanol,

and 70% ethanol for 5 min each. The cells were stained with 0.5% toluidine blue (w/v), and excess dye was washed with water. For the final assembly, 1-2 drops of 50% glycerol were placed on the samples, coverslips were placed on top and sealed with nail varnish. Histological sections were observed under the 5X lens of a Leica DM6 microscope.

6.18 Morphometric analysis.

Morphological traits as plant size, foliar area, fruit diameter, cell diameter and all the morphometric parameters were measured using ImageJ software.

7. BIBLIOGRAPHY

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8. SUPPLEMENTAL MATERIAL

8. SUPPLEMENTAL MATERIAL.

Supplemental Table 1. **Sterol composition in tomato leaves of wt and ami*SlSGT1* lines**

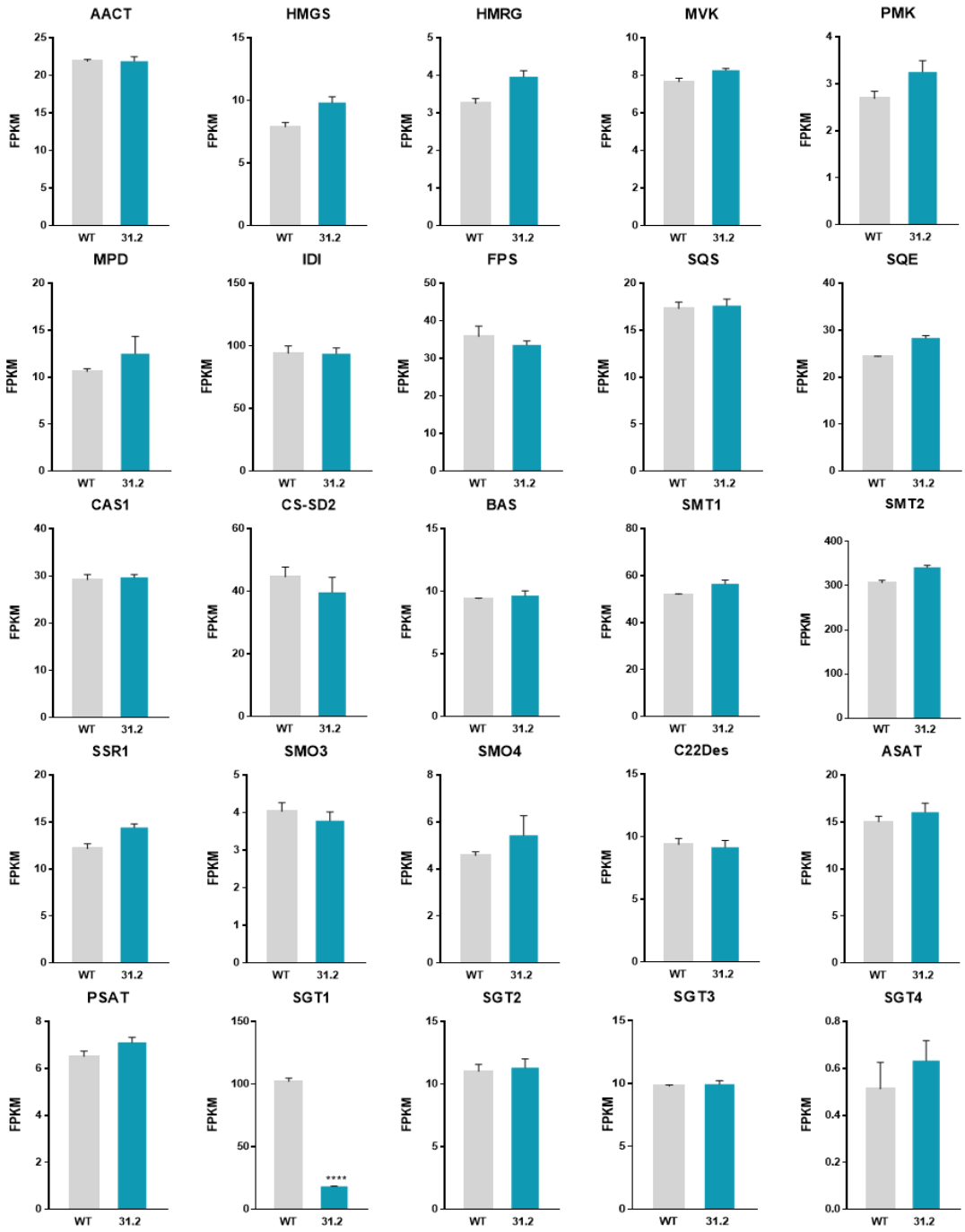
	SG	ASG	(SG+ASG)	FS	SE	Total
Wt	**0.40**[a] ±0.05	**1.51**[a] ±0.31	**1.92**[a] ±0.05	**0.22**[a] ±0.02	**0.10**[a] ±0.00	**2.22**[a] ±0.03
31.2	**0.27**[b] ±0.01	**1.41**[a] ±0.36	**1.67**[b] ±0.38	**0.35**[b] ±0.01	**0.12**[a] ±0.01	**2.12**[b] ±0.03
61.1	**0.23**[b] ±0.02	**1.37**[a] ±0.07	**2.05**[b] ±0.12	**0.34**[b] ±0.06	**0.12**[a] ±0.20	**2.18**[b] ±0.00

Average values are expressed as mean µg sterol per mg of dry weigth of leaf tissue and ±SEM from three biological replicates with three technical replicates each (n=9). Different letters (a or b) represented significant differences determined by t-test (*P<0.05).

Supplemental Table 2. **Major sterol species composition in tomato leaves of wt and ami*SlSGT1* lines *SlSGT1***

	Wt	**31.2**	**61.1**
		SG	
Cholesterol	**0.080**[a] ±0.00	**0.058**[b] ±0.00	**0.060**[b] ±0.00
Campesterol	**0.029**[a] ±0.00	**0.016**[b] ±0.00	**0.014**[b] ±0.00
Stigmasterol	**0.103**[a] ±0.00	**0.044**[b] ±0.00	**0.045**[b] ±0.00
β-sitosterol	**0.285**[a] ±0.02	**0.153**[b] ±0.01	**0.140**[b] ±0.01
		ASG	
Cholesterol	**0.324**[a] ±0.02	**0.292**[a] ±0.01	**0.296**[a] ±0.01
Campesterol	**0.051**[a] ±0.00	**0.068**[a] ±0.02	**0.052**[a] ±0.00
Stigmasterol	**0.634**[a] ±0.05	**0.487**[b] ±0.02	**0.478**[b] ±0.02
β-sitosterol	**0.632**[a] ±0.03	**0.581**[a] ±0.03	**0.569**[a] ±0.02
		FS	
Cholesterol	**0.050**[a] ±0.00	**0.064**[b] ±0.00	**0.065**[b] ±0.00
Campesterol	**0.009**[a] ±0.00	**0.013**[b] ±0.00	**0.014**[b] ±0.00
Stigmasterol	**0.078**[a] ±0.00	**0.151**[b] ±0.01	**0.149**[b] ±0.01
β-sitosterol	**0.077**[a] ±0.00	**0.119**[b] ±0.01	**0.116**[b] ±0.02
		SE	
Cholesterol	**0.036**[a] ±0.00	**0.036**[a] ±0.00	**0.039**[a] ±0.00
Campesterol	**0.012**[a] ±0.00	**0.013**[a] ±0.00	**0.013**[a] ±0.00
Stigmasterol	**0.005**[a] ±0.00	**0.008**[b] ±0.00	**0.010**[b] ±0.00
β-sitosterol	**0.050**[a] ±0.00	**0.059**[a] ±0.00	**0.058**[a] ±0.00

Average values are expressed as mean µg sterol per mg of dry weigth of leaf tissue and ±SEM from three biological replicates with three technical replicates each (n=9). Different letters (a or b) represented significant differences determined by t-test (*P<0.05).



Supplemental Figure 1. **Transcript levels of genes involved in the sterol metabolic pathway (leaves).** Values are represented as fragments per kilobase of exon per million mapped (FPKM) obtained by RNA-seq analysis of the third and fourth leaves of one-month-old plants. Data shown is the mean ± SEM of three biological replicates. Asterisk represents statistical significance determined by t-test (****, $P \leq 0.0001$)

***Supplemental Table 3*. Sterol composition in tomato fruits of wt and ami*SlSGT1* lines**

	Green	Mature green	Breaker +1	Red
		SG		
wt	**0.332**[a] ±0.02	**0.258**[a] ±0.01	**0.160**[a] ±0.01	**0.091**[a] ±0.00
31.2	**0.165**[b] ±0.00	**0.178**[b] ±0.00	**0.174**[a] ±0.01	**0.060**[a] ±0.01
61.1	**0.172**[b] ±0.01	**0.175**[b] ±0.02	**0.156**[a] ±0.02	**0.065**[a] ±0.00
		ASG		
wt	**0.599**[a] ±0.00	**0.538**[a] ±0.02	**0.510**[a] ±0.00	**0.596**[a] ±0.05
31.2	**0.496**[a] ±0.04	**0.420**[a] ±0.04	**0.397**[a] ±0.01	**0.503**[a] ±0.06
61.1	**0.529**[a] ±0.05	**0.420**[a] ±0.02	**0.430**[a] ±0.03	**0.401**[a] ±0.04
		FS		
wt	**0.025**[a] ±0.00	**0.028**[a] ±0.00	**0.045**[a] ±0.02	**0.028**[a] ±0.00
31.2	**0.046**[b] ±0.00	**0.071**[b] ±0.01	**0.074**[a] ±0.00	**0.039**[a] ±0.00
61.1	**0.050**[b] ±0.00	**0.090**[b] ±0.02	**0.070**[a] ±0.00	**0.044**[a] ±0.00
		SE		
wt	**0.011**[a] ±0.00	**0.092**[a] ±0.02	**0.055**[a] ±0.06	**0.002**[a] ±0.00
31.2	**0.012**[a] ±0.00	**0.101**[a] ±0.02	**0.058**[a] ±0.06	**0.004**[a] ±0.00
61.1	**0.012**[a] ±0.00	**0.090**[a] ±0.00	**0.050**[a] ±0.05	**0.006**[a] ±0.00
		Total sterols		
wt	**0.951**[a] ±0.01	**0.811**[a] ±0.02	**0.670**[a] ±0.02	**0.687**[a] ±0.05
31.2	**0.660**[a] ±0.32	**0.598**[a] ±0.06	**0.561**[a] ±0.01	**0.507**[a] ±0.01
61.1	**0.708**[a] ±0.07	**0.595**[a] ±0.04	**0.587**[a] ±0.05	**0.508**[a] ±0.01

Values are expressed as mean µg sterol per mg of dry weigth of fruits and ±SEM from three technical replicates each (n=3). Different letters (a or b) represented significant differences determined by t-test (*P<0.05).

Supplemental Table 4*. Detailed sterol composition by sterol species in tomato leaves of wt and silencing lines *SlSGT1

		Green	Mature green	Breaker +1	Red
			SG		
Cholesterol	wt	**0.018**[a] ±0.00	**0.016**[a] ±0.00	**0.006**[a] ±0.01	**0.003**[a] ±0.00
	31.1	**0.012**[b] ±0.00	**0.011**[b] ±0.00	**0.006**[a] ±0.00	**0.003**[a] ±0.00
	61.1	**0.013**[b] ±0.00	**0.010**[b] ±0.00	**0.006**[a] ±0.00	**0.003**[a] ±0.00
Campesterol	wt	**0.032**[a] ±0.00	**0.018**[a] ±0.00	**0.014**[a] ±0.00	**0.008**[a] ±0.00
	31.1	**0.013**[b] ±0.00	**0.012**[b] ±0.00	**0.011**[a] ±0.00	**0.006**[a] ±0.00
	61.1	**0.011**[b] ±0.00	**0.011**[b] ±0.00	**0.007**[b] ±0.00	**0.006**[a] ±0.00
stigmasterol	wt	**0.038**[a] ±0.00	**0.039**[a] ±0.00	**0.032**[a] ±0.00	**0.025**[a] ±0.00
	31.1	**0.019**[b] ±0.00	**0.022**[b] ±0.00	**0.026**[a] ±0.00	**0.020**[a] ±0.00
	61.1	**0.022**[b] ±0.00	**0.023**[b] ±0.00	**0.031**[a] ±0.00	**0.017**[b] ±0.00
β-sitosterol	wt	**0.233**[a] ±0.02	**0.188**[a] ±0.01	**0.108**[a] ±0.01	**0.051**[a] ±0.00
	31.1	**0.128**[b] ±0.00	**0.133**[b] ±0.00	**0.127**[a] ±0.01	**0.026**[a] ±0.00
	61.1	**0.106**[b] ±0.01	**0.127**[b] ±0.01	**0.112**[a] ±0.02	**0.042**[a] ±0.00
			ASG		
Cholesterol	wt	**0.045**[a] ±0.00	**0.023**[a] ±0.00	**0.016**[a] ±0.00	**0.021**[a] ±0.00
	31.1	**0.029**[b] ±0.00	**0.018**[a] ±0.00	**0.016**[a] ±0.00	**0.020**[a] ±0.00
	61.1	**0.036**[b] ±0.00	**0.019**[a] ±0.00	**0.020**[a] ±0.00	**0.020**[a] ±0.00
Campesterol	wt	**0.026**[a] ±0.00	**0.020**[a] ±0.00	**0.031**[a] ±0.00	**0.046**[a] ±0.00
	31.1	**0.018**[b] ±0.00	**0.017**[a] ±0.00	**0.019**[b] ±0.00	**0.039**[a] ±0.00
	61.1	**0.019**[a] ±0.00	**0.017**[a] ±0.00	**0.025**[a] ±0.00	**0.043**[a] ±0.00
Stigmasterol	wt	**0.152**[a] ±0.01	**0.092**[a] ±0.00	**0.118**[a] ±0.01	**0.205**[a] ±0.00
	31.1	**0.108**[b] ±0.01	**0.073**[a] ±0.01	**0.084**[b] ±0.01	**0.172**[b] ±0.01
	61.1	**0.133**[a] ±0.01	**0.082**[a] ±0.00	**0.104**[a] ±0.01	**0.181**[b] ±0.00
β-sitosterol	wt	**0.376**[a] ±0.01	**0.403**[a] ±0.03	**0.345**[a] ±0.02	**0.265**[a] ±0.01
	31.1	**0.340**[a] ±0.03	**0.312**[b] ±0.03	**0.272**[b] ±0.01	**0.217**[a] ±0.01
	61.1	**0.341**[a] ±0.04	**0.302**[b] ±0.02	**0.282**[a] ±0.02	**0.197**[a] ±0.00

Continue next page (1-2)

Continuation of S. table 4.

		Green	Mature green	Breaker +1	Red
			FS		
Cholesterol	wt	**0.003**[a] ±0.00	**0.004**[a] ±0.00	**0.004**[a] ±0.00	**0.002**[a] ±0.00
	31.1	**0.004**[a] ±0.00	**0.006**[b] ±0.00	**0.003**[a] ±0.00	**0.002**[a] ±0.00
	61.1	**0.004**[a] ±0.00	**0.007**[b] ±0.00	**0.003**[a] ±0.00	**0.002**[a] ±0.00
Campesterol	wt	**0.002**[a] ±0.00	**0.002**[a] ±0.00	**0.004**[a] ±0.00	**0.003**[a] ±0.00
	31.1	**0.002**[a] ±0.00	**0.002**[a] ±0.00	**0.004**[a] ±0.00	**0.005**[a] ±0.00
	61.1	**0.002**[a] ±0.00	**0.003**[a] ±0.00	**0.004**[a] ±0.00	**0.004**[a] ±0.00
Stigmasterol	wt	**0.005**[a] ±0.00	**0.004**[a] ±0.00	**0.016**[a] ±0.00	**0.010**[a] ±0.00
	31.1	**0.012**[b] ±0.00	**0.014**[b] ±0.00	**0.033**[b] ±0.00	**0.020**[b] ±0.00
	61.1	**0.014**[b] ±0.00	**0.019**[b] ±0.00	**0.038**[b] ±0.00	**0.024**[b] ±0.00
β-sitosterol	wt	**0.015**[a] ±0.00	**0.018**[a] ±0.00	**0.065**[a] ±0.03	**0.013**[a] ±0.00
	31.1	**0.029**[a] ±0.00	**0.048**[b] ±0.00	**0.030**[b] ±0.00	**0.011**[a] ±0.00
	61.1	**0.029**[a] ±0.00	**0.059**[b] ±0.01	**0.025**[b] ±0.00	**0.013**[a] ±0.00
			SE		
Cholesterol	wt	**0.004**[a] ±0.00	**0.025**[a] ±0.01	**0.008**[a] ±0.00	**0.005**[a] ±0.00
	31.1	**0.004**[a] ±0.00	**0.023**[a] ±0.02	**0.007**[a] ±0.00	**0.003**[a] ±0.00
	61.1	**0.004**[a] ±0.00	**0.017**[a] ±0.00	**0.005**[a] ±0.00	**0.004**[a] ±0.00
Campesterol	wt	**ND**	**0.005**[a] ±0.00	**0.004**[a] ±0.00	**0.006**[a] ±0.00
	31.1	**ND**	**0.005**[a] ±0.00	**0.006**[a] ±0.00	**0.004**[a] ±0.00
	61.1	**ND**	**0.005**[a] ±0.00	**0.007**[a] ±0.00	**0.005**[a] ±0.00
Stigmasterol	wt	**ND**	**0.003**[a] ±0.00	**0.003**[a] ±0.00	**0.004**[a] ±0.00
	31.1	**ND**	**0.004**[a] ±0.00	**0.005**[a] ±0.00	**0.006**[a] ±0.00
	61.1	**ND**	**0.005**[a] ±0.00	**0.005**[a] ±0.00	**0.009**[a] ±0.00
β-sitosterol	wt	**0.007**[a] ±0.00	**0.047**[a] ±0.01	**0.038**[a] ±0.00	**0.023**[a] ±0.00
	31.1	**0.008**[a] ±0.00	**0.059**[a] ±0.01	**0.041**[a] ±0.00	**0.017**[a] ±0.00
	61.1	**0.008**[a] ±0.00	**0.055**[a] ±0.00	**0.032**[a] ±0.00	**0.024**[a] ±0.00

Values are expressed as mean µg sterol per mg of dry weigth of fruits and ±SEM from three technical replicates each (n=3). Different letters (a or b) represented significant differences determined by t-test (*P<0.05).

Supplemental Table 5. List of common DEGs between amiSGT1-31.2 and amiSGT1-61.1 fruits

Gene	amiSGT1-31.2		amiSGT1-61.1		Description
	Log2FC	FDR	Log2FC	FDR	
Solyc08g062580.1	10.0	1.7E-46	10.8	7.5E-78	Pentatricopeptide repeat-containing protein family
Solyc01g056430.1	7.9	8.3E-15	9.7	7.2E-29	Nodulin / glutamate-ammonia ligase-like protein
Solyc09g059085.1	7.9	1.2E-13	9.2	8.6E-28	DNA-directed RNA polymerase subunit beta
Solyc10g038020.2	4.8	1.1E-25	5.6	2.9E-43	Cellulose synthase family protein
Solyc04g039670.3	4.4	1.2E-02	5.3	6.3E-03	ATP-citrate synthase, putative
Solyc07g017437.1	3.1	7.4E-10	4.0	2.6E-22	3-oxo-5-alpha-steroid 4-dehydrogenase family protein
Solyc09g008670.3	2.6	3.2E-06	3.3	2.2E-02	threonine deaminase
Solyc05g055343.1	3.2	7.0E-33	2.9	9.0E-03	Glycosyltransferase
Solyc02g063000.3	2.8	5.5E-52	2.4	1.5E-34	Glycosyltransferase
Solyc09g091300.1	3.3	3.0E-17	2.4	1.4E-02	S-protein homologue
Solyc01g112230.3	-10.0	3.6E-36	-10.0	1.4E-35	Metallothionein-like protein
Solyc03g096040.3	-9.9	6.5E-31	-9.9	1.0E-30	1-Cys peroxiredoxin
Solyc05g053640.1	-9.6	1.5E-136	-9.6	1.6E-135	Clavata3/ESR (CLE) gene family member MtCLE06
Solyc09g025210.3	-13.1	1.7E-239	-9.6	3.9E-08	alcohol dehydrogenase-2
Solyc07g064210.2	-13.5	5.3E-164	-9.4	7.9E-161	Grain softness protein
Solyc08g080480.3	-12.5	4.0E-108	-9.4	2.5E-102	Monomeric alpha-amylase inhibitor
Solyc06g060840.1	-8.3	0.0E+00	-9.3	0.0E+00	Oleosin
Solyc09g090150.3	-12.1	8.2E-61	-9.1	2.7E-12	11S storage globulin
Solyc09g082330.2	-11.5	0.0E+00	-9.0	0.0E+00	SM80.1 Vicilin
Solyc08g080490.3	-12.1	1.5E-138	-8.9	9.7E-47	Grain softness protein
Solyc09g072560.3	-12.0	1.4E-66	-8.8	1.8E-07	Legumin 11S-globulin
Solyc09g014750.1	-11.0	1.1E-84	-8.7	2.2E-83	Late embryogenesis abundant protein
Solyc06g064650.3	-10.9	7.0E-72	-8.6	1.2E-68	hydroxysteroid dehydrogenase 5
Solyc03g005590.2	-9.3	0.0E+00	-8.3	1.5E-139	Regulator of chromosome condensation (RCC1) family protein
Solyc02g031950.3	-11.2	0.0E+00	-8.2	5.5E-297	pathogenesis-related family protein
Solyc09g098120.3	-10.5	4.5E-36	-8.2	7.5E-36	Oil body-associated protein 1A
Solyc09g065470.3	-12.2	7.8E-37	-8.1	1.4E-39	7S globulin
Solyc06g034040.1	-9.9	2.2E-109	-7.9	7.2E-98	Oleosin
Solyc12g010920.2	-13.1	2.1E-247	-7.9	2.1E-230	Oleosin
Solyc05g005380.2	-7.7	9.8E-41	-7.8	5.8E-40	Transcription factor
Solyc07g006390.2	-9.0	9.5E-101	-7.3	1.9E-98	Blight-associated protein p12
Solyc03g019820.3	-9.3	2.4E-28	-7.0	1.3E-26	tonoplast intrinsic protein 3.2
Solyc09g059610.3	-9.3	8.3E-05	-7.0	2.5E-05	DNA-directed RNA polymerase subunit beta
Solyc02g077340.1	-6.0	3.7E-46	-6.6	3.9E-46	GDSL esterase/lipase
Solyc06g069260.1	-10.2	6.5E-52	-6.5	3.5E-46	Oleosin family protein
Solyc01g100440.2	-6.6	6.9E-49	-6.3	5.7E-47	ATP-dependent DNA helicase
Solyc02g090370.3	-8.7	3.8E-133	-6.3	1.5E-120	Reticulon-like protein
Solyc12g094440.2	-7.6	8.6E-102	-6.2	1.2E-87	High mobility group family
Solyc02g086310.1	-6.2	2.6E-21	-6.2	1.5E-21	lipid-transfer protein 7k-LTP
Solyc05g053630.1	-10.0	1.0E-40	-6.0	4.4E-34	Clavata3/ESR (CLE) gene family member MtCLE06
Solyc06g007920.2	-11.5	6.8E-67	-5.9	3.1E-53	Gibberellin-regulated family protein
Solyc06g011480.2	-10.7	1.9E-46	-5.7	1.2E-23	Dynamin, putative
Solyc05g005440.2	-4.9	8.7E-32	-5.7	2.6E-35	Transcription factor
Solyc07g064790.3	-5.2	7.4E-32	-5.6	1.8E-32	calcium ATPase 2
Solyc02g086490.3	-6.0	4.3E-298	-5.4	1.9E-276	Oleosin
Solyc01g007940.3	-6.4	3.4E-78	-5.3	6.2E-86	Alanine aminotransferase 2
Solyc08g078160.3	-6.9	9.7E-190	-5.2	6.2E-153	Oleosin
Solyc06g069355.1	-4.7	1.4E-65	-5.2	2.8E-65	root meristem growth factor
Solyc01g009510.2	-4.4	8.7E-44	-5.2	1.7E-48	BZIP transcription factor

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Continuation of S. Table 5.

Gene	amiSGT1-31.2		amiSGT1-61.1		Description
	Log2 FC	FDR	Log2FC	FDR	
Solyc06g072130.3	-5.6	5.0E-141	-5.1	4.1E-125	tonoplast intrinsic protein 3.1
Solyc01g006610.2	-9.7	3.1E-29	-5.1	1.1E-22	2-oxoglutarate-dependent dioxygenase-related family protein
Solyc07g052270.3	-3.9	1.2E-22	-5.1	1.9E-27	hydroxysteroid dehydrogenase 1
Solyc11g066710.2	-5.8	8.0E-90	-5.0	6.1E-85	Phospholipid:diacylglycerol acyltransferase
Solyc12g006110.2	-5.5	3.0E-08	-5.0	1.9E-08	Fasciclin-like arabinogalactan protein
Solyc01g109920.2	-4.7	1.1E-20	-5.0	5.7E-22	Dehydrin protein
Solyc04g006983.1	-6.2	3.5E-54	-5.0	2.7E-42	low-molecular-weight cysteine-rich 56
Solyc03g005980.3	-4.7	2.7E-25	-5.0	2.1E-26	NOD26-like intrinsic protein 1.1
Solyc07g053750.1	-6.5	1.9E-66	-4.7	1.4E-53	Zinc finger transcription factor 50
Solyc09g092370.1	-4.8	5.5E-54	-4.7	1.4E-39	Plant self-incompatibility protein S1 family
Solyc04g007570.2	-5.9	3.1E-55	-4.7	4.4E-47	GDSL esterase/lipase 6
Solyc12g089170.2	-6.3	5.6E-38	-4.7	3.8E-30	Myb family transcription factor family protein
Solyc10g049370.2	-4.8	1.1E-66	-4.6	3.2E-64	Pectinesterase
Solyc07g054940.2	-5.4	1.2E-157	-4.6	1.4E-141	2-oxoglutarate (2OG) and Fe (II)-dependent oxygenase superfamily protein
Solyc12g006680.2	-5.0	1.9E-52	-4.6	1.5E-47	Early nodulin 93 protein
Solyc09g091380.1	-5.2	1.2E-53	-4.5	7.8E-43	WUSCHEL-related homeobox 3A
Solyc04g051280.3	-4.4	1.4E-171	-4.5	7.8E-179	Transmembrane protein, putative
Solyc12g098130.1	-4.2	5.5E-17	-4.5	3.4E-17	Glucose-6-phosphate isomerase, cytosolic 1
Solyc09g008690.2	-4.8	8.7E-35	-4.4	1.0E-41	GDSL esterase/lipase
Solyc01g067340.2	-5.7	9.4E-34	-4.4	6.3E-20	bHLH transcription factor 075
Solyc02g087910.2	-4.2	3.0E-111	-4.3	2.8E-125	Non-specific lipid-transfer protein
Solyc07g065570.2	-4.8	3.9E-58	-4.3	7.1E-53	Nuclear transcription factor Y subunit B
Solyc03g112440.1	-4.0	7.1E-162	-4.3	3.1E-181	Oleosin
Solyc06g083600.2	-5.3	1.3E-45	-4.2	5.3E-40	B3 domain-containing transcription factor ABI3-like protein
Solyc01g008940.2	-5.8	2.0E-25	-4.2	1.3E-17	ATPase E1-E2 type family protein / haloacid dehalogenase-like hydrolase family protein
Solyc08g079160.3	-4.5	2.8E-60	-4.2	3.6E-59	Vacuolar-processing enzyme
Solyc02g072290.1	-4.9	3.3E-95	-4.2	2.0E-79	Subtilisin-like protease
Solyc12g096930.2	-4.8	8.1E-136	-4.1	6.4E-98	Caleosin
Solyc04g014775.1	-4.4	4.2E-84	-4.1	1.2E-77	Cysteine proteinase inhibitor
Solyc02g094460.2	-5.0	2.2E-27	-4.1	2.9E-23	B3 domain transcription factor
Solyc02g068410.2	-3.6	1.7E-25	-4.1	2.2E-28	Polygalacturonase QRT3-like protein
Solyc08g074870.2	-5.2	2.8E-31	-4.0	1.7E-25	carboxyl-terminal peptidase (DUF239)
Solyc06g017860.2	-3.3	4.7E-13	-4.0	7.3E-17	Carboxypeptidase
Solyc11g043115.1	-3.8	1.5E-33	-4.0	3.8E-34	Cytochrome c biogenesis protein CcsA
Solyc07g008290.3	-3.2	2.0E-18	-4.0	1.5E-22	O-fucosyltransferase family protein
Solyc03g116390.3	-5.3	7.2E-49	-4.0	4.7E-39	Late embryogenesis abundant protein
Solyc01g091540.2	-3.6	7.0E-19	-4.0	5.3E-21	Growth-regulating factor
Solyc03g121350.3	-4.8	1.6E-68	-4.0	3.2E-48	anoctamin-like protein
Solyc06g069220.1	-3.9	1.0E-107	-4.0	1.1E-107	Eukaryotic aspartyl protease family protein
Solyc07g007745.1	-4.0	5.9E-24	-4.0	2.1E-21	Defensin-like protein
Solyc10g009440.3	-4.2	6.0E-31	-3.9	1.2E-28	Transcription factor
Solyc02g068980.1	-4.1	3.3E-21	-3.9	5.3E-20	Zinc finger family protein
Solyc03g097440.3	-5.0	2.5E-30	-3.9	7.8E-22	Dehydrogenase/reductase SDR family protein 7-like protein
Solyc09g009610.2	-4.3	1.4E-42	-3.8	5.2E-39	Purple acid phosphatase
Solyc11g043110.2	-3.8	1.2E-26	-3.7	1.7E-23	GDSL esterase/lipase
Solyc07g045145.1	-2.8	3.3E-19	-3.7	2.6E-25	transmembrane protein
Solyc07g045140.3	-3.5	3.3E-20	-3.7	1.9E-21	transmembrane protein
Solyc09g011100.1	-3.9	1.3E-43	-3.6	4.4E-41	Plant self-incompatibility protein S1 family
Solyc08g080700.2	-3.3	9.5E-60	-3.6	3.6E-66	carboxyl-terminal peptidase (DUF239)

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Continuation of S.Table 5

Gene	amiSGT1-31.2		amiSGT1-61.1		Description
	Log2FC	FDR	Log2FC	FDR	
Solyc11g069560.2	-3.7	8.4E-32	-3.6	4.5E-25	Glycosyl transferase, family 2
Solyc03g033690.2	-3.8	6.5E-48	-3.5	1.6E-44	Peroxidase
Solyc11g013430.2	-3.6	9.5E-29	-3.5	6.9E-27	Bushy growth protein
Solyc08g083510.2	-3.5	1.5E-30	-3.4	1.3E-31	Cytochrome P450 family protein
Solyc10g006080.2	-3.6	5.7E-20	-3.4	6.6E-19	Sodium/hydrogen exchanger
Solyc11g065280.2	-3.9	2.9E-89	-3.4	7.6E-61	unknown
Solyc10g075010.1	-3.3	1.1E-25	-3.4	1.2E-28	F-box family protein
Solyc02g085590.3	-4.3	3.6E-66	-3.4	9.2E-25	BAC19.13
Solyc08g066040.1	-3.0	1.6E-20	-3.4	1.5E-33	Oleosin
Solyc07g062360.1	-3.1	1.0E-99	-3.3	1.9E-64	Duplicated homeodomain-like superfamily protein
Solyc10g049940.1	-2.9	7.8E-70	-3.3	2.3E-57	Myb/SANT-like DNA-binding domain protein
Solyc04g064600.1	-3.1	3.5E-30	-3.3	3.8E-22	Structural maintenance of chromosomes protein
Solyc11g072380.2	-3.2	2.1E-92	-3.2	3.2E-140	Vicilin-like antimicrobial peptides 2-2
Solyc03g115300.2	-4.6	6.7E-31	-3.2	8.0E-22	Expansin
Solyc09g083310.2	-2.3	1.1E-10	-3.2	1.6E-17	E3 ubiquitin ligase DRIP2-like protein
Solyc01g080000.1	-2.8	2.2E-36	-3.2	1.6E-36	Eukaryotic aspartyl protease family protein
Solyc10g019220.1	-2.8	6.5E-12	-3.2	7.1E-14	Sulfotransferase
Solyc10g085530.2	-4.1	1.6E-43	-3.2	4.4E-173	unknown
Solyc05g023830.1	-3.5	3.8E-12	-3.2	2.7E-10	Malate dehydrogenase [NADP], chloroplastic
Solyc01g098280.3	-9.4	1.0E-33	-3.1	1.0E-16	DNAJ heat shock N-terminal domain-containing protein, putative
Solyc09g091320.1	-3.7	6.5E-31	-3.1	1.0E-25	Plant self-incompatibility S1
Solyc07g006460.1	-4.2	1.0E-43	-3.1	1.2E-30	Eukaryotic aspartyl protease family protein
Solyc08g075150.3	-3.5	8.9E-29	-3.1	5.3E-24	Coiled-coil domain-containing 73
Solyc07g049780.2	-2.7	1.7E-12	-3.1	1.5E-15	Cell differentiation protein rcd1, putative
Solyc01g081350.1	-3.5	1.1E-64	-3.1	7.1E-53	Eukaryotic aspartyl protease family protein
Solyc02g079460.2	-2.5	7.8E-16	-3.1	6.5E-20	unknown
Solyc12g021150.2	-3.0	3.7E-36	-3.0	2.0E-40	unknown
Solyc06g009600.1	-4.6	1.4E-30	-3.0	1.7E-20	HIT zinc finger, PAPA-1-like conserved region, putative
Solyc05g010565.1	-2.9	1.7E-42	-3.0	1.1E-38	Nodule Cysteine-Rich (NCR) secreted peptide
Solyc03g058370.2	-3.2	1.2E-35	-3.0	7.3E-34	Glycosyltransferase
Solyc00g044750.2	-3.4	5.1E-22	-3.0	8.4E-19	RNA-binding family protein
Solyc09g075210.3	-4.6	2.6E-20	-3.0	1.8E-04	Late embryogenesis abundant protein Lea5
Solyc10g019210.1	-3.0	2.5E-27	-3.0	1.0E-25	Sulfotransferase
Solyc10g005150.2	-2.8	4.0E-11	-3.0	1.8E-08	Purine permease-related family protein
Solyc01g100880.3	-2.3	6.2E-20	-3.0	6.1E-20	WAT1-related protein
Solyc11g005990.2	-3.1	9.4E-45	-3.0	6.2E-46	Transcriptional corepressor SEUSS-like protein
Solyc07g061800.3	-2.7	1.2E-09	-3.0	2.0E-15	SOUL heme-binding family protein
Solyc01g104380.3	-3.4	3.2E-118	-2.9	1.0E-89	Basic blue protein
Solyc02g062310.1	-2.2	1.6E-15	-2.9	7.8E-63	BURP domain protein RD22
Solyc00g170510.2	-2.4	1.1E-35	-2.9	5.2E-42	Pectinesterase
Solyc01g006770.2	-3.5	2.1E-15	-2.9	7.2E-17	transferring glycosyl group transferase (DUF604)
Solyc01g013995.1	-3.1	5.1E-10	-2.8	4.5E-06	B3 domain-containing protein family
Solyc06g048430.3	-3.1	9.7E-05	-2.8	9.4E-04	ATP synthase epsilon chain
Solyc03g111990.3	-2.4	1.5E-17	-2.7	3.0E-19	Cytochrome P450
Solyc08g080790.1	-3.4	2.5E-04	-2.7	7.0E-05	carboxyl-terminal peptidase (DUF239)
Solyc07g007740.1	-3.0	5.2E-52	-2.7	1.4E-47	Defensin-like protein
Solyc06g065010.3	-3.5	8.4E-30	-2.7	7.3E-23	Lipase, GDSL
Solyc05g011900.1	-2.5	2.1E-17	-2.7	9.6E-19	Sulfotransferase
Solyc06g009890.2	-9.6	1.5E-25	-2.7	2.1E-10	PHD finger family protein

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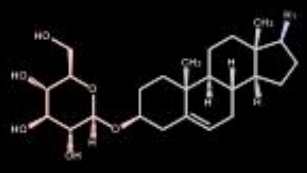
Continuation of S. Table 5

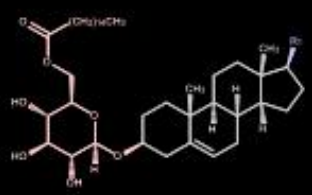
Gene	amiSGT1-31.2		amiSGT1-61.1		Description
	Log2FC	FDR	Log2FC	FDR	
Solyc03g019750.3	-2.8	5.3E-41	-2.7	1.7E-40	Alpha-1,4-glucan-protein synthase [UDP-forming]
Solyc02g092070.3	-3.2	9.3E-15	-2.7	7.7E-10	Growth-regulating factor
Solyc12g056673.1	-2.2	5.1E-06	-2.7	1.9E-08	Serine/threonine protein phosphatase 7 long form isogeny
Solyc05g043380.1	-3.3	1.0E-07	-2.7	5.6E-12	phosphatidylinositol 4-kinase gamma-like protein
Solyc11g010940.2	-2.9	1.5E-20	-2.7	7.8E-19	Dof zinc finger protein
Solyc01g096865.1	-3.0	1.2E-17	-2.7	9.1E-15	AP2-like ethylene-responsive transcription factor
Solyc07g064190.2	-4.3	1.1E-11	-2.7	1.9E-02	unknown
Solyc10g009610.2	-3.3	1.0E-61	-2.7	8.0E-48	Auxin-responsive GH3 family protein
Solyc08g081020.1	-3.1	6.9E-20	-2.7	3.3E-17	carboxyl-terminal peptidase (DUF239)
Solyc08g005830.2	-2.1	6.6E-27	-2.7	6.2E-35	unknown
Solyc03g078360.2	-4.2	4.5E-20	-2.7	1.9E-12	unknown
Solyc09g089950.1	-2.7	6.5E-47	-2.7	3.5E-58	BEL1-like homeodomain 10
Solyc11g007050.1	-3.2	6.8E-39	-2.7	3.3E-21	Serine protease inhibitor, potato inhibitor I-type family protein
Solyc07g047940.3	-2.5	3.9E-26	-2.6	1.4E-26	Zinc finger transcription factor 49
Solyc05g018370.1	-3.2	4.4E-109	-2.6	1.5E-86	Leguminosin group485 secreted peptide
Solyc02g071050.3	-2.6	7.4E-29	-2.6	2.2E-27	Purine permease
Solyc02g014730.3	-3.2	8.5E-04	-2.6	2.2E-04	Cytochrome P450
Solyc08g067640.1	-2.8	1.0E-94	-2.6	2.5E-78	Zinc finger C-x8-C-x5-C-x3-H type family protein
Solyc08g083500.2	-2.8	3.6E-60	-2.6	2.1E-123	Cytochrome P450 family protein
Solyc11g006140.1	-3.1	2.5E-25	-2.6	7.8E-22	Chaperone DnaJ-domain protein
Solyc05g012540.3	-2.7	1.5E-18	-2.6	3.0E-12	Heparanase, putative
Solyc05g047510.2	-2.8	1.1E-55	-2.5	1.8E-47	Sulfotransferase
Solyc09g014310.3	-3.2	1.1E-08	-2.5	1.3E-07	Desiccation-related PCC13-62
Solyc09g030370.3	-3.1	2.1E-53	-2.5	6.8E-36	GDSL esterase/lipase
Solyc01g107390.3	-2.6	1.6E-15	-2.5	1.1E-14	GH3
Solyc08g082350.3	-2.2	2.8E-15	-2.5	4.0E-17	F-box family protein
Solyc10g047500.2	-3.2	9.3E-12	-2.5	1.3E-08	transmembrane protein
Solyc08g067990.2	-2.7	1.2E-107	-2.5	1.7E-100	Subtilisin-like protease
Solyc01g095720.3	-2.7	2.9E-64	-2.4	6.7E-39	Lipase
Solyc05g009430.3	-2.4	1.8E-29	-2.4	3.6E-30	Nuclease S1
Solyc11g051090.1	-3.0	7.3E-13	-2.4	1.9E-14	Sulfotransferase
Solyc00g212260.2	-3.4	5.7E-38	-2.4	8.1E-18	Auxin-responsive GH3 family protein
Solyc05g051380.3	-2.5	6.0E-15	-2.4	5.6E-14	AP2-like ethylene-responsive transcription factor
Solyc10g055420.2	-2.2	1.1E-16	-2.4	1.0E-17	Malate dehydrogenase, cytoplasmic
Solyc07g017970.2	-2.8	1.6E-35	-2.4	6.9E-08	Pathogenesis-related thaumatin family protein
Solyc05g013930.2	-2.1	5.7E-09	-2.4	1.3E-11	RNA-binding family protein
Solyc12g044520.2	-2.6	2.2E-38	-2.4	2.4E-30	Glutathione S-transferase
Solyc03g006940.2	-2.2	8.7E-11	-2.4	6.8E-13	Protein phosphatase 2C family protein
Solyc10g047570.2	-2.1	2.5E-14	-2.4	1.2E-23	Pectin lyase-like superfamily protein
Solyc11g050930.1	-2.3	1.5E-29	-2.4	1.2E-28	Sulfotransferase
Solyc03g115310.1	-2.5	5.5E-41	-2.4	2.6E-38	Expansin
Solyc03g006950.2	-2.2	8.8E-09	-2.3	3.1E-10	Protein phosphatase 2C family protein
Solyc03g097870.3	-3.3	3.7E-18	-2.3	7.2E-08	Bidirectional sugar transporter SWEET
Solyc11g012370.1	-2.7	6.3E-64	-2.3	3.4E-45	Cysteine proteinase inhibitor
Solyc08g076250.2	-2.6	1.2E-16	-2.3	5.6E-45	Cytochrome P450
Solyc01g096860.2	-2.7	3.3E-47	-2.3	3.7E-38	AP2-like ethylene-responsive transcription factor
Solyc02g032140.2	-2.9	3.8E-15	-2.3	6.3E-11	Ninja-family protein
Solyc02g089150.3	-2.4	7.4E-30	-2.3	8.6E-28	PI-PLC X domain-containing protein
Solyc02g021220.1	-2.5	1.8E-69	-2.3	7.1E-66	Subtilisin-like protease

Continue next page (4-5)

Continuation of S. Table 5

Gene	amiSGT1-31.2		amiSGT1-61.1		Description
	Log2FC	FDR	Log2FC	FDR	
Solyc10g055250.1	-2.6	2.3E-31	-2.3	1.4E-20	cold shock domain protein 1
Solyc10g017970.1	-2.4	2.4E-04	-2.2	2.8E-03	Chitinase
Solyc00g007030.2	-2.3	3.2E-21	-2.2	3.8E-19	Glutathione S-transferase
Solyc09g065260.2	-2.4	5.1E-98	-2.2	3.1E-91	Early nodulin-like protein
Solyc03g097580.3	-2.1	8.7E-26	-2.2	6.1E-17	Bidirectional sugar transporter SWEET
Solyc05g014120.1	-2.7	1.3E-18	-2.2	2.6E-14	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
Solyc09g091635.1	-2.8	3.9E-23	-2.2	7.8E-14	F-box family protein
Solyc09g074530.3	-2.4	5.9E-79	-2.2	4.0E-94	Nodulin MtN3 family protein
Solyc08g065590.2	-2.3	1.5E-20	-2.2	2.8E-19	Vacuolar processing enzyme
Solyc07g044820.1	-2.3	1.8E-15	-2.2	3.6E-16	Hexosyltransferase
Solyc08g083410.2	-2.2	1.8E-41	-2.2	1.8E-81	unknown
Solyc10g012110.2	-2.4	2.2E-90	-2.2	1.0E-25	Non-specific lipid-transfer protein
Solyc03g097730.2	-2.4	6.9E-10	-2.2	6.0E-09	CASP-like protein
Solyc10g083580.2	-2.6	1.8E-22	-2.2	4.2E-14	Phytosulfokine 3, putative
Solyc04g014790.1	-2.1	1.1E-47	-2.1	1.7E-52	Cysteine proteinase inhibitor
Solyc04g071615.1	-3.0	1.6E-82	-2.1	1.8E-08	ASR4
Solyc09g090900.3	-3.0	2.5E-25	-2.1	1.3E-16	Aconitate hydratase
Solyc08g065570.2	-2.5	1.1E-99	-2.1	2.1E-87	Vacuolar processing enzyme
Solyc12g013820.2	-2.5	3.1E-72	-2.1	1.5E-54	Ubiquitin conjugating enzyme, putative
Solyc07g064730.2	-2.5	1.5E-20	-2.1	3.1E-19	GDSL esterase/lipase
Solyc02g086300.3	-2.6	1.8E-29	-2.1	1.4E-08	Class I glutamine amidotransferase-like superfamily protein
Solyc05g053460.1	-2.6	1.2E-20	-2.1	3.3E-14	Plant self-incompatibility protein S1 family
Solyc08g022130.2	-3.6	1.8E-19	-2.1	2.6E-05	F-box-like/WD repeat-containing protein TBL1XR1
Solyc12g089300.2	-2.4	7.7E-13	-2.1	1.5E-10	Gibberellin-regulated protein 2
Solyc10g012120.1	-2.6	5.1E-78	-2.0	8.8E-53	Non-specific lipid-transfer protein
Solyc06g007180.3	-3.2	1.6E-33	-2.0	2.9E-17	asparagine synthetase 1
Solyc08g061970.3	-2.1	1.1E-51	-2.0	2.6E-37	putative spermine synthase
Solyc11g069940.1	-2.3	1.4E-64	-2.0	2.2E-56	Glutaredoxin
Solyc07g055440.2	-2.2	6.8E-27	-2.0	4.6E-19	Cytochrome P450
Solyc11g027800.1	-2.0	1.2E-12	-2.0	6.6E-12	Sulfotransferase





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