MANIPULATION OF PLANT STEROL PROFILE AND ITS EFFECT ON INSECTS

A Dissertation

by

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ABSTRACT

Sterols are crucial membrane components in all eukaryotic cells. Cholesterol is the most common sterol in animals, including insects, and it is the required precursor of the insect steroid hormone that regulates molting and developmental processes. However, insects are sterol auxotrophs and require a dietary sterol source. Herbivorous insects obtain phytosterols to meet their needs, but only metabolize certain types of phytosterols into cholesterol. To explore whether this sterol metabolic constraint in insects can be exploited to control insect herbivore pests, I manipulated phytosterol profile and investigated its effects on two different insect herbivores: a chewing insect (*Plutella xylostella*) and a sucking insect (*Myzus persicae*).

In this study I modified the sterol profile of *Arabidopsis thaliana* by silencing the *HYD1* gene, which encodes a $\Delta^{8,7}$-sterol isomerase that converts Δ^{8} sterols to Δ^{7} sterols. Arabidopsis lines with > 95% transcript reduction exhibited normal growth, but contained less sterol, half of which showed a Δ^{8} configuration uniquely detected in the RNAi lines. Notably, Δ^{8} sterols were not detected in the phloem even in RNAi lines. I then examined growth and reproduction for *P. xylostella* (caterpillar) and *M. persicae* (aphid). Both insects displayed reduced survival, growth and reproduction when reared on plants with > 95% transcript reduction, and this pattern was consistent over successive generations. Caterpillars reared on Arabidopsis lines with > 95% transcript reduction showed reduced cholesterol and high levels of Δ^{8} sterols compared to caterpillars from control plants. Aphids on Arabidopsis lines with > 95% transcript reduction showed reduced sterol

content (> 60%) compared to aphids on control lines. I then compared aphid feeding behavior across the lines but found no difference. Finally, I used our collective data to estimate insect population growth on different Arabidopsis lines. Compared to controls, caterpillar populations on lines with > 95% transcript reduction were 70% reduced after 3 generations, while aphid populations were 60% reduced after 4 generations.

I also investigated *M. persicae* performance of various sterol-modified plants targeting different steps of plant sterol biosynthesis. Aphids reared on lines that accumulated atypical sterols (*hyd2, ste1* and *cas1-1*, which exhibit $\Delta^{8,14}$, Δ^{7} sterols, and 2,3-oxidosqualene, respectively) showed significantly reduced body mass and reproduction compared to *M. persicae* reared on control lines. Additionally, *M. persicae* on lines that accumulated campesterol instead of β-sitosterol were also negatively affected. However, aphids reared on lines that displayed high levels of suitable sterols, but with normal sterol profiles, were similar to control aphids. Insects with poor performance consistently showed reduced sterols in their body. Collectively, these results demonstrate the potential of using sterol-modified plants as a novel strategy for controlling insect herbivore pests.

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NOMENCLATURE

20E	20-exdysteroid
BRs	Brassinosteroids
CAS1	Cycloartenol synthase 1
DBM	Diamondback moths (*Plutella xylostella*)
EPG	Electrical penetration graph
GC-MS	Gas chromatography–mass spectrometry
GPA	Green peach aphids (*Myzus persicae*)
HMG1	3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR)
HYD1	C-8,7 sterol isomerase
HYD2	Sterol C-14 reductase (FACKEL)
MS	Murashige & Skoog
PSAT1	Phospholipid:sterol acyltransferase 1
SMT2	Sterol-C-24-methyltransferase
STE1	Δ^7-sterol-C-5-desaturase (DWF7)
TMSI	Trimethylsilylimidazole

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CHAPTER I

INTRODUCTION

Herbivorous insects obtain nutrients, such as carbohydrates, proteins, and lipids from plants to complete their whole life span. Sterols are one type of lipid that play important roles in many physiological processes of insects (Joop et al 2005). However, comparing to other nutrients involved in insect-plant interactions, such as macronutrients or plant secondary metabolites, sterols are relatively less studied, in spite of their importance in insect growth, development and reproduction (Behmer & David Nes 2003). The common structure of sterols contains three domains: (A) a hydroxyl-group at C3 position, (B) a tetracyclic ring, and (C) a side hydrocarbon chain (Fig. 1.1.). One of the major roles of sterols is to construct the cellular membranes (Bloch 1983, Nes 1974). The C3 hydroxyl-group of sterols (the polar part) in the phospholipid bilayers can form hydrogen-bonds with the hydrophilic part of the phospholipids (Xu et al 1988). The non-polar tetracyclic ring and side-chain of sterols interact and align adjacently with the phospholipid fatty acid tails (Hofsäss et al 2003). The interaction between sterols and phospholipids helps maintain a certain fluidity, increases the rigidity and reduces the permeability of the bilayers. These membrane functions of sterols are widely recognized in all eukaryotic organisms, including insects (Carvalho et al 2010, Simon-Plas et al 2011). Sterols are also precursors of some critical hormones, such as the insect molting hormone ecdysteroid (Gilbert & Warren 2005, Huang et al 2008) and mammalian androgens (Attard et al 2009). Sterols have also been discovered participating in the hedgehog signaling,

which is involved in regulating growth and development in both insects and mammals (Ingham 2001, Ogden et al 2004).

For both insects and mammals, cholesterol is the major sterol, and also recognized as the most common zoosterol. However, unlike mammals, insects lack the ability to synthesize sterols *de novo*, and most of them depend on an exogenous source for dietary sterol requirements (Robbins et al 1971, Vinci et al 2008). For carnivorous and omnivorous insects, cholesterol can be obtained from their prey directly since the animal tissues they consume are generally rich in cholesterol. Insects that are bearing endosymbionts can also obtain sterols from fungal symbionts, but not from bacterial symbionts, as bacteria do not synthesize sterols (Akman Gündüz & Douglas 2009). For herbivorous insects, plants are the major source for sterols (Behmer & David Nes 2003). However, in most plants, cholesterol is produced at a very low level or is undetectable. The major sterols produced by plants are phytosterols, which need to be converted into cholesterol by insects (Benveniste 1986).

In plants, there are more than 100 different sterols that have been identified. Different plant species can also produce some unique sterols, but the most common and abundant phytosterols are the pathway end-product Δ^5 sterols: β-sitosterol (24α-ethyl), stigmasterol (24β-ethyl) and campesterol (24α-methyl) (Benveniste 2004). In *Arabidopsis thaliana* ecotype Columbia, a typical sterol profile has been illustrated, which contains 60~80% β-sitosterol, 10~15% campesterol, 1~3% stigmasterol and cholesterol, and the remaining are stanols and many other sterol biosynthetic pathway intermediates (Schrick

K et al 2004, Schrick et al 2002). In addition, brassinosteroids (BRs) are plant steroid hormones synthesized from campesterol. The levels of BRs are much lower than typical phytosterols, but they are essentially plant hormones regulating the growth and development of plants (Chung & Choe 2013). Many sterol intermediates are hard to detect because they are generally near trace levels, and some are structurally unstable. The amount of cholesterol in plants is rather low as stated, which requires phytophagous insects to convert phytosterols into cholesterol to meet their requirements. Both cholesterol and the major plant sterols are Δ^5 sterols (C5 double bond in the tetracyclic ring). The main differences between cholesterol and the major plant sterols are, a) the side chain unsaturation, a typical example is the C-22 double bond in stigmasterol. b) the extent of C24-alkylation in the side chain. For example, the main difference between sitosterol/campesterol and cholesterol is the presence of an ethyl/methyl group at the C24-position. (Fig. 1.1.). Although in most plant species, the major phytosterols are Δ^5 sterols, exceptions do exist when atypical sterols become dominant. For example, Δ^7 and Δ^8 sterols (with C7 and C8 double-bond in tetracyclic ring, respectively) are sterol intermediates which are usually undetectable in most plant species. However, spinasterol (a Δ^7 sterol) was found to accumulate in plants such as spinach, alfalfa and squash. In many Cucurbitaceae plants, Δ^7 sterols are the dominant sterols, and Δ^8 sterols also detectable (Akihisa et al 1986a, Akihisa et al 1986c, Schmelz et al 2000).

Among the sterols produced by plants, only very limited types can be utilized by insects by converting them into cholesterol, which are defined as suitable sterols. Previous studies using radiolabeled phytosterols to track metabolites in insect bodies, and found

that for most insects, only Δ^5 phytosterols (β-sitosterol, campesterol, stigmasterol) are suitable sterols. Atypical sterols, such as Δ^7 and Δ^8 sterols, are unsuitable sterols which cannot be metabolized by insects (Behmer & David Nes 2003, Corio-Costet et al 1989, Ritter 1986). The incapability of metabolizing Δ^7 and Δ^8 sterols is consistent with the fact that insects do not have a post-squalene sterol biosynthesis pathway (Svoboda et al 1995). However, for Δ^5 phytosterols, the ethyl or methyl group on C24 side chains of β-sitosterol and campesterol can be removed through the C24-dealkylatioin process to synthesize cholesterol (Fujimoto et al 1985). An additional E_t^{22} sterol reductase is involved to remove the C22 double bond of stigmasterol (Ikekawa et al 1993). Although Δ^5 phytosterols can be dealkylated by most insects species, some insect that do not possess E_t^{22} sterol reductase are incapable of using stigmasterol (Ikekawa et al 1993, Lafont et al 2012). For example, stigmasterol is unsuitable for grasshoppers and aphids (Behmer et al 1999b, Bouvaine et al 2012). In addition to different types of sterols, different forms of sterols are usually present in plants: free sterols and sterol conjugates (sterol esters, sterol glycosides and acyl steryl glycosides). Free sterols and sterol esters are found to be the most abundant forms in plants (Ferrer et al 2017). For insects, the form of sterols appears to have no effect on the suitability of a specific sterol. In the gut lumen, esterases and glycosidases are synthesized and released to remove the conjugated part from sterols and help maintain sterol homeostasis in insects (Terra et al 1996).

However, unmetabolized unsuitable sterols, could have severe impacts on insect performance. Previous dietary studies using artificial diets containing different types, amounts, and ratios of sterols revealed: (1) a minimum threshold of suitable sterols is

required for insects, (2) when the presence of unsuitable sterols reaches a certain ratio, the performance of insects can be negatively affected, even when suitable sterols are above the minimum threshold. For example, studies on grasshoppers (*Schistocerca americana*) found that when above 50% of the sterols in the diet are unsuitable sterols, grasshoppers failed to complete development, although the amount of suitable sterols alone could have supported the normal growth (Behmer & Elias 1999, Behmer & Elias 2000). A study on caterpillars also found that the developmental time of *Helicoverpa zea* was significantly delayed when the proportion of unsuitable sterols increased to 50%, and the growth rate decreased when there were more than 30% unsuitable sterols in the diet (Nes et al 1997). However different insect species have shown different sterol metabolisms. A recent dietary study on *Myzus persicae* showed that the unsuitable steroids (ketosteroids) significantly reduced the aphid lifespan and fecundity, relative to aphids on diet containing cholesterol and β-sitosterol. However, aphids reared on diet with equal amounts of cholesterol and cholest-4-en-3-one showed fecundity equivalent to aphids on diet containing only cholesterol (Bouvaine et al 2014). However, precaution should be taken when concluding that the ratio is less critical for aphids, since the two unsuitable steroids tested were different from actual sterols. Ketosteroids are steroids that were synthesized from sterol oxidation, and they do not have the C3 hydroxyl-group, which is important for many physiological functions of sterols. It is unclear if they can be absorbed by insects like other unsuitable sterols. Interestingly, other studies have revealed that aphids have a very narrow range of suitable sterols. Although β-sitosterol can be utilized by aphids, the

capacity of dealkylation is low compared to other insects. Stigmasterol, on the other hand, is an unsuitable sterol for aphids (Bouvaine et al 2012, Campbell & Nes 1983).

The constraints of insect sterol metabolism have been extensively studied using artificial diets. The objective of this dissertation is to demonstrate new aspects of plant sterols involved in insect-plant interaction via modified sterol profiles. Modifying the ratio of different sterols rather than depleting plant sterols can have significant effects on insect performance (Behmer 2017, Jing et al 2014). One way is to use sterol biosynthesis inhibitors. However, the drawback is that most inhibitors have multiple targets in the sterol pathway (Benveniste et al 1984). To target an enzyme of interest in plant sterol biosynthetic pathway specifically, a better approach is to silence its encoding gene. In this dissertation, I firstly generated the sterol-modified plants *Arabidopsis thaliana* by RNAi, and then I examined the effects of the silenced plants on two different insect herbivores, a leaf-chewing caterpillar (*Plutella xylostella*) and a phloem sap-feeding aphid (*Myzus persicae*). Both the sterol profiles of plants and insects were measured to determine whether and how the two insects are affected by sterol-modified plants. Furthermore, I took advantage of the rich genomic information and transgenic line resources of Arabidopsis, and used a series of genetically modified lines targeting different enzymes in the plant sterol biosynthetic pathway, and measured the performance of aphids on each of these lines with different sterol profiles. The collective results, prospective directions and future application are discussed in the last chapter.

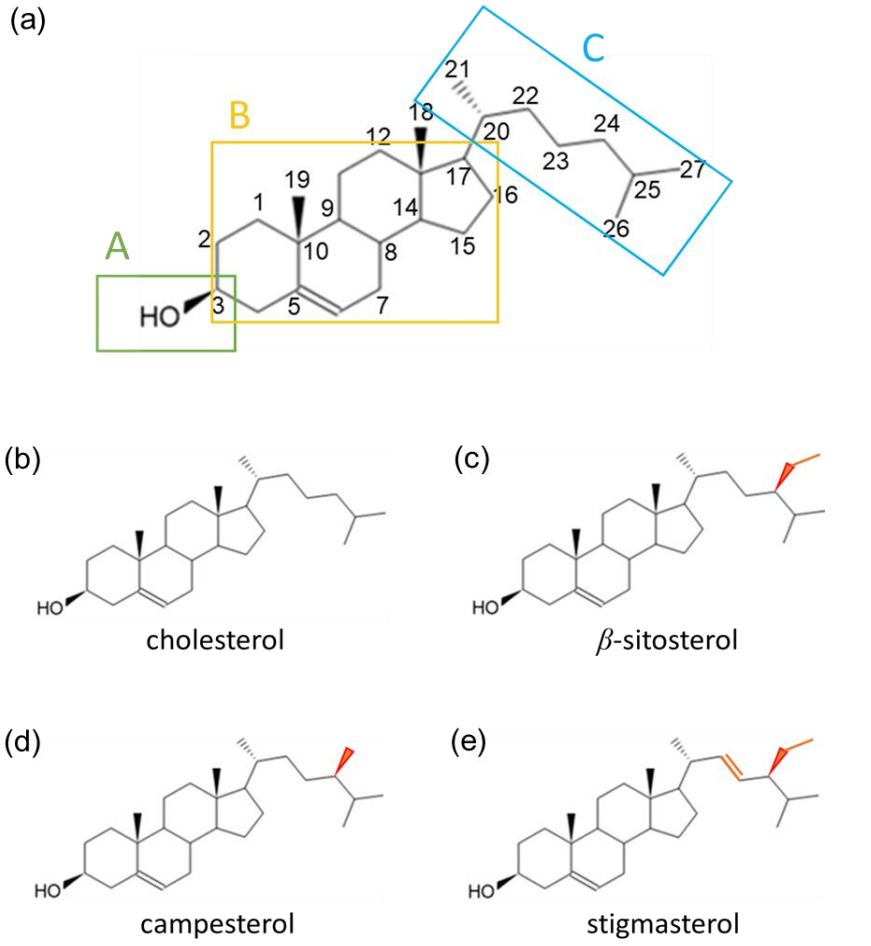

Figure 1-1 Sterol numbering system and structure of representative sterols.
(a) A, B, C are three major structure domains of sterols, numbers labelled were references for carbon positions in sterols. (b) Cholesterol is the most common sterol in insects. It is a Δ5 sterol with a C5 double bond in the tetracyclic ring. *β*-Sitosterol (c), campesterol (d) and stigmasterol (e), are the most common sterols in plants, and they are also Δ5 sterols. However, all these three typical phytosterols have a C24 alkyl group (red side chain on C24 position). In addition, stigmasterol has a C22 double bond.

CHAPTER II

SILENCING HYD1 MODIFIES PLANT STEROL PROFILES AND NEGATIVELY IMPACTS INSECT HERBIVORE PESTS

2.1 Introduction

Sterols are found in all eukaryotes, where they serve vital physiological roles as structural components in the membranes of cells and organelles (Behmer & David Nes 2003, Bloch 1983), as precursors to steroid hormones, vitamins and bile acid (Costet et al 1987, Tabas 2002) and as signaling compounds (Simons & Toomre 2000). All sterols share in common three features (Figure 1-1): (A) a hydroxyl group at the C3-position, (B) a tetracyclic ring structure and (C) and an aliphatic side chain. Cholesterol (Figure 1-1) is the best known and most studied sterol, and is the dominant sterol found in animals (Behmer & David Nes 2003, Bloch 1991). Sterols in plants are known as phytosterols, and more than 200 different sterols have been documented from plants (Benveniste 2004). Sitosterol, campesterol and stigmasterol (Figure 1-1) are the major phytosterols. Different from cholesterol, they have an alkyl group on the side chain at the C24 position, and they can have double bonds on the side chain (e.g. at C22) and/or in the tetracyclic ring (e.g. at C7, C8 or C14). Sterols also occur in fungi. Fungal sterols are similar to phytosterols, but they tend to have multiple double bonds. Ergosterol, the most common fungal sterol, has double bonds at C5, C7 and C22). In contrast to the majority of animals, plants and fungi tend to have multiple types of dominant sterols. However, the sterol profile of plants and fungi is dominated by a small number of structures, often comprising 70-90% of the total

sterol profile; the remaining sterols tend to occur at very low or trace levels (Piironen et al 2003, Schaller 2004).

For reasons still not understood, arthropods are unique among eukaryotes in that they are unable to synthesize sterols *de novo*; arthropods lack squalene synthase and other enzymes that are necessary for sterol biosynthesis (Grieneisen 1994, Kurzchalia & Ward 2003). Consequently, arthropods depend on an exogenous source of sterols. Cholesterol is the dominant sterol recovered from most animals, so carnivorous and omnivorous arthropods have ready access to it. In contrast, because plants typically do not contain much cholesterol – with the exception of some species in Solanaceae that have been shown to produce 10-30% cholesterol (Schaller 2004, Sonawane et al 2016) – the cholesterol recovered from the great majority of herbivorous arthropods (mostly insects) is generated from phytosterols. For insect herbivores ingesting β-sitosterol and campesterol (two commonly encountered phytosterols), this happens via dealkylation of the C24 alkyl group (Figure 2-1) and involves three enzymatic steps (Ciufo et al 2011, Ikekawa et al 1993). In the case of stigmasterol, another relatively common phytosterol, an additional reduction process is necessary to remove the double bond at position C22 (Lafont et al 2012). However, there is significant variation among insect herbivores in their ability to metabolize phytosterols to cholesterol (Behmer & David Nes 2003). For example, most caterpillars can readily convert sitosterol, campesterol and stigmasterol to cholesterol. In contrast, grasshoppers can generate cholesterol from sitosterol and campesterol, but they cannot produce it from stigmasterol because they lack the enzymes that reduce C22-double bonds (Behmer et al 1999b). Additionally, some metabolic constraints are ubiquitous

among insect herbivores. For example, the inability to modify double bonds that reside within the tetracyclic ring (Corio-Costet et al 1989, Ikekawa et al 1993, Ritter 1986).

Insect herbivores reared on artificial diets containing phytosterols, that cannot be readily converted to cholesterol, show high rates of mortality, slow growth and reduced reproduction (Behmer & Nes 2003). However, some plant sterols can be metabolized to cholesterol while others cannot. For a number of years, it was thought that the ratio of dietary sterols available to an insect wasn't important as long as a small pool of cholesterol was available for metabolic purposes (e.g. hormone production where there is an absolute requirement for cholesterol). However, studies on the caterpillar *Helicoverpa zea* (Nes et al., 1997) and the grasshopper *S. americana* (Behmer & Elias, 1999) have shown that when the proportion of sterol that could not be metabolized to cholesterol represented 50% and 30% of the total sterol profile, respectively, growth and development was significantly delayed and mortality was high. The work on grasshoppers demonstrated this effect was independent of the absolute amount of sterol that could be metabolized to cholesterol. A recent re-evaluation of these two studies, plus an additional study, indicated the negative effects associated with a mixed-sterol diet are a function of the types of dietary sterols present and their relative ratios (Behmer 2017, Jing et al 2014).

Given the requirement of insects for dietary source of sterol, and insect metabolic constraints with respect to converting some phytosterols to cholesterol, there is real potential to exploit insect sterol metabolic constraints for pest management. One strategy is to modify plant sterol profiles by manipulating the sterol biosynthetic steps that determine the position of double-bonds in the tetracyclic ring. The biosynthesis of sterols

in plants is initiated from squalene produced via the acetate/mevalonate (MVA) pathway, followed by multiple enzymatic steps including two critical double bond migration events: 1) C8 to C7 via $\Delta^{8,7}$-sterol-isomerase (8,7 SI), and 2) C7 to C5 via Δ^{7}-sterol-C5(6)-desaturase and $\Delta^{5,7}$-sterol-Δ^{7}-reductase (Figure 2-1). A number of sterol intermediates are quickly processed in this pathway, which includes sterols with C7 and C8 double bonds. Both Δ^{7} and Δ^{8} phytosterols (sterols with C7 and C8 double bonds) are considered atypical and in most plant species they are usually undetectable (Benveniste 2004, Schaller 2003). There are of course exceptions. Plants in the *Amaranthaceae* (which includes spinach) and *Cucurbitaceae* (which includes cucumber) have sterol profiles dominated by Δ^{7} sterols, while Δ^{8} sterols have been isolated from the seeds of several *Cucurbitaceae* plants (Akihisa et al 1986b, Piironen et al 2003). These atypical sterols also accumulate in plants treated with sterol biosynthesis inhibitors. For example, plants treated by AY-9944 or fenpropimorph exhibit dramatic increases in Δ^{8} sterols and decreases in Δ^{5} sterols, while cyanide treatment led to an accumulation of Δ^{7} sterols (Benveniste 1986).

For this study we focused on Arabidopsis 8,7 SI gene, *HYDRA1* (*HYD1*). Previous studies showed that the *HYD1* is important for embryonic development – the *hyd1* seedlings have short hypocotyls and roots, and the number and size of cotyledons is variable (Grebenok et al 1998, Souter et al 2002, Topping et al 1997). *hyd1* plants also show a drastically modified sterol profile. *β*-sitosterol and campesterol are greatly reduced (99%) relative to wild-type, and nearly half of the total sterols are abnormal sterols, including three $\Delta^{8,14}$ sterols and an unknown stimasta-monoen-3β-ol (39% of total sterols) (Schrick K et al 2004). Therefore, the loss-of-function mutant is highly defective both

morphologically and physiologically, furthermore, the homozygotes are seedling-lethal, so its value as a genetic resource for studying plant-insect interactions is limited. Alternatively, the *HYD1* gene can be knocked-down. To this purpose, we used RNAi interference (RNAi) to modulate the gene expression level of *HYD1* and manipulate phytosterol composition. I expressed double-stranded *HYD1* mRNA in Arabidopsis and screened for $HYD1_{RNAi}$ lines that varied in gene expression and sterol profiles (while maintaining normal phenotypes). Next, in a series of experiments, I presented our different plant lines to a caterpillar (*Plutella xylostella*; the diamondback moth) and an aphid (*Myzus persicae*; the green peach aphid) to feed on and measured their growth and reproduction, plus characterized sterol profiles in insect carcasses and excreta. I also compared the feeding behavior of aphids on the different plant lines. Collectively our data suggest there is significant potential for using sterol-modified plants to manage insect herbivore pests.

2.2 Materials and Methods

2.2.1 Plant growth and insect rearing

Vernalized seeds of *Arabidopsis thaliana* ecotype Columbia 0 (Col-0) were planted on LP5 potting medium (Sun Gro Horticulture, Bellevue, WA) and grown in an environmental chamber (EGC Shelf Lit Rooms, Environmental Growth Chambers, Chagrin Falls, Ohio). The growth conditions were set at 23°C (day)/21°C (night), 65% relative humidity (RH), with a 12h light/12h dark photoperiod and light intensity of 80-100 μmol m^{-2} s^{-1} (photon flux density) (Lei et al 2014). The Col-0 plants used for

transformation were grown in 10 cm x 10 cm pots (~20 plants per pot), and each week starting from week 3 these plants received fertilizer (Miracle Gro, Marysville, OH). Seedlings used for gene expression and sterol analyses, plus caterpillar and aphid bioassays were planted in 6 cm x 6 cm pots (one plant per pot). The growing conditions were similar to those previously described, except for fertilization.

A caterpillar (which eats plant vegetative tissues) and an aphid (which feeds on phloem) were used in this study. The caterpillar, *Plutella xylostella* (the diamondback moth, DBM), feeds exclusively on plants in the family Brassicaceae. DBM eggs were purchased from Benzon Research Inc. (Carlisle, PA), and newly hatched larvae from these eggs were used in a number of different performance and sterol bioassays. All experiments involving DBM were performed using insect growth chambers (Refrigerated Incubator, Thermo Scientific, Waltham, MA) maintained at 24°C and 65% RH, with a 14h light/10h dark cycle. The aphid, *Myzus persicae* (the green peach aphid, GPA), is recorded feeding on plants from a number of different families, including Brassicaceae. The GPA used in this study originated from a long-term laboratory colony maintained at Texas A&M University. The colony was reared on 6-10-week-old Chinese cabbage (*Brassica oleracea*) in a growth room maintained at 23°C and 65% RH, under a 12h light/12h dark cycle. All GPA bioassays were performed under identical environmental conditions.

2.2.2 RNAi constructs and plant transformation

The *AtHYD1* (AT1G20050) cDNA clone (TAIR accession: 4516197542) from the vector LIC/pYL436 was obtained from the Arabidopsis Biological Resource Center

(ABRC). A 264 bp fragment was PCR amplified from the cDNA template using 2 sets of primers with different restriction enzymes at each end of the fragment (Table 2-1). The PCR fragments were cloned into pGFC1008 plasmid at the *Asc*I/*Swa*I restriction site in the antisense direction, and the *Bam*HI/*Spe*I site in the sense direction, respectively. The two insertion sites were separated by a *GUS* spacer sequence to produce a hairpin RNA (hpRNA) (no intron included in pFGC1008). The pFGC1008-*HYD1* construct was then transformed into *E. coli* DH5α and plated onto LB plates containing chloramphenicol at 34 mg L^{-1}. After DNA sequencing analysis verified that the sequence was correct, the construct was introduced into an *Agrobacterium tumefaciens* GV3101 strain via electroporation (Koiwa et al 2003, Mohanpuria et al 2011).

A single *Agrobacterium* transformant was then cultured in 500 mL YEP medium containing 25 mg L^{-1} of chloramphenicol, and then incubated at 28 °C, with shaking (150 rpm), until the OD_{600} reached 1.5. Next, cells were centrifuged at 4,000 x g for 10 min, after which the cell pellet was gently resuspended in 500 mL of freshly prepared dipping solution containing 5% sucrose and 0.02% Silwet L-77. Next, the floral dipping method (Zhang et al 2006) was used to perform the transformation. The dipped plants (including the pot) were then wrapped with clear plastic wrap to maintain moisture and kept in the dark for 24 h, and then returned to normal growth condition. Seeds (T1) were harvested from these plants approximately 4 weeks later, and kept at 4 °C. To screen for transformants, T1 seeds were sterilized with 70% ethanol, followed by 25% bleach, and then plated on ½ Murashige & Skoog (MS) media (Phyto Technology Laboratories, Lenexa, KS) containing carbenicillin (100 mg L^{-1}) and hygromycin B (25 mg L^{-1}). Plates

were then kept at 23°C in a tissue culture room under low light (40-60 µmol m^{-2} s^{-1}) for 7-10 days. Out of the 5000 plated seeds, 96 seedlings showed green cotyledons and extended roots; these were transferred onto ½ MS media without antibiotics. One week later, these seedlings were moved to potting soil and grown individually until seeds were produced. At least 100 T2 seeds from each individual line were plated on ½ MS medium containing 25 mg L^{-1} hygromycin. T2 seedlings that showed a 3:1 segregation ratio at day 10 were individually grown to obtain T3 seeds. T3 seeds showing no segregation were used for all experiments (details below).

2.2.3 RT-PCR and qPCR analysis

Six 4-week-old seedlings from each of the four T3 homozygous lines (*HYD1*$_{RNAi}$7, 10, 12 and 25), and Col-0, were collected and ground into powder using a mortar and pestle, plus liquid nitrogen. Total RNA was extracted using RiboZol^{TM} RNA extraction reagent (AMRESCO, Radnor, PA) and quantified using a Nanodrop spectrometer (Thermo Scientific, Waltham, MA). DNase I (New England Biolabs, Ipswich, MA) was added to remove gDNA; this was followed by another RNA extraction. The quality of RNA was verified by agarose gel electrophoresis. To obtain the first cDNA strand for quantitative RT-PCR, 1 µg RNA was reverse transcribed using random primers and a ProtoScript® II First Strand cDNA Synthesis Kit (NEB). qPCR reactions were performed using a SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA) on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) following a two-step PCR protocol (denaturation at 95 °C for 10 s, followed by annealing/elongation

at 59 °C for 40 s, repeated for 40 cycles). Dissociation curves were used to check amplification specificity; *Ubiquitin10* (*UBQ10*) served as an internal control. Primer sequences used for *HYD1* and *UBQ10* are provided in Table 2-3. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001). Each PCR reaction was run in duplicate, and three biological replicates were performed for statistical analysis.

2.2.4 Leaf and phloem sterol analysis

Sterol analysis was first performed on vegetative tissues. We collected leaves from 4-week-old plants from five selected genotypes (Col-0 and *HYD1*$_{RNAi}$7, 10, 12, 25), and ground them into a fine powder under liquid nitrogen using mortar and pestle. Each sample (20 mg) was extracted with 4.5 mL $CHCl_3$-MeOH (2:1, v/v), containing the internal standard, 5α-cholestane (1 µg/mL, Sigma-Aldrich, St. Louis, MO), and homogenized in a Branson 1510 Ultrasonic Cleaner (Branson-Emerson) at 50 ºC for 45 min. From each extract, 2.5 mL was collected using a rotary evaporator (Fisher Scientific). The dried sample was then base-saponified with 1 mL of 5% KOH in MeOH at 55 ºC for 2 h to release the base hydrolysable sterol esters, followed by 3 rounds of extraction with 0.5 mL hexane. 0.3~0.35 mL of supernatant was collected each time. Approximately 1 mL total supernatant was combined and dried using a rotor evaporator, resuspended in 0.2 mL ether-hexane (9:1, v/v) and applied to a silica gel column (40-63 µm, pre-washed with 3 mL MeOH and 3 ml ether subsequently) (Suza & Chappell 2016, Suzuki et al 2004). The eluent was dried with a rotor evaporator and resuspended in 60 µL of pyridine followed by the addition of 40 µL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide with 1%

trimethylchlorosilane (Sigma-Aldrich) and a 1 h incubation at 60 ºC. Trimethylsilylated (TMSI) sterols were analyzed by a single quadrupole gas chromatograph-mass spectrometer (GCMS-QP2010 SE, Shimadzu, Japan) equipped with a ZB-35HT column (30 m × 0.25 mm, 0.25 µm film thickness; Phenomenex Zebron, Torrance, CA). Samples were injected in the splitless mode with an injection temperature of 220 ºC. The oven temperature, starting at 80 ºC for 1 min, was raised to 180 ºC at a rate of 20 ºC min^{-1} and then to 320 ºC at a rate of 5 ºC min^{-1}, and held constant at 320 ºC for 2 min. The sterol quantity was estimated based on their peak area comparing to the internal standard.

We next analyzed the sterol profile of the phloem from three lines (Col-0, plus *HYD1*$_{RNA}$10 and 25). To collect the phloem exudate, the petioles of leaves were cut near the proximal end of 4-week-old rosettes and immediately inserted into 500 µL Eppendorf tubes containing 100 µL EDTA (15 mM, pH 7.5), with petioles at the cut end immersed in EDTA (one leaf per tube). The tubes were then placed in the dark with 100% relative humidity for 6 h, during which a 2 mm segment of the petioles was cut after the first 3 h. Exudate from 500 leaves was pooled as one replicate and dried to 5 mL with an Organomation N-EVAP 111 nitrogen evaporator (Organomation Associates, Inc., Berlin, MA) prior to lyophilization; for each line there were two replicates. The sterol extraction and GC-MS system followed the method described previously (Behmer et al 2013).

2.2.5 Diamondback moth growth and reproduction bioassays

This experiment was conducted over two generations, to take into account potential maternal and paternal diet effects. In the first generation, caterpillars were reared

from hatch on leaves from one of five different Arabidopsis lines (Col-0, *HYD1*$_{RNAi}$7, 10, 12, 25). To synchronize the start of this experiment, one rosette from each selected line was placed on a moistened paper towel in a 15 x 100 mm petri dish and 200-300 ready-to-hatch DBM eggs (when the yellow eggs turn to black) were inoculated on the rosette; after 6h (enough time for a large number of neonates to establish) the egg sheet was removed. Upon reaching the second instar, larvae were individually transferred to wells in a 24-well-plate (48 larvae per line, 10 plates in total); each well contained an Arabidopsis leaf appropriate for that plate. The plates were checked twice daily to ensure caterpillars were never without food; when necessary, old leaves were removed, and fresh leaves were added. For each caterpillar we recorded whether it pupated, and if so the time to pupation and its pupal mass. Pupae were then transferred individually to Eppendorf tubes and monitored for eclosion; we recorded if eclosion happened, and the time from pupation to eclosion. When eclosion occurred, females were transferred into a mating cup containing two recently eclosed males (Behmer & Grebenok 1998, Jing et al 2013). Each mating cup contained an Eppendorf tube with a 10% sucrose solution (on distilled water) plugged by a cotton wick, and a 4 x 4 cm wrinkled aluminum foil (pre-coated with cabbage extract) for collecting eggs. Egg sheets were replaced daily, and egg numbers were recorded over a period of three days.

Neonates for a given line in the second generation (n = 48 per line) came from eggs collected from first-generation adults reared as caterpillars on leaves from that given line. Neonates, pupae and adults were handled as described above in the first-generation.

2.2.6 Sterol analysis of DBM adults and larval feces

DBM neonates were reared individually on leaves from one of five lines (Col-0 or *HYD1*$_{RNAi}$7, 10, 12, 25) using the 24-well-plate approach described above. Upon pupating, pupae were collected and transferred to a new plate until eclosion. Every 2nd-day adults (1 female and 1 male) were combined as one replicate (n = 6 for each line). Additionally, as the larvae grew, feces were collected (every 2 days; stored at – 80 °C). Feces from six larva were pooled as one replicate, and for each line there were 3 total replicates. Both adult bodies and feces were lyophilized and weighed prior to sterol extraction.

Dried samples were ground in a 1.5 mL Eppendorf tube and then extracted with 1 mL $CHCl_3$-MeOH (1:1, v/v); each sample also contained 5α-cholestane (10 µg/mL, Sigma-Aldrich) as an internal standard, plus 2 glass beads (DWK Life Sciences, Vineland, NJ). Each sample was then homogenized for 30 min using a Pneumatic paint shaker (Central Pneumatic, Camarillo, CA). Following homogenization, 0.5 mL H_2O was added to each sample and vortexed for 30 s and left to incubate for 12 h. Next, from each extraction, 1 mL was collected and dried under nitrogen using a nitrogen evaporator (Organomation Associates). Saponification, hexane extraction and conversion of TMSi derivatives followed the methods stated above. Trimethylsilylated sterols were analyzed in an Agilent 6890 Networked GC-fid (Agilent Technologies, Santa Clara, CA), equipped with a DB-17 column (30 m × 0.25 mm, 0.25 µm film thickness; Agilent Technologies). Samples were injected with a 280 ºC inlet temperature and 290 ºC detector temperature. The oven temperature started at 80 ºC, and then ramped to 240 ºC at a rate of 25 ºC min^{-1} and to 290 ºC at a rate of 5 ºC min^{-1}, and held constant at 290 ºC for 20 min. The quantity

of detected sterols was determined by respective peak areas in comparison to the internal standard.

2.2.7 Green peach aphid growth and reproduction bioassays

In this set of experiments aphid development and reproduction were tested on one of five lines: Col-0 or *HYD1*$_{RNAi}$7, 10, 12, 25. We initiated this experiment by taking adult aphids maintained on cabbage and transferring them to each of five Arabidopsis lines. For each line, 30 adults were distributed on five 4-week-old plants. 12 h later, adults were removed, but any newly born nymphs were left. Once these nymphs became adults and started to produce offspring, we initiated the experiment. For this first generation, synchronized 1st-instar nymphs (produced within 12 h) were inoculated onto a new batch of 4-week-old plants of the same line (one nymph per plant, 16 replicates per line). Developmental time to adult of each aphid was recorded, and offspring were counted (and removed daily) during the first seven days of the reproductive period. For a separate group of aphids, we also measured body mass on each line, using the same rearing approach described above. Each replicate consisted of 10 adults, and for each line there were three replicates.

We then extended this experiment for two additional generations, but only for Col-0 and *HYD1*$_{RNAi}$10. As with the caterpillar study, we did this to account for potential maternal and paternal diet effects. However, for the aphid experiment we collected data across three generations because aphids produce offspring that have already started to produce their own offspring (referred to as telescoping generations). Offspring for the 2nd-

generation came from adult aphids from the Col-0 and *HYD1*$_{RNAi}$10 lines, and these offspring were individually transferred to new plants of the corresponding lines. Experimental design and data collection followed methods outlined above, and for each line there were 16 replicates. Adult mass for a separate group of aphids (from Col-0 and *HYD1*$_{RNAi}$10) was also collected. We then repeated this process one more time (3rd-generation) for the Col-0 and *HYD1*$_{RNAi}$10 lines.

2.2.8 Sterol analysis of GPA adults

In a separate experiment we reared aphids on one of five lines (Col-0 or *HYD1*$_{RNAi}$7, 10, 12, 25) and then analyzed the sterol profile of adult aphids. For the sterol analysis of GPA adults, we collected 20~30 2nd day adults, lyophilized and weighed them prior to sterol extraction as described previously (Behmer et al 2011).

2.2.9 Aphid feeding behavior experiments

We conducted two aphid feeding behavior experiments. The first was a choice test using two lines: Col-0 and *HYD1*$_{RNAi}$10. Here we placed the two experimental plants side by side (each plant centered in a 6 cm x 6 cm pot). For each pair of pots, a 1 cm gap was left between the two pots, and leaves from the two plants were not allowed to come into contact. Next, a 2 cm x 4 cm parafilm bridge was used to connect the two pots, and on the center of the bridge we released 30 adult aphids. The number of aphids settled on each plant was recorded at 4 h, 24 h and 48 h. The arrangement of the plant pairs was varied to control for any potential positional bias. All bioassays were repeated three times (6 pairs

each time, for a total of 18 replicates). This experiment was conducted in a growth chamber using previously described conditions.

Our second experiment used a Giga-8dd Basic 8 channel EPG recording system (EPG systems, Wageningen University, Netherlands) to explore the finer details of green peach aphids feeding on four different lines: Col-0 or *HYD1*$_{RNAi}$7, 10, 12 (using 4-week-old plants). To prepare the experimental insects, GPA adults from cabbage colonies were transferred to a new cabbage plant for 12 h to produce synchronized nymphs (so that the age of aphids used were similar). The newborn nymphs were kept on cabbage until the second day of adulthood and then transferred to an empty petri dish and starved for one-hour. Next, we made an insect electrode. Here each individual aphid had a golden wire (2~3 cm in length, 18 μm in diameter) attached to its dorsal side using a water-based conductive glue (EPG systems). We also placed a plant electrode into the moistened soil of the potted plant but did so prior to placing the wired aphid on the plant. Next, the wired aphid was placed on the abaxial side of the leaf, and we started recording. All recordings took place with the plants (and aphids) under the protection of a Faraday cage. The EPG data and waveforms were recorded and analyzed using Stylet^{+} v01.23 (Wageningen, Netherlands). When aphids are on plants, they exhibit non-probing (np) behavior and six different types of probing behavior; EPG represents these different behaviors as seven unique waveform events. The six waveform events associated with probing behavior are: (1) a pathway period (C), which involves stylet penetration; (2) a potential drop (pd), which occurs briefly following cell puncture by the stylets; (3) derailment of the stylets (F), where the stylets experience difficulty with penetration; (4) xylem ingestion (G); (5)

phloem salivation (E1); and (6) phloem ingestion (E2). The identification of waveforms followed the methods described in previous studies (Bastias et al 2017, Machado-Assefh & Alvarez 2018). For each replicate, one aphid was inoculated on one plant; 21 replicates were conducted for each line. Aphids that did not display any probing activity for ≥ 1h were not considered as valid replicates.

2.2.10 Statistical analysis

JMP 11.0 software (Cary, NC) was used for data analysis. All datasets were checked for normal distributions using the Shaprio Wilk test, and homogeneity of variances by the Levine and Bartlett tests. Analysis of variance (ANOVA) was performed for the following datasets that were normally distributed with homogenous variance: quantitative RT-PCR, sterol profile of plants and insects, phenotypic trait of plants; Dunnett`s test was followed to test the statistical significance comparing to Col-0.

For DBM and GPA bioassays that were conducted on multiple lines and more than one generation, a full factorial model was applied to evaluate the single effect and cross-effect of lines and generations. For body mass and reproduction of DBM and GPA, which were normally distributed, Least Square Means were calculated for the effect test by ANOVA, and the difference between each treatment and control was compared by Dunnett`s test. For days to adult, parametric survival fit (Weibull distribution) was applied in the model, and the effect of each factor (lines, generations, lines*generations) was evaluated by the Effect Likelihood Ratio test. Steel`s test was then used to compare the difference between each treatment and control. For DBM pupation success and survival

to adult, an ordinal logistical fit was applied to the model, and each factor was analyzed by the Effect Likelihood Ratio test. For GPA bioassays that were solely performed in one generation, the body mass and reproduction was analyzed by ANOVA, followed by Dunnett`s test to determine the significant difference between each line and Col-0. The GPA developmental time was analyzed by nonparametric methods using the Kruskal-Wallis test followed by Steel`s test. The choice test of GPA which was binomially distributed, was analyzed by Likelihood Ratio Chi-Square test.

For EPG analysis, the datasheets of all the valid replicates obtained from Stylet^{+} v01.23 were imported into the Excel Workbook EPG_ParProc_JKI_mult (by Edgar Schliephake, Julius Kühn Institute, Germany, 2014). The variables of all the occurred waveform events were auto-calculated in the program for each replicate. For each of the variables, the difference among all the lines was calculated by Kruskal-Wallis, and Steel`s test was followed to compare the difference between aphids on Col-0 and a *HYD1*$_{\mathrm{RNAi}}$ line. Fisher`s exact test was performed to analyze the percentage of aphid numbers that displayed each activity. All the data above were presented as means with standard errors, and the statistical significance was determined when $P < 0.05$.

2.3 Result

2.3.1 Knockdown of HYD1 does not drastically impair Arabidopsis growth and development

Ninety hygromycin-resistant lines were recovered from the initial T1 screening. Three of these lines resembled the *hyd1* mutant phenotype – dwarfed seedlings with

extremely short hypocotyls and petioles, small leaves with irregular margins or spoon-shaped, and a growth habit that was "cabbage-like" (Topping et al 1997); these three lines also failed to produce viable seeds. We thus collected T2 seeds from the remaining eighty-seven lines, and seven of these (*HYD1*$_{RNAi}$7, 10, 12, 14, 22, 25, 29) exhibited a 3:1 segregation for hygromycin-resistance. These seven lines were then grown to collect seeds for the T3 generation (which are homozygote). Compared to the wild-type (Col-0), six lines (*HYD1*$_{RNAi}$10, 12, 14, 22, 25, 29) were slightly smaller at early growth stage (2-week to 4-week after sowing). In contrast, early growth for the seventh line (*HYD1*$_{RNAi}$7) was comparable to Col-0.

For each of our seven *HYD1*$_{RNAi}$ lines we conducted quantitative RT-PCR analysis to measure *HYD1* expression levels (Figure 2-2 A). The 314 bp PCR fragment was within the *HYD1* coding region but outside the dsRNA sequence used in the RNAi construct. *HYD1*$_{RNAi}$7 showed the lowest silencing effect (84%), while *HYD1*$_{RNAi}$10 showed the highest (99%). The remaining five lines (*HYD1*$_{RNAi}$12, 14, 22, 25, 29) achieved approximately 95% transcript repression.

We further characterized the plant phenotypes (relative to Col-0) for the least silenced line (*HYD1*$_{RNAi}$7), the most silenced line (*HYD1*$_{RNAi}$10), and two intermediate lines (*HYD1*$_{RNAi}$12, 25). Notably, 4-week old *HYD1*$_{RNAi}$10, 12 and 25 plants appeared to be smaller than Col-0 plants. However, this was mostly a function of significantly shortened petioles (Figure 2-2 B). Interestingly, as seedlings continued to grow, the 'short petiole' trait in *HYD1*$_{RNAi}$10, 12 and 25 diminished, and by week 6 no significant difference was detected in petiole length between these three lines and Col-0 (Figure 2-2

B). For *HYD1*$_{RNAi}$7, shorter petioles in early development was not observed (Figure 2-2 B). No obvious changes were observed in biomass, leaf number, days to bolting, and silique number and length between Col-0 and *HYD1*$_{RNAi}$ plants (Table 2-2).

2.3.2 Sterol profiles are altered in HYD1$_{RNAi}$ *lines with >95% levels of transcript silencing*

The leaf sterol phenotype of Col-0 and *HYD1*$_{RNAi}$7, 10, 12, 25 was characterized using GC-MS analyses (Figure 2-3 A). The sterol chromatograms of Col-0 and *HYD1*$_{RNAi}$7 (lowest silencing effect) showed three peaks: *β*-sitosterol, campesterol and cholesterol. These three sterols were also found in *HYD1*$_{RNAi}$10, 12 and 25 (where there is high silencing), but two additional peaks were observed: Δ^8 sitostenol and Δ^8 campestenol [based on the MS *m/z* (relative abundance of molecular and prominent fragment ions) published previously (Rahier et al 2008)].

Next, we quantified the amount of each sterol from our different lines, plus their summed total. In terms of leaf total sterol amount, we observed a significant difference between lines ($F_{4,15}$ = 15.5, P = 0.0002). A Dunnett's post hoc test – using Col-0 as the control – showed that *HYD1*$_{RNAi}$ 10, 12 and 25, but not *HYD1*$_{RNAi}$7, exhibited significantly reduced total sterols (Figure 2-3 B). Next, we compared the amounts of the two dominant sterols in Col-0. With respect to *β*-sitosterol (~80% of the sterol in Col-0), there was a significant difference between the lines ($F_{4,15}$ = 57.6, P < 0.0001). A Dunnett's post hoc test revealed no difference between Col-0 and *HYD1*$_{RNAi}$7, but *β*-sitosterol amounts were significantly reduced (~60-70%) in *HYD1*$_{RNAi}$ 10, 12 and 25 compared to Col-0. The amount of campesterol (~16% of the sterol in Col-0) also differed between the lines ($F_{4,15}$

= 70.6, $P < 0.0001$). In *HYD1*$_{RNAi}$ 10, 12 and 25 they were significantly lower (~70%) compared to Col-0, but in *HYD1*$_{RNAi}$7 campesterol amount did not differ compared to Col-0. Finally, when we considered β-sitosterol + campesterol as a percentage of the total sterol pool (in Col-0 this is ~ 97%), we observed a significant difference between the lines ($F_{4,15}$ = 1972.7, $P < 0.0001$). For *HYD1*$_{RNAi}$7, there was no difference compared to Col-0. In contrast, the β-sitosterol + campesterol pools in *HYD1*$_{RNAi}$ 10, 12 and 25 were significantly reduced (~50%) compared to Col-0. Notably, Δ^8 sterols were approximately 50% in *HYD1*$_{RNAi}$ 10, 12 and 25 lines (Table 2-3); in Col-0 and *HYD1*$_{RNAi}$7 these two Δ^8 sterols were undetected.

Sterols extracted from phloem sap of Col-0 plus *HYD1*$_{RNAi}$ 10 and 25 were also identified, as three different classes (Table 2-4): (1) total sterols [pooled free sterols + acylated sterols + glycosylated sterols]; (2) lipophilic sterols [free sterols and acylated sterols], and (3) hydrophilic sterols [glycosylated sterols]. For each of the three sterol classes we express the proportion of each phloem sterol because it is difficult to quantify the concentration of sterol in the phloem exudate, because it is difficult to quantify the volume of phloem collected for each sample. However, a number of findings concerning phloem are fairly straightforward. First, despite variation in relative amounts of sterol type in phloem, cholesterol and β-sitosterol were the dominate phloem sterols. Second, the proportion of cholesterol in phloem was much higher compared to that recovered from leaf tissue. Third, with respect to glycosylated sterols, cholesterol was the dominant sterol, presenting a higher proportion than β-sitosterol in all lines. Fourth, campesterol was detected in phloem from Col-0 plus *HYD1*$_{RNAi}$ 10 and 25. However, campesterol was only

present as glycosylated-campesterol in *HYD1*$_{RNAi}$ 10 and 25 phloem. Finally, no Δ^8 sterols were found in phloem collected from *HYD1*$_{RNAi}$ 10 and 25, despite large amounts of Δ^8 sterols being recovered from leaf tissues in these lines.

Based on these collective sterol analyses, we classify *HYD1*$_{RNAi}$ 10, 12 and 25 as sterol-modified lines. In contrast, we consider *HYD1*$_{RNAi}$7 a transgenic control, even though this line displays an 84% reduction of the *HYD1* transcript.

2.3.3 DBM growth and reproduction are reduced on HYD1$_{RNAi}$ *lines with modified sterols*

For two successive generations we measured pupation success, pupal mass, eclosion success, time to adult development and egg production on five different lines – Col-0 (control), *HYD1*$_{RNAi}$7 (a silenced plant with normal sterol profiles – a second control) and *HYD1*$_{RNAi}$10, 12, 25 (silenced lines with modified sterol profiles). Measuring these traits over two successive generations gave us insights into potential paternal diet effects. We first examine two larval traits. Pupation success was significantly affected by plant line ($\chi_4^2 = 24.7$, $P < 0.0001$). In both generations, it was 20-25% lower on *HYD1*$_{RNAi}$10, 12, 25 compared to Col-0 (Figure 2-4 A); *HYD1*$_{RNAi}$7 did not differ compared to Col-0 (Figure 2-4 A). However, there was no generation effect ($\chi_1^2 = 2.0$, $P = 0.1587$) and the line-by-generation interaction was not significant ($\chi_4^2 = 0.40$, $P = 0.9825$). DBM pupal mass was also significantly affected by plant line ($F_{4,199} = 90.0$, $P < 0.0001$). It was significantly decreased (by 10~15%) in both generations on *HYD1*$_{RNAi}$10, 12, 25, relative to Col-0; no difference was observed between Col-0 and *HYD1*$_{RNAi}$7 (Figure 2-4 B). There was no significant effect of generation ($F_{1,199} = 0.77$, $P = 0.3825$), but there was a

significant line-by-generation effect ($F_{4,199}$ = 4.23, P = 0.0026), with the magnitude of the differences being slightly smaller in the second generation (Figure 2-4 B).

We also assessed the effects of the different plant lines on three adult traits. DBM eclosion success was significantly affected by plant line ($\chi_4^2 = 32.3$, $P < 0.0001$). Compared to Col-0, eclosion success was significantly lower on *HYD1*$_{RNAi}$10, 12, 25 in the first generation, and *HYD1*$_{RNAi}$10, 25 in the second generation (Figure 2-4 C; always below 50%); no difference was observed between Col-0 and *HYD1*$_{RNAi}$7 in either generation (Figure 2-4 C). There was no generation effect ($\chi_1^2 = 2.7$, P = 0.0998), nor was the line-by-generation interaction significant ($\chi_4^2 = 0.37$, P = 0.9848). Plant line did not significantly affect development time to the adult stage ($\chi_4^2 = 3.7$, P = 0.4447), even though it was slightly delayed on *HYD1*$_{RNAi}$10, 12, 25 (Figure 2-4 D). Development was slightly, but significantly longer in the second generation ($\chi_1^2 = 42.5$, $P < 0.0001$; Figure 2-4 D), but there was no significant line-by-generation interaction ($\chi_4^2 = 0.90$, P = 0.9247). Finally, there was a significant effect of plant line on egg production ($F_{4,61}$ = 90.0, $P < 0.0001$). In both generations, egg production on *HYD1*$_{RNAi}$10, 12, 25 was often reduced by > 50% compared to Col-0 (Figure 2-4 E).

2.3.4 Δ^8 sterols are recovered from DBM reared on lines with modified sterols

We characterized the sterol profile of DBM reared on different plant lines (expressed as ug/mg dry mass), as well as the sterol profile of feces produced by DBM caterpillars (also expressed as ug/mg dry mass). First, we examined sterol amounts and profiles from newly eclosed adult DBMs. We detected cholesterol and sitosterol in all

caterpillars, but Δ^8 sterols were only recovered from caterpillars reared on *HYD1*$_{RNAi}$10, 12, 25 plants (Figure 2-5 A). In terms of total sterol amounts, differences were observed for caterpillars reared on different plant lines ($F_{4,31}$ = 4.1, P = 0.0106), but compared to Col-0 no differences were observed (Figure 2-5 A). We also compared cholesterol content of DBM from different Arabidopsis lines and found significant differences ($F_{4,31}$ = 4.5, P = 0.0036). Amounts were lower in *HYD1*$_{RNAi}$10, 12, 25 reared moths compared to Col-0 moths (Figure 2-5 A), but there was no difference between *HYD1*$_{RNAi}$7 and Col-0 (Figure 2-5 A). Furthermore, we examined cholesterol as a proportion of the total sterol profile and found significant differences between lines ($F_{4,31}$ = 33.6, P < 0.0001). In moths reared on Col-0 and *HYD1*$_{RNAi}$7, cholesterol was > 60% of the total sterol profile, but for moths fed with *HYD1*$_{RNAi}$10, 12, 25 plants it was < 40% (Figure 2-5 A).

The sterol profiles of DBM feces were similar to DBM bodies for the corresponding lines (Figure 2-5 B), but total sterol amounts in the feces did not differ as a function of plant line ($F_{4,15}$ = 3.0, P = 0.0735). For caterpillars on Col-0 and *HYD1*$_{RNAi}$7, β-sitosterol was the dominant sterol in feces for (> 50%). In contrast, for DBM reared on *HYD1*$_{RNAi}$10, 12, 25, Δ^8 sterols dominated the feces, making up > 60% (Figure 2-5 B).

2.3.5 GPA growth and reproduction are reduced on HYD1$_{RNAi}$ *lines with modified sterols*

We also tested how *HYD1*$_{\mathrm{RNAi}}$ plants affected green peach aphid growth and reproduction. Plant line significantly affected time to reach the adult stage ($\chi_4^2 = 60.1$, P < 0.0001) and adult body mass ($F_{4,30}$ = 18.5, P < 0.0001). Adult development was delayed on *HYD1*$_{RNAi}$10, 12, 25 compared to Col-0 (Figure 2-6 A), but *HYD1*$_{RNAi}$7 did not differ

compared to Col-0 (Figure 2-6 A). A similar pattern, only more dramatic, was observed for adult body mass (Figure 2-6 B); body mass for GPA feeding on *HYD1*$_{RNAi}$10, 12, 25 was reduced approximately 30% compared to Col-0 fed GPA. Reproduction also differed on the different plant lines ($F_{4,60}$ = 10.4, P < 0.0001). It was reduced 20-30% on *HYD1*$_{RNAi}$10, 12, 25 compared to Col-0 (Figure 2-6 C), but no significant difference was observed between Col-0 and *HYD1*$_{RNAi}$7 (Figure 2-6 C).

Aphids are parthenogenic and show telescoping generations (i.e., daughters bear parthenogenetic granddaughters) while growing inside their mothers. Thus, on two lines – Col-0 and *HYD1*$_{RNAi}$10 – we reared aphids for three successive generations. This allowed us to test for potential maternal diet effects. In the first (F1) generation, GPAs on *HYD1*$_{RNAi}$10 exhibited significantly delayed development, reduced body mass and decreased reproduction (Figure 2-3, Table 2-5). In the second (F2) and third (F3) generations, nymphs on *HYD1*$_{RNAi}$10 again took significantly longer to develop into adults, were smaller and produced fewer offspring compared to nymphs reared on Col-0 (Figure 2-7; Table 2-5). Generally, GPA performance in the second and third generations was similar to the first generation (Figure 2-7), although development time decreased slightly (but significantly) by the 3rd generation (Table 2-5). For all three measured traits, there was no line-by-generation interaction (Table 2-5).

2.3.6 Total sterol amount is reduced in GPA fed HYD1$_{RNAi}$ *lines with modified sterols*

We used GC-MS to identify and quantify the body sterol profile of GPA reared on Col-0 (control), *HYD1*$_{RNAi}$7 (a second control) and *HYD1*$_{RNAi}$10, 12, 25 (lines with

modified sterols). The total amount of sterols recovered from aphids differed significantly ($F_{4,19}$ = 23.7, P < 0.0001). For aphids reared on *HYD1*$_{RNAi}$10, 12, 25 lines, total sterol amount was < 50% compared to Col-0 (Figure 2-8 A); there was no difference between *HYD1*$_{RNAi}$7 and Col-0 (Figure 2-8 A). Three sterols were recovered from aphids on all the lines: β-sitosterol was the most abundant (approximately 70% of total sterols), followed by cholesterol and then campesterol. Also notable was the lack of Δ^8 sterols in aphid bodies. Generally, the proportions of β-sitosterol, cholesterol and campesterol in aphids was similar across the 5 plant lines tested. However, there was a difference in the absolute amount of cholesterol recovered from these aphids ($F_{4,19}$ = 34.1, P < 0.0001). For GPA feeding on *HYD1*$_{RNAi}$10, 12, 25, cholesterol amounts were reduced by > 50% compared to Col-0 (Figure 2-8 A).

2.3.7 Feeding behavior of GPA is not affected by HYD1$_{RNAi}$ lines

We used the electronical penetration graph (EPG) technique to characterize aphid feeding behavior on 4 Arabidopsis lines: Col-0 (control), *HYD1*$_{RNAi}$7 (a second control) and *HYD1*$_{RNAi}$10, 12 (modified sterol lines). Among all the EPG variables were measured and analyzed; no difference was observed between our different plant lines. In Table 2-6, we presented summary statistics for 40 variables that characterize seven key EPG events: nonprobing (Np), pathway (C), cell puncture (Pd), derailed stylet mechanics (F), xylem ingestion (G), phloem salivation (E1) and phloem ingestion (E2). The only significant difference was in mean pathway (C) duration, but if we apply a Bonferroni correction this

variable is no longer significant. In Figure 2-8 B we show a breakdown of probing activity; for all four lines, the time spent in pathway (C) and phloem ingestion (E2) dominated.

2.4 Discussion

For insect herbivores, plant phytosterol composition and quantity can have significant negative repercussions on development, survival and reproduction (Behmer 2017, Behmer & David Nes 2003). Given these constraints, there is potential to modify plant sterol profiles to manage insect pests. In this study, we silenced Arabidopsis *HYD1*, a gene controlling a critical double-bond shifting event in the plant sterol biosynthetic pathway, i.e. converting Δ8 to Δ7 sterols. In three of our *HYD1*-silenced lines (*HYD1*$_{RNAi}$10, 12 and 25) we observed two effects: (1) a reduction in the total amount of sterols produced, and (2) the accumulation of Δ^8 sterols, which are unsuitable for insects (Corio-Costet et al 1989, Ritter 1986). These changes did not affect plant morphology, but they did negatively impact the performance of two insect herbivores – the diamondback moth and the green peach aphid. Interestingly, the sterol profiles of these two insect species differed (Figure 2-10). This is likely linked to the sterol profile of the plant tissues they consumed (leaf tissues for the caterpillar and phloem for the aphid), but it could also reflect differences in metabolic activities between the two species. Our results suggest that Δ^8 sterols in *HYD1*$_{RNAi}$10, 12, 25 may have exerted a direct toxic effect on DBM, whereas insufficient Δ^5 sterols likely explain poor performance of GPA.

We demonstrated the advantage of an RNAi technique to acquire *HYD1*-silenced plants over the established *HYD1* mutants directly. Selected lines (*HYD1*$_{RNAi}$10, 12, 25)

produced leaf sterol profiles that accumulated unsuitable sterols, plus contained typical end-product phytosterols. This latter observation is important because the depletion of typical end-product Δ^5 sterols is known to have detrimental impacts on plants. Previous studies have shown that the *HYD1* mutant exhibits embryonic defects and seedling death when the two major phytosterols, *β*-sitosterol and campesterol, were reduced to only 0.2% of the wild-type (Schrick K et al 2004). In *HYD1*$_{RNAi}$10, 12, 25, however, the reduction was much less dramatic with Δ^5 sterols dropped to 30~40%. In fact, this reduction level is similar to a weak allele of *ste1* mutants reported previously, in which the mutant produced 30% of Δ^5 sterols without showing a negative morphological phenotype (Husselstein et al 1999, Silvestro et al 2013). Whether 30% is the threshold for the normal growth and development of plants remains unknown. However, it appears that wild-type plants tend to produce more sterols than they need. Previous studies revealed that overproduced sterols were acylated into sterol esters via phospholipid: sterol acyltransferase (PSAT) and stored as lipid droplets to maintain the sterol homeostasis (Banas et al 2005, Benveniste 2004). It should be noted that sterol quantity is not the only factor that could affect plant growth and development. An altered ratio of campesterol to sitosterol also results in substantial negative effects on plant morphology even though there is no reduction in the total sterol pool (Schaeffer et al 2001). By using an RNAi approach, we were able to select *HYD1*-silenced lines without arrested growth, development and fertility, which are ideal material for the following insect studies.

We also characterized the sterol profile of the phloem in three of our lines (Col-0, plus *HYD1*$_{RNAi}$10, 25). Cholesterol and sitosterol were the dominant sterols recovered in

the three lines (Table 2-4), and this is similar to what has been reported for beans (*Vicia faba*, *Phaseolus vulgaris*) and tobacco (*Nicotiana tabacum*) (Behmer et al 2011, Behmer et al 2013, Bouvaine et al 2012). Additionally, sterols existed in the phloem in three forms – as free sterols; conjugated to fatty acids; and conjugated to sugars; this too is consistent with results reported for the phloem sterol studies listed above. Perhaps most notable in our study was that we never observed Δ^8 sterols in the phloem of *HYD1*$_{RNAi}$10 and 25. This suggests the transportation of sterols to the phloem is conducted in a highly specific manner with only specific sterols being transported. In mammalian systems, cholesterol plays a crucial role in cellular signaling network and lipid transfer systems. Cholesterol trafficking is highly complex and contains various tightly controlled stages, with multiple proteins organized by apoliproteins (Barter et al 2007, Ikonen 2008, Mahley 1988). In contrast, since neither cholesterol nor major phytosterols has been found to have any signaling function in plants, sterol transport in plants is understudied. In addition, the regulatory roles of cholesterol observed in mammals appear not to apply in plants since plants do not have dietary sterol intake, and the excess sterols have not been found negatively affecting plants (Lange et al 2015). Our study, however, demonstrates the importance of plant sterol transport, and the fact that only certain types of sterols are carried into the phloem. In fact, studies on sterol biosynthetic mutants have suggested potential signaling roles of plant sterols, even though no direct evidence has been demonstrated (Clouse 2002). It will be interesting to investigate whether Δ^5 sterols in phloem play a role in long-distance signalling. Additionally, future investigations into

plant sterol transporters will increase our understanding of how and why Δ^8 sterols are excluded from phloem.

The different sterol contents in GPA and DBM on *HYD1*$_{RNAi}$10, 12, 25 indicates these two insects were affected through different mechanisms. Apparently, DBM could not utilize Δ^8 sterols since these sterols cannot be converted to cholesterol. The accumulation of Δ^8 sterols in DBM showed a strong association with reduced survival, suggesting Δ^8 sterols have a toxic effect on DBM. As a vital membrane component in animals, including insects, cholesterol regulates cell membrane organization, maintaining its fluidity and rigidity and it is critical for the formation of highly ordered domains (Simons & Ikonen 1997). When cell membrane cholesterol is depleted, or replaced by other sterols (*e.g.* Δ^7 or Δ^8 sterols), it can drastically change membrane properties including ion homeostasis, stiffness and fluidity, which in turn affects the function of membrane proteins (Grouleff et al 2015, Haines 2001, Wang et al 2004). For example, when cell membranes incorporate zymosterol (a Δ^8 sterol) instead of cholesterol, organization of the lipid raft is weakened (Hąc-Wydro et al 2014). Since incorporation of Δ^8 sterols could modify cell membrane properties, we speculate the presence of Δ^8 sterols in DBM alters cell membrane structure and environment. Cell membrane damage can significantly interfere with the proper functioning of various insect tissues. Previous studies had compared sterol profiles of whole insects to midguts and suggested that unmetabolized sterols tend to accumulate in the midgut (Behmer et al 1999b). This accumulation is likely due to the extensive cell membrane tissues and the rapid proliferation and regeneration of the midgut epithelium during each instar (Hakim et al

2009). Keeping a healthy midgut epithelium is highly important for the completion of various cell membrane activities, which are responsible for nutrient assimilation, digestive enzyme production, and providing barriers against plant defensive compounds and pathogenic microbes (Huang et al 2015). In caterpillars, the apical surface of the midgut is exposed to a highly alkaline environment, while the basal surface is exposed to a neutral or slightly acidic environment. This steep pH gradient is driven by proton pumping via the plasma membrane vacuolar-ATPase (V-ATPase) in the midgut, which also powers the epithelial ion transporter (Terra et al 2006, Wieczorek et al 2003). However, when insects are reared on unsuitable sterols, the expression of V-ATPase is upregulated, in addition to the differential gene expressions of various ion transporters (transcriptome analysis of *Helicoverpa zea*) (Jing et al 2012b). The stimulation of ATP pump is very likely caused by cation leaking, which is generally inhibited by the appearance of cholesterol in membrane as a mechanism to prevent wasting metabolic ATP energies (Haines 2001). The incorporation of unsuitable sterols into cell membranes can also affect other physiological processes, including immune responses and nutrient absorption (Jing et al 2012b). The amino acid transporters of lepidopterans, for instance, rely on V-ATPase and an antiporter to provide the drive force for the absorption of amino acids (Castagna et al 1997). The inefficient food absorption caused by incorporation of unsuitable sterols could severely affect insect growth. To further investigate the exact effects of Δ^8 sterols on insect membrane structure and membrane transporters, it will be valuable to study the transcriptome of insects fed on Δ^8 sterols in the future.

In contrast to DBM, unsuitable sterol was absent in GPA after feeding on *HYD1*$_{RNAi}$10, 12, 25. However, the drastically decreased sterol quantity suggests that the observed delayed development and reduced reproduction could be linked to insufficient sterols. When the minimum requirement for sterols is not met, the lack of sterols as nutritional components could directly affects aphid growth. A deficiency in cholesterol could also lead to reduced ecdysteroid biosynthesis (Costet et al 1987). As the insect moulting hormone, the decreasing of the ecdysteroid titer could delay expression of molting genes in every nymphal stage (Christiaens et al 2010), which could explain delayed development on *HYD1*$_{RNAi}$10, 12, 25. Additionally, the deficiency in both cholesterol and ecdysteroids could severely affect GPA reproduction. During oogenesis, sufficient nutrients, including cholesterol and other lipids, are required (Klowden 2013). A prioritized sterol allocation to reproduction was demonstrated previously in pea aphids (*Acyrthosiphon pisum*), and higher cholesterol density was detected in insect embryos than in aphids whole body. Pea aphids on both insufficient and unsuitable sterol diets display decreased number of embryos and reduced embryo protein contents (Bouvaine et al 2012), indicating the crucial role of sterols in oogenesis and embryogenesis. Furthermore, ecdysteroids might also play a role in reproduction since silencing of the 20E receptor during oogenesis and embryogenesis leads to a significantly reduced reproduction in grain aphids (*Sitobion avenae* F.) and brown citrus aphids (*Aphis citricidus*), indicating 20E signal is required for the reproduction of these two insects (Shang et al 2018, Yan et al 2016).

Apart from unsuitable sterols, the compositions of suitable sterols in DBM and GPA varies greatly, indicative of different sterol metabolic pathways adopted by these two insects. Effective dealkylation at C-24 of phytosterol is generally true for caterpillars or grasshoppers, but not for aphids (Behmer & David Nes 2003, Nes et al 1997). Similarly, in our study, the ratio of cholesterol to phytosterol (RCP) in DBM body was approximately 1.5~2. This suggests DBM have are very efficient at converting phytosterol to cholesterol, given the RCP in the rosette leaves was as low as 0.02~0.03. In contrast, the RCP in GPA was only 0.25~0.3. Given that phloem has a RCP of 0.4~0.6 RCP, this suggests that the C-24 dealkylation capacity of aphids is relatively low. Previous studies in other aphid species have revealed similar findings. That is, that compared to chewing insects, a much larger proportion of phytosterols was recovered from aphids feeding on diet containing phytosterols, and these studies suggested aphids were able to use phytosterol as well as cholesterol for constructing cell membranes (Behmer et al 2011, Campbell & Nes 1983, Janson et al 2009). It is interesting that GPA on *HYD1*$_{\text{RNAi}}$10, 12, 25 displayed proportionally decreased phytosterol and cholesterol, which indicates GPA did not prioritize sterol deacylation even when cholesterol level is largely reduced.

We did not observed differences in feeding behavior of aphids feeding on different Arabidopsis lines, as revealed by electronical penetration graph (EPG) methods. EPG analysis can reveal a sequence of stylet activities during aphid probing and feeding, as each behavior is followed by the assessment of different physical or chemical cues (Powell et al 2005). When aphids are released on plants, their antennae make contact with plant surfaces, and assess a variety of cues, such as texture, volatiles, and trichome exudates

(Müller & Riederer 2005). This so called "time to first probing (min)" reflects the period of time from contacting to starting probing when aphids might perceive repellent cues from plants (Goławska & Łukasik 2012). However, no difference in "time to first probing" was observed among different lines in this study, indicating cues from plant surfaces of *HYD1*-silenced lines were indifferentiable to aphids. After making contact, aphids initiate stylet penetration to further assess their hosts before phloem feeding. The non-phloem peripheral cues acquired during penetration are critical for aphids to determine acceptance of their the host plants (Powell et al 2005). On one hand, chemical cues (*e.g.* sugars, amino acids, plant secondary compounds, etc.) from apoplast or epidermis are ingested with small quantities by aphids, resulting in accepting or rejecting the plant. On the other hand, physical cues (*e.g.* size and shape of plant cells, thickness of the cell walls, etc.) can also lead to the changes of feed behaviors (Matsiliza & Botha 2002). Taken together, stylet penetration before phloem ingestion is critical for aphids to discriminate their host, and our EPG results displayed 60% of the overall time was spent in this period, including C-pathway penetration, Pd-cell puncture and F-derailed stylet mechanics. However, no difference was observed in variables associated with these activities, indicating chemical and physical cues given by *HYD1*-silenced plants and the wild-type were similar to GPA. Finally, aphids start phloem salivation and ingestion (E1 and E2) to obtain food. Generally, a sustained ingestion over 10 min represents the acceptance of phloem content by aphids (Powell et al 2005, Tjallingii 1994). In our case, GPA accepted all the lines equally after phloem ingestion and decided to continue for a much longer period of time. Although we could not quantify sterols in the phloem, the reduced total sterol recovered

from aphid body suggests reduced levels of sterol in the phloem. In addition, it appears that sterols in phloem have no effect on aphids feeding behaviors. Previous studies on grasshoppers have shown a similar result. Specifically, feeding behavior is only modified when unsuitable sterols are present, and changes in feeding behavior to unsuitable sterols occur post-ingestively, and involve learning (Behmer et al 1999a, Cook 1977, Harley & Thorsteinson 1967).

As an environmentally friendly alternative to chemical pesticides, genetically engineered crops have been playing an important role in pest management. Our study demonstrated that we can develop sterol-modified plants to control insects, by utilizing the constraints of insects in sterol synthesis and metabolism. The long-term impact of *HYD1*-silenced plants on insects was demonstrated, by estimating the effect on insects at the population level (Figure 2-11). We found the impact was accumulated on both insects, particularly on chewing insects. To simplify the model, we applied a previously published method, using 100 individuals as a start population with no other biotic or abiotic stress considered (Behmer & Grebenok 1998, Jing et al 2012a). It is notable that for both insects on sterol-modified plants, population size was decreased to less than 50% of the starting population within only two to three generations. In field conditions, we expect to see the enhanced negative effect of *HYD1*-silenced plants, as longer developmental times force insects to spend more days in the less mobilized larvae/nymph stages, which increases the exposure to other biotic and abiotic stresses. One of the current successful examples of transgenic plants for pest control (particularly for chewing insects) is the *Bacillus thuringiensis* (Bt) crops. Introducing two or more genes that synergistically defend plants

against insects is becoming a major interest in developing second generation Bt-crops. An example is transgenic cotton producing two Bt toxins, Cry1Ac and Cry2Ab. Most of the combinations of Cry1Ac and Cry2Ab exhibited synergism between these two toxins and increased the motility of cotton bollworm (*Helicoverpa armigera*) strains that were resistant to a single Bt toxin (Wei et al 2015). Apart from combining different Bt toxins, the integration of *HYD1* silencing and Bt toxins in transgenic crops can be a novel strategy. Most Bt toxins create holes on insect gut membranes by interacting with specific receptors on the host cell surface or interacting with membrane lipids directly (Bravo et al 2007). With the membrane environment modified by Δ^8 sterols as we suggested previously, it will be interesting to investigate: first, whether the 'leaky' membranes with Δ^8 sterols make it more vulnerable for hole formation on midgut epithelium; second, whether the presence of Δ^8 sterols changes the interaction between Bt toxins and membrane receptors. Presumably, the impact of Δ^8 sterols on insects is systematic, and to develop resistance, would require insects to evolve the enzyme $\Delta^{8,7}$ sterol isomerase or even the whole sterol biosynthetic pathway. This is very different from developing resistance against Bt toxins, in that mutations on a single receptor gene is sufficient for insect to annihilate the toxicity. By knocking down *HYD1* gene and expression Bt toxins simultaneously, it might be a good strategy to increase the effectiveness and durability of the transgenic crops.

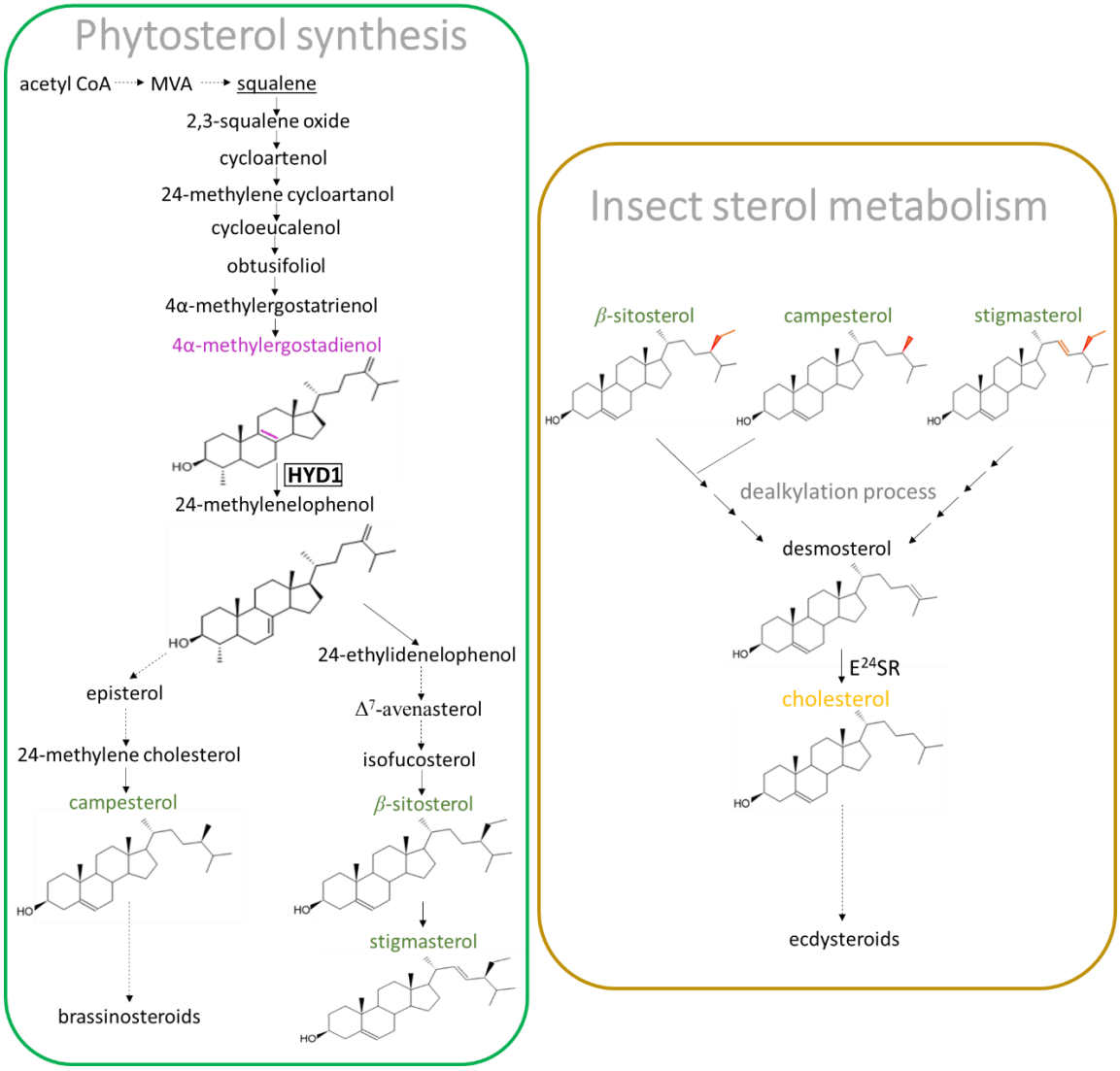

Figure 2-1 The sterol biosynthesis pathway in plants and its relationship to sterol metabolism in insect herbivores.

Arrows with solid line mean one step, dotted arrows mean multiple steps involved. The green box shows the phytosterol biosynthesis pathway - the early steps involves part of the mevalonate (MVA) pathway, and leads to the synthesis of squalene. In post-squalene biosynthetic pathway. HYD1 - $\Delta^{8,7}$ sterol isomerase isomerizes the C8 double bond to C7 double bond and forms 24-methylenelophenol, and eventually leads to the synthesize of *β*-sitosterol, campesterol and stigmasterol. The brown box shows the insect sterol metabolism pathway. The three predominant phytosterols are up-taken by insects and converted into desmosterol via several steps of dealkylation process, and then desmosterol is converted into cholesterol via C24 sterol reductase. The sidechain and double bond removed during dealkylation process were marked in red color. Some of the cholesterol acts as the precursor of ecdysteroids.

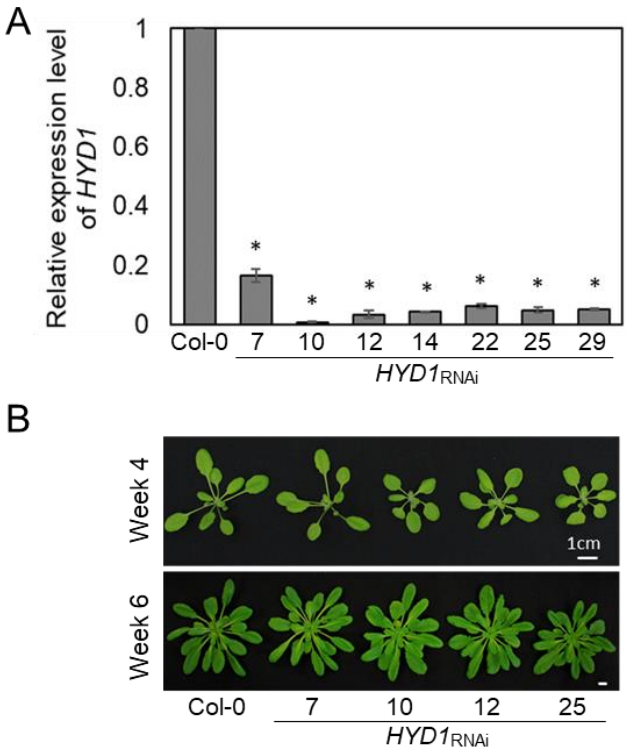

Figure 2-2 *HYD1$_{RNAi}$* lines did not display drastically changed phenotype while *HYD1* expression level was repressed.

A, qPCR of the silenced lines *HYD1$_{RNAi}$*7, 10, 12, 25 showed the relative fold change ± SEM (n = 3) of *HYD1* transcripts were largely reduced in comparison to Col-0. Asterisks indicate significant difference from Col-0 as calculated by Dunnett`s test (asterisk indicates *P* value < 0.001). **B,** Rosette of *HYD1$_{RNAi}$* Arabidopsis lines as they were placed with wild-type Col-0 side by side at 4-week and 6-week old. Silenced line *HYD1$_{RNAi}$*10, 12, 25 showed smaller size at 4-week old, the difference became smaller at 6-week old. The size of rosette and petiole length were measured and presented in Table 2-2.

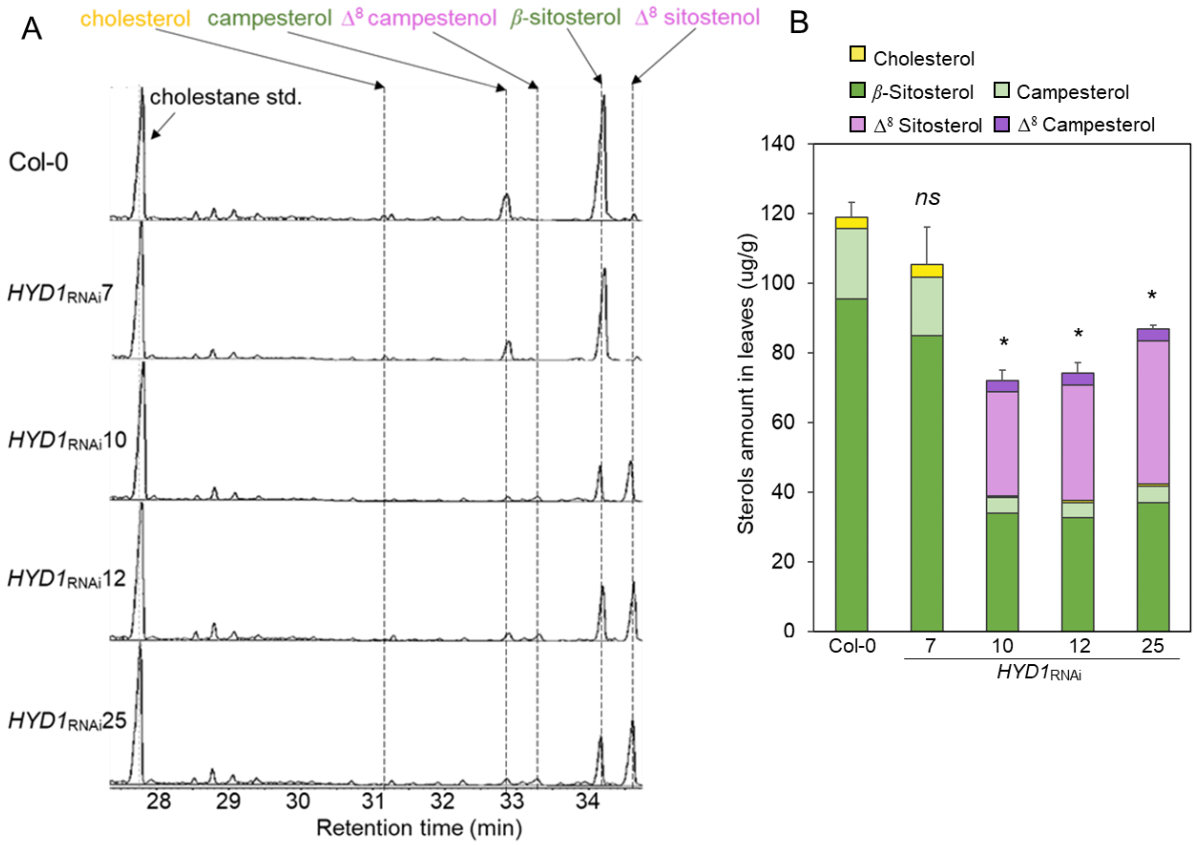

Figure 2-3 Sterol profile of *HYD1*$_{\text{RNAi}}$ Arabidopsis was modified except for line *HYD1*$_{\text{RNAi}}$7.

A, GC-MS spectra of *HYD1*$_{RNAi}$ lines and Col-0 showed similar pattern between *HYD1*$_{RNAi}$7 and Col-0, while *HYD1*$_{RNAi}$10, 12 and 25 exhibited distinct sterol profile from Col-0. TIC was displayed to show the relative abundancy of the main sterols detected, with two new sterols - Δ^8 sitostenol and Δ^8 campestenol that were only observed in *HYD1*$_{RNAi}$10, 12 and 25. **B,** Absolute amount (µg sterol in per mg lyophilized vegetative sample) of the predominant sterols in *HYD1*$_{RNAi}$ lines and Col-0 was determined by GC-MS, values in the panels indicate mean ± SEM (n = 3~4). Error bars indicate the standard error calculated for the total sterols amount. Asterisk indicates the total sterol amount in a silenced line is significantly different from Col-0 calculated by Dunnett`s test ($P < 0.05$), *ns* indicates no significant difference was observed compared to Col-0.

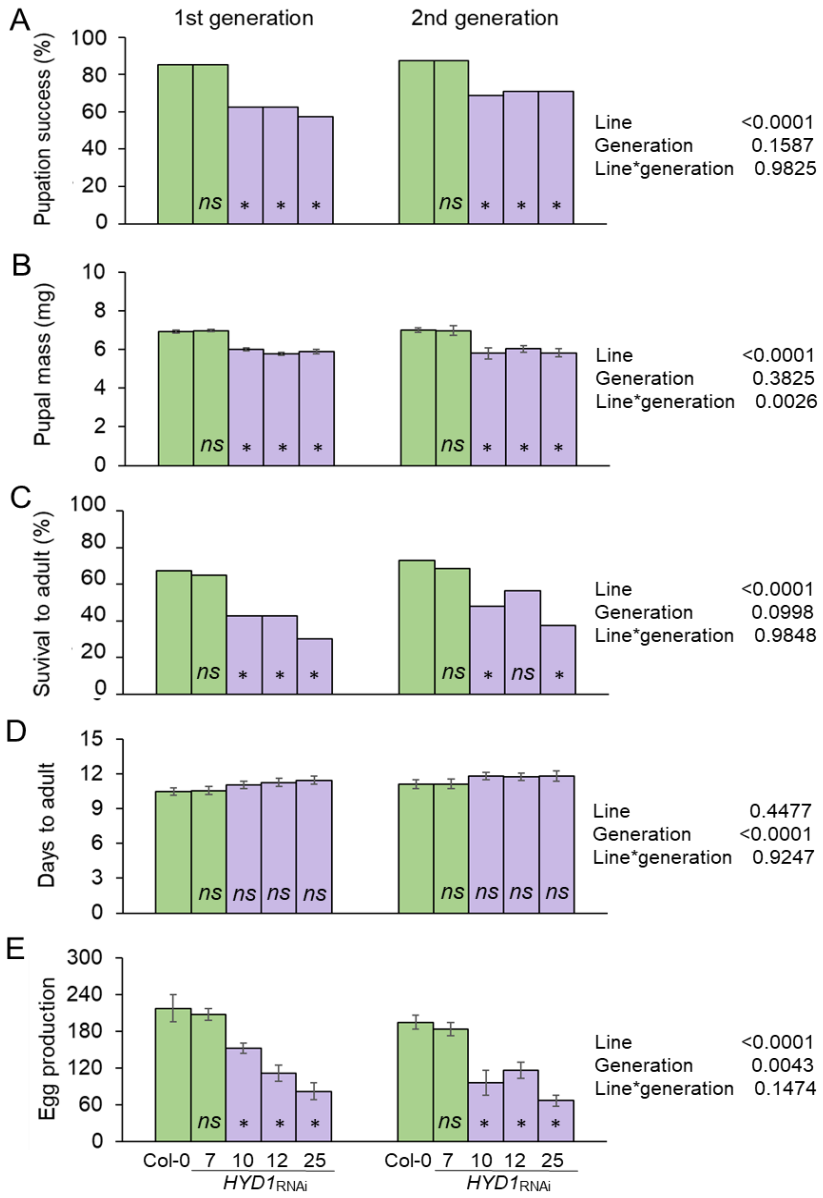

Figure 2-4 The performance of *P. xylostella* on *HYD1*$_{RNAi}$10, 12 and 25 was decreased in both two generations.

A, the percentage of neonates that successfully became pupae. **B**, fresh weight of pupa in the second day of pupation, mg ± SEM (n = 28-38). **C**, the percentage of neonates that successfully survived to adult stage. **D**, amount of time from hatching to 1st day adult, days ± SEM (n = 20-34). **E**, total egg number produced per female adult in the first three reproductive days, mean ± SEM (n = 5-10). The value shows the *P* value of the effect of line, generation and line*generation. Likelihood Ratio Chi-Square test was used for pupation rate and survival rate. Dunnett`s test was performed for pupa mass and egg production, and Steel`s test was performed for days to adults comparing to control. Asterisks indicate the significant difference from Col-0 ($P < 0.05$).

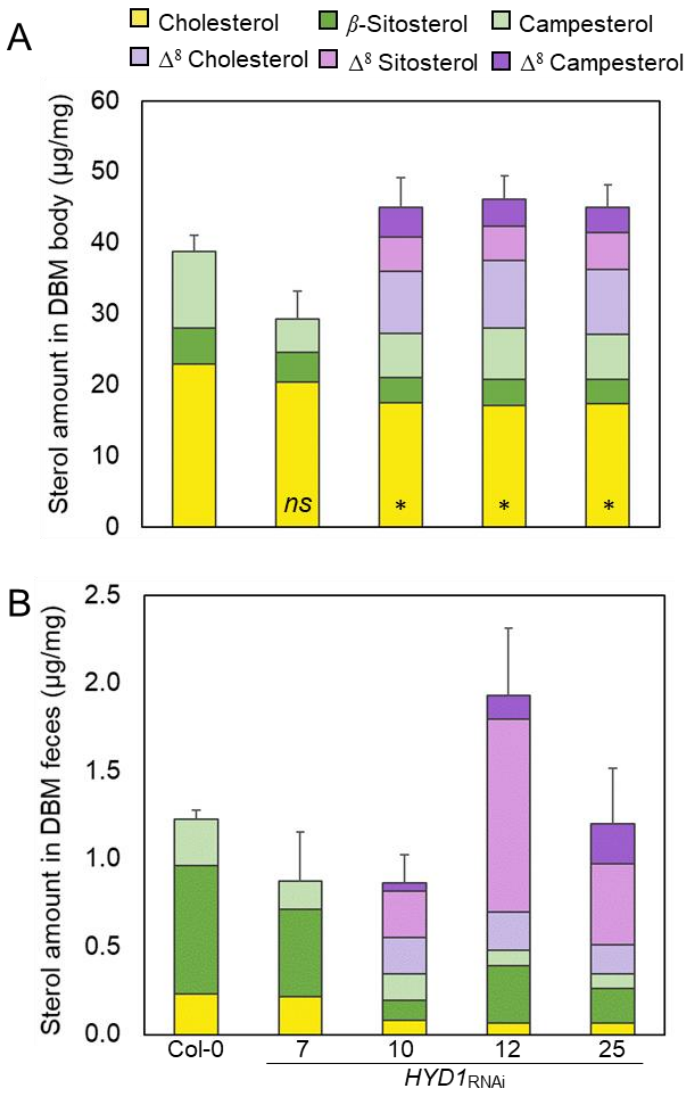

Figure 2-5 Sterol profile of *P. xylostella* body and feces were altered after feeding on *HYD1*$_{RNAi}$10,12,25.

A, three atypical sterols - Δ^8 sitostenol, Δ^8 campestenol and Δ^8 cholestenol were only observed in the adult body of DBM on *HYD1*$_{RNAi}$10, 12 and 25. Asterisks presented in the yellow series indicate the percentage of cholesterol in total sterol was significantly changed compared to Col-0, *ns* means there was no significant difference calculated by Dunnett`s test ($P < 0.05$). The percentage of each sterol was calculated in Table 2-3. **B.** DBM feces showed the presence of Δ^8 sitostenol, Δ^8 campestenol and Δ^8 cholestenol after feeding on *HYD1*$_{RNAi}$10, 12 and 25. The level of cholesterol and *β*-sitosterol in feces was also significantly reduced on these lines. Error bars in both panels indicate the standard error of total sterol amount. Significant difference was calculated by Dunnett`s test comparing to Col-0 ($P < 0.05$). The absolute amount of total sterols in DBM body and feces from each silenced line did not show significant difference compared to that DBM reared on Col-0.

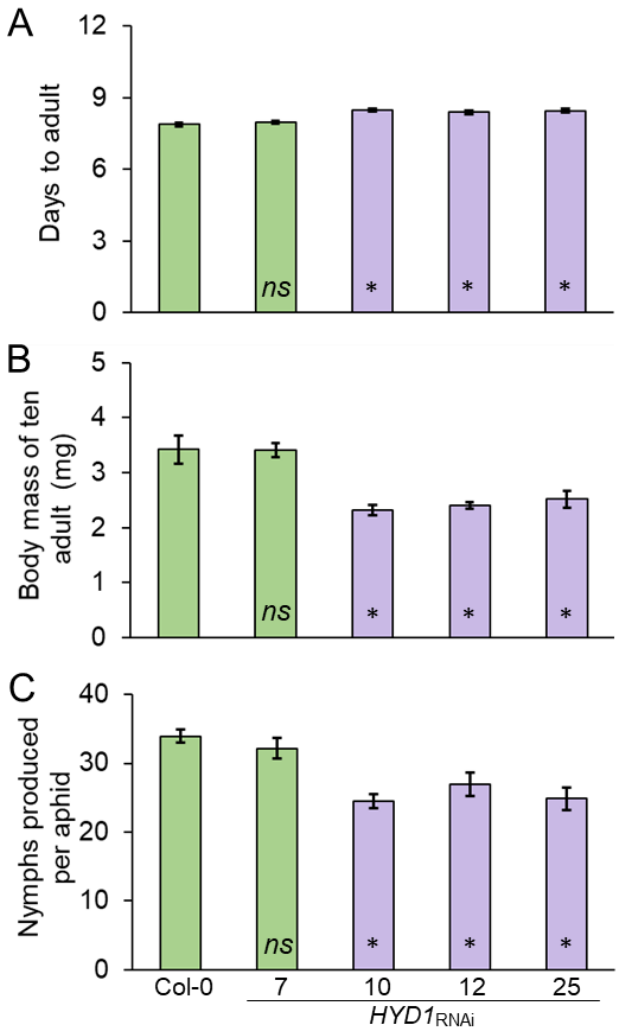

Figure 2-6 *M. persicae* displayed delayed development and reduced reproduction on *HYD1*$_{RNAi}$10, 12, 25.

A, developmental time of *M. persicae*, from born (1st instar nymph) to adult (n = 16). **B**, fresh weight (mg) of every ten adult aphids combined from each line, mg ± SEM (n = 6). **C**, offspring produced per adult in the first seven days of reproduction, mean ± SEM (n = 16). Statistically significant difference of days to adults were determined by Steel`s test; body weight and nymphs produced were analyzed by Dunnett`s test. Asterisks indicate the significant difference between one *HYD1*$_{RNAi}$ line and Col-0 ($P < 0.05$).

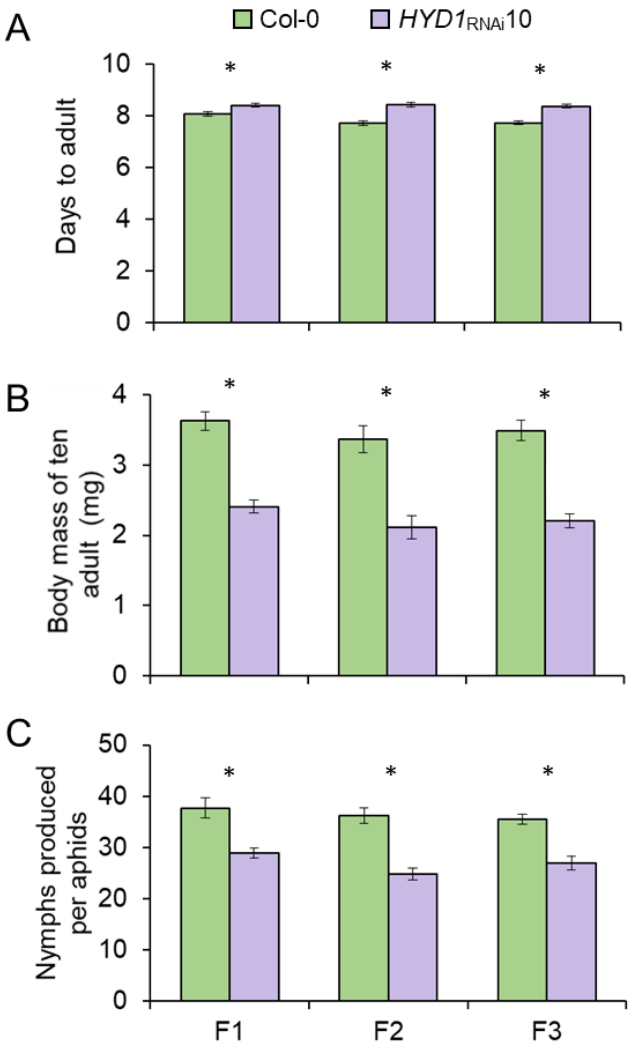

Figure 2-7 *HYD1*$_{RNAi}$10 exhibited similar effects on GPA in three generations.
A, developmental time of GPA, from born (1st instar nymph) to adult (n = 16). **B,** fresh weight (mg) of every ten adult aphids combined that were reared on each line, values indicate mean ± SEM (n = 6). **C,** offspring produced by each adult in the first seven days of reproduction, mean ± SEM (n = 16). Statistically significant difference of days to adults were determined by Steel`s test; body weight and reproduction were analyzed by Dunnett`s test. Asterisks indicate the significant difference between one *HYD1*$_{RNAi}$10 and Col-0 in each generation ($P < 0.05$). The effect of line, generation and line*generation was presented in Table 2-5.

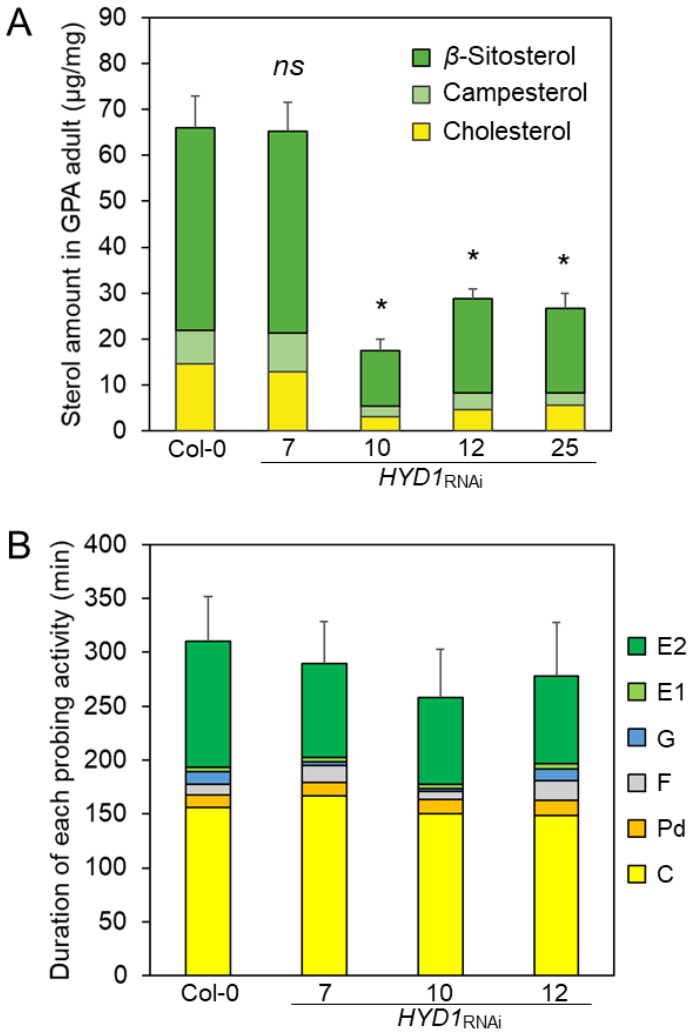

Figure 2-8 *HYD1*$_{RNAi}$ Arabidopsis altered the sterol profile of *M. persicae* without changing the aphid probing behavior.

A, levels of cholesterol and *β*-sitosterol in aphids were reduced in aphids reared on *HYD1*$_{RNAi}$10, 12 and 25 in comparison to Col-0 and *HYD1*$_{RNAi}$7. Values in this panels indicate the amount (µg) of sterols in per mg dry weight pooled aphid (n = 3). Error bars indicate standard error calculated for total sterols. Asterisks indicate significant difference between a silenced line and Col-0 calculated by Dunnett`s test ($P < 0.05$), *ns* indicates no significant difference was observed. **B**, total duration of each aphid probing activities on Col-0 and *HYD1*-silenced lines, during 6h recording (n = 17~19). C, pathway; Pd, cell puncture; G, xylem ingestion; F, derailed stylet mechanics; E1, phloem salivation; E2, phloem ingestion. Error bars stand for the standard of total probing time, calculated by summing all the activities presented above. Statistical significance was determined by Kruskal-Wallis test followed by Steel`s test ($P < 0.05$)

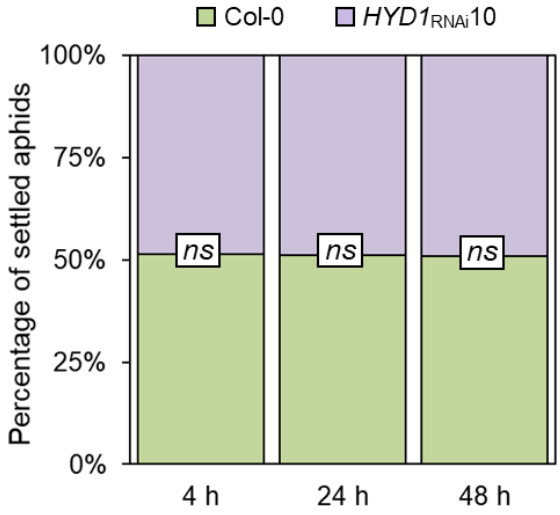

Figure 2-9 GPA did not show preference between *HYD1*$_{RNAi}$10 and Col-0, in 4 h, 24 h and 48 h.

Settled aphid number from the same genotype was combined and Likelihood Ratio Chi-Square was calculated for significant difference ($P < 0.05$), *ns* indicates no significant difference was observed.

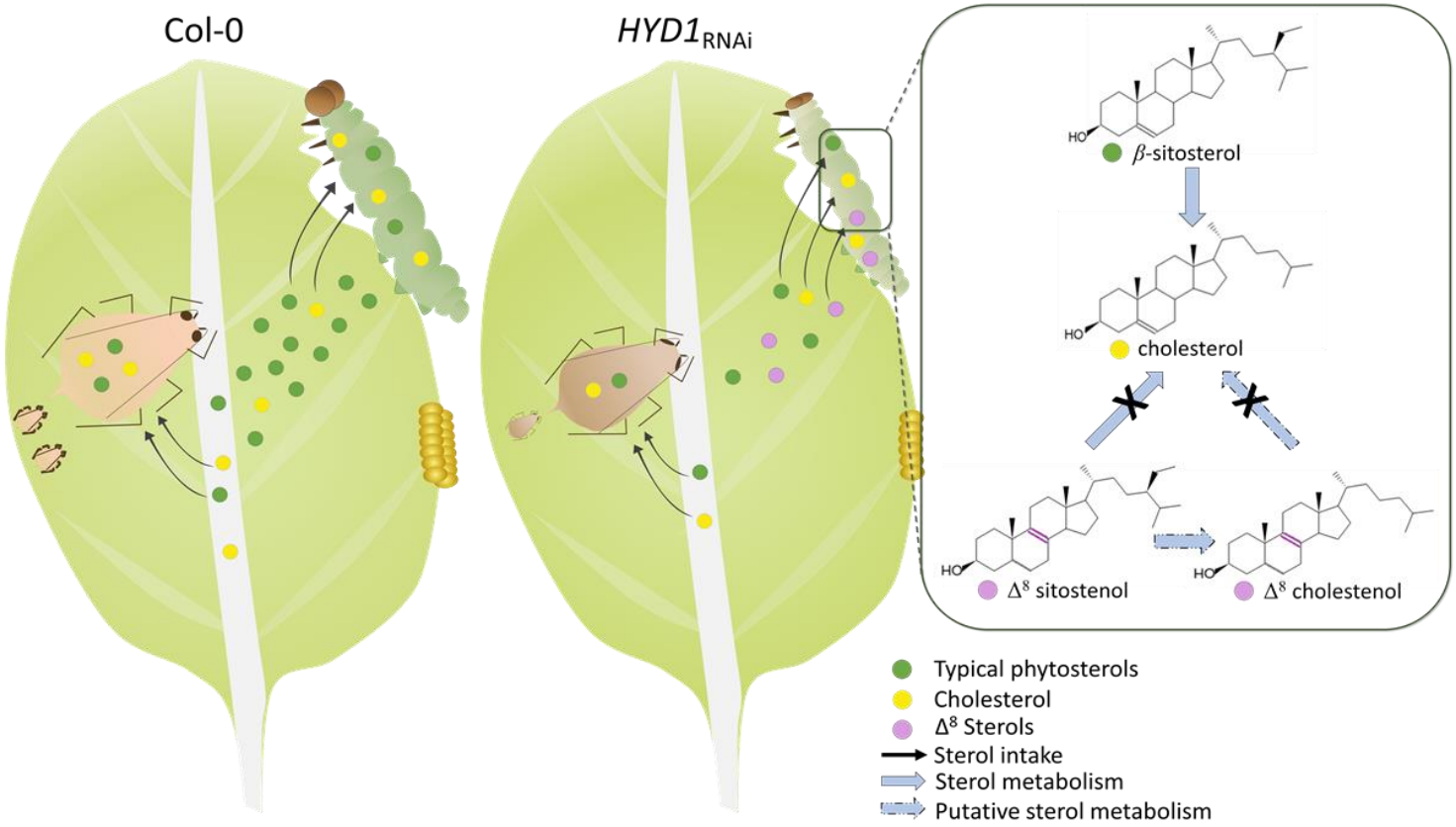

Figure 2-10 *HYD1*-silenced plants affected DBM and GPA through different mechanisms.
The typical phytosterols produced by Col-0 are suitable sterols for insects. *HYD1*-silenced lines *HYD1*$_{RNAi}$10, 12, 25 accumulate Δ^8 sterols and produced decreased sterols. However, Δ^8 sterols was absent from phloem. The performance of GPA and DBM on *HYD1*$_{RNAi}$10, 12, 25 was negatively affected, but different sterols were recovered from insects. Δ^8 sterols was only accumulated in DBM, but not in GPA. GPA exhibited decreased total sterols. The lack of sterols in GPA might be the key factor for the delayed development and reduced reproduction of this insect. The Δ^8 sterols accumulated in DBM might have a toxic effect on this chewing insect

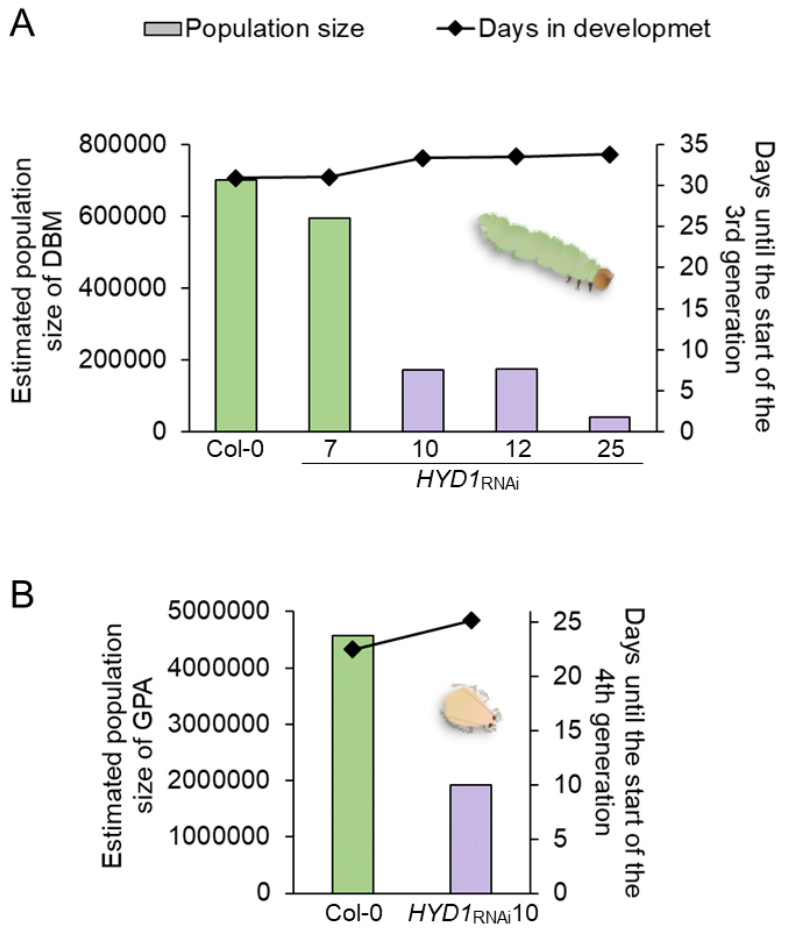

Figure 2-11 Enlarged impacts by *HYD1*-silenced plants at the population level.
Staring population with 100 individuals and 1:1 sex ratio was applied as the starting population for both DBM and GPA, and the estimation assumes the growth condition is ideal and no other factor contribute to the mortality. **A,** estimated population size and developmental time of DBM after two generations. **B,** estimated population size and developmental time of GPA after three generations.

Table 2-1 Primers used in this study.

Gene name	Forward primer (5'→3')	Reverse primer (5'→3')
For RNAi construct		
HYD1 (set1)	GGGGCGCGCC(*Asc*I)ACGGAAAGAAATGAGTGAAGG	CGATTTAAAT(*Swa*I)GCAGTTGTATCTGTTGAAGG
HYD1 (set2)	CGGGATCC(*Bam*HI)GCAGTTGTATCTGTTGAAGG	GGACTAGT(*Spe*I)ACGGAAAGAAATGAGTGAAGG
For Quantitative RT-PCR		
HYD1	ACCAATCTCAATGTCAATGTCC	CAACGATAACAGCAGTGATCC
UBQ10	AGATCCAGGACAAGGAAGGTATTC	CGCAGGACCAAGTGAAGAGTAG

Table 2-2 Silencing of *HYD1* gene moderately changed the growth and reproductive traits in Arabidopsis.

	Age	Col-0	*HYD1*$_{RNAi}$7	*HYD1*$_{RNAi}$10	*HYD1*$_{RNAi}$12	*HYD1*$_{RNAi}$25
Rosette diameter (mm)	4-week	63.00 ± 1.32	63.20 ± 0.44	44.60 ± 1.18*	52.70 ± 1.30*	46.50 ± 0.40*
	6-week	92.44 ± 2.30	87.33 ± 1.28	80.11 ± 1.94	85.30 ± 1.33	76.10 ± 2.96*
Petiole length[1] (mm)	4-week	16.15 ± 0.33	14.65 ± 0.29	10.15 ± 0.49*	10.80 ± 0.39*	9.50 ± 0.42*
	6-week	21.19 ± 0.50	21.02 ± 0.86	18.23 ± 0.70	18.40 ± 0.69	12.29 ± 0.51*
Above-ground biomass[2] (g)	4-week	0.24 ± 0.01	0.22 ± 0.00	0.14 ± 0.01*	0.21 ± 0.01	0.18 ± 0.01*
	6-week	1.21 ± 0.06	1.14 ± 0.04	1.03 ± 0.03	1.14 ± 0.02	1.01 ± 0.04
Days to bolting		35.50 ± 0.13	37.40 ± 0.15	40.33 ± 0.20*	37.74 ± 0.21	38.94 ± 0.33
Leaf number by Bolting day		21.00 ± 0.53	22.67 ± 0.49	22.56 ± 0.44	22.90 ± 0.31	23.20 ± 0.42
Total number of filled siliques	12-week	153.60 ± 6.32	159.50 ± 6.82	120.40 ± 4.62*	140.60 ± 6.20	138.00 ± 5.58
Siliques length (mm)	12-week	8.94 ± 0.06	8.91 ± 0.05	8.48 ± 0.12	8.82 ± 0.06	8.50 ± 0.11

Mean ± SEM. * Significantly different from Col-0 (Dunnett`s test, $P<0.001$)

[1] Petiole length of two largest leaves from each plant under standard growth conditions

[2] Fresh weight of above-ground tissue

Table 2-3 Percentage of each sterol in Arabidopsis rosette leaves, DBM body and DBM feces.

Tissue	Sterols (%)	Col-0	*HYD1*$_{RNAi}$			
			7	10	12	25
Plant	cholesterol	3	3	1	1	1
	campesterol	17	16	**6**	**6**	**6**
	β-sitosterol	80	81	**47**	**44**	**43**
	Δ^8 campestenol	0	0	**5**	**5**	**4**
	Δ^8 sitostenol	0	0	**41**	**44**	**47**
	Δ^5 sterols	100	100	**54**	**51**	**49**
	Δ^8 sterols	0	0	**46**	**49**	**51**
DBM body	cholesterol	59	70	**39**	**37**	**39**
	campesterol	28	16	14	16	14
	β-sitosterol	13	14	8	8	8
	Δ^8 cholestenol	0	0	**19**	**21**	**20**
	Δ^8 campestenol	0	0	**9**	**8**	**8**
	Δ^8 sitostenol	0	0	**11**	**10**	**12**
	Δ^5 sterols	100	100	**61**	**61**	**60**
	Δ^8 sterols	0	0	**39**	**39**	**40**
DBM feces	cholesterol	19	25	**9**	**3**	**6**
	campesterol	22	19	18	**4**	**7**
	β-sitosterol	60	56	**13**	**17**	**16**
	Δ^8 cholestenol	0	0	**24**	**11**	**14**
	Δ^8 campestenol	0	0	**5**	**7**	**19**
	Δ^8 sitostenol	0	0	**31**	**57**	**38**
	Δ^5 sterols	100	100	**40**	**25**	**29**
	Δ^8 sterols	0	0	**60**	**75**	**71**

Values presented as mean percentage (%). Value of Δ^5 sterols sums up the percentage of cholesterol, campesterol and *β*-sitosterol combined. Value of Δ^8 sterols sums up the percentage of all the detected Δ^8 sterols combined. Bold numbers indicate significant difference compared to Col-0.

Table 2-4 Δ8 sterols were not found in the phloem of *HYD1* silenced Arabidopsis

Arabidopsis line	Batch	Sterol content [% (w/w) of total in each sterol form]				
		cholesterol	*β*-sitosterol	campesterol	stigmasterol	Δ^8 sterol
Total sterols (free and conjugated)						
Col-0	1	29.3	49.6	9.1	12.0	n.d.
	2	50.3	32.9	16.2	0.5	n.d.
*HYD1*RNAi10	1	77.9	13.0	9.1	n.d.	n.d.
	2	13.5	86.5	n.d.	n.d.	n.d.
*HYD1*RNAi25	1	92.5	5.6	2.0	n.d.	n.d.
	2	39.3	56.8	3.8	n.d.	n.d.
Free sterols and acylated sterols						
Col-0	1	28.0	50.6	9.2	12.2	n.d.
	2	60.0	36.1	2.9	1.0	n.d.
*HYD1*RNAi10	1	88.2	11.8	n.d.	n.d.	n.d.
	2	8.9	91.1	n.d.	n.d.	n.d.
*HYD1*RNAi25	1	95.9	4.1	n.d.	n.d.	n.d.
	2	39.8	60.1	n.d.	n.d.	n.d.
Glycosylated sterols						
Col-0	1	100.0	n.d.	n.d.	n.d.	n.d.
	2	39.9	29.5	30.6	n.d.	n.d.
*HYD1*RNAi10	1	39.8	17.4	42.9	n.d.	n.d.
	2	100.0	n.d.	n.d.	n.d.	n.d.
*HYD1*RNAi25	1	46.4	25.0	28.6	n.d.	n.d.
	2	33.3	19.2	47.4	n.d.	n.d.

n.d. – not detected

Table 2-5 Statistical analysis of three-generation GPA on *HYD1*$_{RNAi}$10 and Col-0.

Variable	Effect test		
	Line	Generation	Line * generation
Days to adult (days)	$\chi_1^2 = 31.49$ ($P < 0.0001$)	$\chi_2^2 = 6.75$ ($P = 0.0342$)	$\chi_2^2 = 3.23$ ($P = 0.1991$)
Body mass (mg)	$F_{1,35} = 20.70$ ($P < 0.0001$)	$F_{2,35} = 0.20$ ($P = 0.8217$)	$F_{2,35} = 0.04$ ($P = 0.9575$)
Reproduction (number per adult)	$F_{1,100} = 106.26$ ($P < 0.0001$)	$F_{2,100} = 2.61$ ($P = 0.0791$)	$F_{2,100} = 2.55$ ($P = 0.0837$)

Table 2-6 EPG variables for aphids on Col-0 and *HYD1*$_{RNAi}$7,10,12, during 6h.

Activity (waveform)	Variable	Unit	Col-0 (n = 17)	*HYD1*$_{RNAi}$7 (n = 17)	*HYD1*$_{RNAi}$10 (n = 17)	*HYD1*$_{RNAi}$12 (n = 19)	*H*	*P*
Nonprobing (Np)	Total duration	min	61.28 ± 13.07	79.84 ± 19.44	114.46 ± 19.44	94.6 ± 11.49	6.87	0.0763
	Number	#	22.76 ± 3.54	34.06 ± 3.67	29.35 ± 3.65	32.95 ± 3.08	5.76	0.1240
	Mean duration	min	2.37 ± 0.24	2.57 ± 0.37	4.04 ± 0.58	3.02 ± 0.37	6.36	0.0846
Probing (all waveforms)	Total duration	min	298.72 ± 13.07	280.16 ± 14.55	245.54 ± 19.44	265.4 ± 11.49	6.87	0.0763
	Number	#	22.59 ± 3.50	33.88 ± 3.64	29.18 ± 3.65	32.68 ± 3.09	5.88	0.1176
	Mean duration	min	24.03 ± 5.40	11.26 ± 2.25	16.75 ± 5.45	10.82 ± 1.92	4.89	0.1803
	Time to 1st probe	min	3.28 ± 0.78	1.77 ± 0.40	2.14 ± 0.40	3.16 ± 0.58	6.67	0.0833
Pathway (C)	Total duration	min	155.83 ± 6.96	166.41 ± 12.17	150 ± 17.07	148.85 ± 10.32	1.41	0.7036
	Number	#	27.53 ± 3.66	41.29 ± 3.97	36 ± 3.89	39.47 ± 3.10	6.74	0.0806
	Mean duration	min	8.33 ± 1.71	4.51 ± 0.45	4.89 ± 0.77	4.02 ± 0.32*	9.19	0.0269*
Cell puncture (pd)	Total duration	min	11.66 ± 0.78	12.75 ± 1.06	13.1 ± 1.26	13.49 ± 1.10	2.11	0.5500
	Number	#	136.00 ± 10.44	152.35 ± 13.96	158.06 ± 16.96	151.58 ± 10.97	2.02	0.5690
	Mean duration	min	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.65	0.8854
	Time to 1st pd	min	0.99 ± 0.29	1.46 ± 0.78	1.67 ± 0.82	0.50 ± 0.16	3.85	0.2777

To be continued

Table 2-6 Continued

Activity (waveform)	Variable	Unit	Col-0 (n = 17)	*HYD1*$_{RNAi}$7 (n = 17)	*HYD1*$_{RNAi}$10 (n = 17)	*HYD1*$_{RNAi}$12 (n = 19)	*H*	*P*
Derailed stylet mechanics (F)	Total duration	min	10.35 ± 7.02	16.01 ± 8.08	7.83 ± 4.64	18.3 ± 13.94	0.65	0.8838
	Number	#	0.47 ± 0.17	0.88 ± 0.41	0.88 ± 0.30	1.16 ± 0.50	0.84	0.8397
	Mean duration	min	15.99 ± 5.37	18.46 ± 3.16	7.56 ± 3.02	9.6 ± 3.30	2.75	0.4313
	Aphids with F	# (%)	6 (35)	5 (29)	8 (47)	6 (32)	1.37	0.7132
Xylem ingestion (G)	Total duration	min	11.67 ± 7.42	3.34 ± 1.94	2.81 ± 1.69	11.36 ± 8.03	0.62	0.8919
	Number	#	0.35 ± 0.15	0.41 ± 0.19	0.35 ± 0.0.17	0.58 ± 0.26	0.33	0.9549
	Mean duration	min	38.32 ± 12.02	7.28 ± 1.35	7.32 ± 1.08	18.25 ± 4.17	1.17	0.7612
	Aphids with G	# (%)	5 (29)	4 (24)	4 (24)	6 (32)	0.46	0.9280
Phloem salivation (E1)	Total duration	min	4.23 ± 0.58	3.81 ± 0.69	3.7 ± 0.50	4.51 ± 0.72	0.76	0.8590
	Number	#	4.88 ± 0.69	6.12 ± 1.03	5.88 ± 0.90	5.42 ± 0.67	1.32	0.7234
	Mean duration	min	0.98 ± 0.10	0.69 ± 0.08	0.75 ± 0.09	0.83 ± 0.08	6.17	0.1036
	Time to 1st E1	min	118.61 ± 20.23	126.11 ± 27.73	109.97 ± 21.30	113.65 ± 24.54	0.78	0.8532
	Aphids with E1	# (%)	16 (94)	14 (82)	16 (94)	17 (89)	1.67	0.6441
Single E1 (sgE1)	Total duration	min	0.19 ± 0.13	0.13 ± 0.07	0.26 ± 0.18	0.39 ± 0.22	2.68	0.4432
	Number	#	0.12 ± 0.08	0.24 ± 0.11	0.29 ± 0.14	0.47 ± 0.18	3.18	0.3643
	Mean duration	min	1.64 ± 0.01	0.54 ± 0.10	0.73 ± 0.13	0.66 ± 0.09	5.66	0.1294
	Aphids with sgE1	# (%)	2 (12)	4 (24)	4 (24)	7 (37)	3.19	0.3639

To be continued

Table 2-6 Continued

Activity (waveform)	Variable	Unit	Col-0 (n = 17)	*HYD1*$_{RNAi}$7 (n = 17)	*HYD1*$_{RNAi}$10 (n = 17)	*HYD1*$_{RNAi}$12 (n = 19)	*H*	*P*
Phloem ingestion (E2)	Total duration	min	116.42 ± 18.78	87.02 ± 14.87	80.52 ± 19.32	81.57 ± 15.20	3.05	0.3839
	Number	#	4.65 ± 0.68	5.88 ± 1.02	5.59 ± 0.85	4.84 ± 0.62	1.35	0.7177
	Mean duration	min	36.53 ± 12.71	19.26 ± 4.69	22.23 ± 9.49	22.15 ± 7.27	5.29	0.1520
	Time to 1st E2	min	120.73 ± 20.23	126.87 ± 27.64	111 ± 21.30	115.81 ± 24.80	0.9	0.8243
	Aphids with E2	# (%)	16 (94)	14 (82)	16 (94)	17 (89)	1.67	0.6441
Sustained E2 (sE2)	Total duration	min	104.34 ± 19.40	72.02 ± 13.90	62.96 ± 20.14	73.65 ± 15.21	3.78	0.2865
	Number	#	2.35 ± 0.34	2.59 ± 0.44	2.06 ± 0.38	2.47 ± 0.41	1.37	0.7118
	Mean duration	min	52.19 ± 13.05	31.98 ± 5.84	32.08 ± 10.11	41.49 ± 14.57	5.39	0.1651
	Aphids with sE2	# (%)	15 (88)	13 (76)	14 (82)	16 (84)	0.86	0.8359

Values presented as Mean ± SEM
Sustained E2 > 10 min. Single E1 stands for phloem salivation (E1) was not followed by phloem ingestion (E2) immediately.
Non-parametric Kruskal-Wallis`s followed by Steel`s test for comparing the difference between control and *HYD1*$_{RNAi}$7, 10, 12.
Likelihood ratio test was calculated for the percentage of aphids with F, G, E1, single E1, E2 and sustained E2, respectively. *$P < 0.05$.

CHAPTER III

APHID GROWTH AND REPRODUCTION ON PLANTS WITH ALTERED STEROL PROFILES: NOVEL INSIGHTS USING ARABIDOPSIS MUTANT AND OVEREXPRESSION LINES

3.1 Introduction

Sterols are important membrane components and essential steroid precursors in eukaryotes, including insects (Bloch 1983, Gordon 2003). However, unlike most eukaryotes, insects and all other arthropods are unable to synthesize sterols (Robbins et al 1971). As such, insects and other arthropods generally require a dietary source to meet this requirement (Behmer & David Nes 2003). Cholesterol (Figure 3-1) is the most common sterol in the majority of insects (Robbins et al 1971). The bulk is found in cellular membranes, where it regulates their fluidity, rigidity and permeability, plus it interacts with and modulates various membrane proteins and peptides (Bloch 1983, Grouleff et al 2015). Additionally, a small amount is required as a precursor for ecdysteroids, a class of insect steroid hormones that regulates and controls crucial insect physiological processes including molting, oogenesis and diapause (Denlinger 2002, Lafont et al 2012, Swevers & Iatrou 2009). Cholesterol is also used in the Hedgehog signaling pathway, which regulates animal development (Ingham 2001, Rodenfels et al 2014).

The sterol profile of most insect herbivores is dominated by cholesterol, even though plants typically contain only small amounts of cholesterol relative to the total sterol pool. Insect herbivores generate most of their cholesterol by metabolizing phytosterols

(Ikekawa et al 1993). However, from the hundreds of different sterols found in plants (some examples are shown in Figure 3-1), the majority of insects can only convert a limited number to cholesterol. For example, most caterpillars (Behmer & David Nes 2003, Jing et al 2012a) can generate cholesterol from β-sitosterol, campesterol and stigmasterol (Figure 3-1 A); in plants these are the three major end-products of the plant sterol biosynthetic pathway (see Figure 3-2). Specifically, β-sitosterol and campesterol are metabolized to cholesterol using a three-step dealkylation process that removes the C-24 alkyl-group (Ikekawa et al 1993). The conversion of stigmasterol to cholesterol by caterpillars is a 5-step process (Lafont et al 2012, Tomazic et al 2011). Here the C-22 double bond (via a 2-step desaturation process) is removed prior to side-chain dealkylation. However, not all insects can perform the reduction at C-22 (Svoboda 1999). For example, grasshoppers and aphids can generate cholesterol from β-sitosterol and campesterol, but not stigmasterol. Biochemical studies suggest that grasshoppers lack the desaturase enzymes required to remove the C-22 double bond on stigmasterol, and when stigmasterol is present above a threshold level in the diets of grasshoppers (Behmer & Elias 1999) and aphids (Bouvaine et al 2014), they show high mortality and low reproduction. Insects are also generally unable to convert Δ^7 and Δ^8 sterols to cholesterol because they lack enzymes that remove the double bonds in the tetracyclic ring (Behmer & David Nes 2003, Ritter 1986). Insects eating diets containing Δ^7 and Δ^8 sterols above a threshold amount also show high mortality and reduced reproduction (Behmer 2017, Ritter 1986). Finally, phytosterols can exist in both free and conjugated forms (Ferrer et al 2017). However, because insects possess digestive enzymes to cleave conjugated sterols (either

a fatty acid or sugar attached at the C-3 position), sterol conjugation is not thought to impact insect sterol uptake or use (Terra et al 1996).

The sterol profile of individual plants is comprised of many sterols because sterol production in plants follows a multi-step biosynthetic pathway (Figure 3-2). Typically, there is one phytosterol that dominants and a few that occur at intermediate levels; these are usually the end-products of the sterol biosynthetic pathway (Figure 3-2). Additionally, there are multiple sterols that occur at very low to trace levels. For example, in the leaves of *Arabidopsis thaliana* (wildtype Col-0), *β*-sitosterol is the dominant sterol (64%), followed by campesterol (17%), stigmasterol (4.8%), cholesterol (4.3%), sitostanol (3.3%), isofucosterol (2.9%) and brassicasterol (1.5%); the remaining 3.7% of the total sterol profile is comprised of 15 different sterols (Schrick et al. 2004). Many of the sterols that occur at very low to trace levels are intermediates in the plant sterol biosynthetic pathway (see Figure 3-2); they exist only briefly before being modified further during each successive step of the pathway. How insect herbivores use and metabolize different phytosterols is often investigated using artificial diets (Behmer & Nes, 2003); this approach has worked particularly well for grasshoppers, beetles, flies and caterpillars because the incorporation of sterols into artificial diets for these insects is relatively straightforward. In contrast, incorporating sterols into aphid artificial diets is less straightforward because there is always some degree of uncertainty related to the extent to which sterols solubilize in the aqueous-based artificial diets used to investigate aphid nutritional physiology.

In the current study we explore sterol use by aphids *in vivo*. We use five Arabidopsis lines with mutations in the sterol biosynthetic pathway to study the effects of atypical plant sterol profiles. We also use two overexpression lines to explore the effects of increasing the total sterol pool in plants and shifting the ratio of sterol end-products. Specifically, we rear green peach aphids (*Myzus persicae*) from neonates on each Arabidopsis lines, plus their respective wildtype control. Three separate experiments were performed. In one experiment we reared neonates to the adult stage and measured their mass. In the second we tracked adult reproduction over seven successive days. Finally, we analyzed the sterol profiles of aphids reared on four of the different plant lines. We hypothesized that aphids reared on plants expressing atypical sterols would perform poorly, but that aphids on plants that overexpressed typical sterols would show no enhancement. We use our aphid sterol analysis results to infer potential mechanisms impacting aphid performance. We also discuss the potential of using plants with modified sterols to control insect herbivore pests.

3.2 Materials & Methods

3.2.1 Plant growth and insect rearing

All *Arabidopsis thaliana* seeds were cold-treated in 4°C for three days before sowing. Stratified seeds were then sown in 10 cm x 10 cm x 6 cm pots (20 to 25 seeds per pot) filled with LP5 potting medium (Sun Gro Horticulture, Bellevue, WA). Pots were then transferred to a tray and covered with a clear plastic lid to maintain the moisture for germination. Next, ten-day-old seedlings were transplanted in 6 cm x 6 cm x 6 cm pots

(one plant per pot) filled with the same potting medium. Plants were kept in an environmental chamber with the photoperiod of 12h light/12h dark and light intensity of 80~100 μmol m^{-2} s^{-1} (photon flux density). The temperature in the growth chamber was set at 23°C day/21°C night, with 65% relative humidity (RH) (Lei et al 2014).

The green peach aphids (*Myzus persicae*) used in this study came from a parthenogenic colony maintained at Texas A&M University. The colony was reared on 6~10-week-old cabbage (*Brassica oleracea*), and maintained at 23°C and 65% RH, under a 12h light/12h dark photo regime. All aphid bioassays described below were performed under these conditions.

3.2.2 Arabidopsis lines

A total of seven unique Arabidopsis lines were used in this study (*HMG1*-OE, *cas1-1*, *smt2*, *SMT2*-OE, *hyd2*, *ste1-1* and *psat1*-2). These lines differ in their leaf sterol profiles and represent 6 different positions along the plant sterol biosynthetic pathway, as shown in Figure 3-2.

The first line is *HMG1*-OE (shown as HMG1/HMGR in Figure 3-2); it has a Col-0 background. *HMG1*-OE overexpresses 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG1), which is the rate-limiting step of sterol biosynthesis (Chappell et al 1995). The total sterol content of *HMG1*-OE is 3.5x greater compared to Col-0 (Lange et al 2015). Additionally, this line has reduced *β*-sitosterol and increased isofucosterol (expressed on a percent basis) compared to Col-0 (see Table 3-1).

The second line is *cas1-1* (shown as CAS1 in Figure 3-2); it has a C24 background. Cycloartenol synthase 1 (CAS1) catalyzes the conversion of 2,3-oxidosqualene to cycloartenol and forms the tetracyclic ring of sterols. *Cas1-1* mutant do not show reduced Δ^5 sterols. Instead, they show an accumulation of 2,3-oxidosqualene, especially in stems, flowering shoots and siliques (Babiychuk et al 2008).

The third line is *hyd2* (shown as FACKEL/HYD2 in Figure 3-2); it has a Col-0 background. The *HYD2* gene encodes C-14 reductase (FACKEL/HYD2). The mutant of this gene is *hyd2* (*fk*-J3158). It accumulates $\Delta^{8,14}$ sterols, which account for ~80% of the total sterol profile (Qian et al 2013).

The last Arabidopsis line is *psat1*-2; it has a Col-0 background. It is a mutant of the *PSAT1* gene (see Figure 3-2), which encodes phospholipid:sterol acyltransferase 1 (PSAT1). PSAT1 controls sterol homeostasis by converting free sterols into sterol esters (Banas et al 2005). In *psat1*-2 plants, the ratio of free sterols relative to sterol esters is greater compared to Col-0. Additionally, the total amount of free sterol and sterol ester are increased compared to Col-0 (Bouvier-Navé et al 2010).

3.2.3 Aphid adult size and reproduction

We conducted two separate experiments, using the plant lines described above, to measure aphid adult mass and reproductive output. For both we used a similar pre-experiment protocol, that aimed to minimize potential maternal effects. To start, we transferred adults that had previously been maintained on cabbage to each of our seven plant lines, plus their respective wild-type. For each line there were five 4-week-old plants,

and each plant received 6 adult aphids. They were left for 12 h and allowed to reproduce; after 12 hours the adults were removed. The nymphs were left to feed until they became adults and started to reproduce. Next, we transferred these adults to new plants of the same line; there were five plants per line, and each plant received 6 adult aphids. After 12 h, all of the adults were removed from the plants; all newly produced neonates were left on these plants for 24 h (enough time for them to become 2nd instars). These 2nd instar nymphs were then inoculated onto a new batch of 4-week-old plants of the corresponding line (6 nymphs per plant, 8 replicates per genotype) and checked daily for survival. Once these nymphs became adults, we measured their adult mass and reproductive output.

For the first experiment, which explored the effects of the different plant lines on adult body mass, we collected 20 adults (each 2-days old), pooled them, and weighed them (this was a single replicate); three replicates were measured for each line. The second experiment examined how the different plant lines affected reproduction. Here the number of nymphs produced on an individual plant group was counted daily for seven consecutive days. The newly produced nymphs were removed daily while counting. We report these data in two ways. One, total reproduction/aphid for seven days. Two, daily reproduction/aphid (over seven days).

3.2.4 Sterol quantification of aphids

The sterol profile of aphids on each plant line was also characterized and quantified. For this experiment, 30 adult aphids from each line were obtained as described above and pooled as a single sample; the dry weight of each sample was measured

following lyophilization. The extraction of aphid sterols followed the method published in (Behmer et al 2011). The dried aphids were homogenized with 1:1 chloroform:methanol in the presence of #5 glass beads (Sigma, St Louis, MO, USA), shaking for 5 min at maximum speed. The sample was then incubated at room temperature for 24 h, evaporated and resuspended in 3 mL of hexane. The hexane fraction was washed with three volumes of 70% methanol:water-equilibrated hexane. The hexane sample was further processed and examined for sterol content. All free sterols / steroids, including those produced following base saponification, were converted to TMSI derivatives and identification and quantification by GC-MS following Jing et. al. (2012).

3.2.5 Statistical analysis

JMP 11.0 software (Cary, NC) was used for all data analyses. Normal distribution of each dataset was checked using the Shaprio Wilk test, and homogeneity of variances was determined by the Levine and Bartlett tests. Both body weight and reproduction were normally distributed with homogenous variance. Aphid body mass and total reproduction on five of the lines (*HMG1*-OE, *psat1-2*, *hyd2*, *ste1-1* and *cas1-1*) was analyzed using a student`s t test, with each line compared to its corresponding wild type. We used analysis of variance (ANOVA) for the *smt2* and *SMT2*-OE lines (with Col-0 as the control), because these three lines were run as a single experiment; a Dunnett`s test, with Col-0 as the control, was used to make post hoc comparisons. Finally, we analyzed daily reproductive rate using repeated measures ANOVA. For daily reproductive rate, we also used a student's t test to conduct post hoc tests for each day. Here we used a Bonferroni

correction (α = 0.05 / 7 comparison = 0.007) to reduce the likelihood of committing a Type I error.

3.3 Result

We report adult aphid mass, reproduction in four groups, based on the published sterol profiles of our plant lines and their potential suitability for aphids. We also note, for all the different plant lines, there was no mortality difference from the 2nd instar to 13-day-old adult stages; approximately 95% of the aphids used in the bioassays reported below successfully developed into adults.

3.3.1 Aphid body mass and reproduction

Group I – *hyd2* and *ste1-1* lines

These Arabidopsis lines accumulate atypical sterols relative to their controls (see Table 3-1); these plants also show retarded performance in comparison to their corresponding controls. The body mass of aphids collected from *hyd2* (t_4 = 25.72, P < 0.0001) and *ste1-1* (t_4 = 13.87, P < 0.0001) was significantly decreased compared to aphids from control plants; on both lines, aphid mass was about 70% compared to controls (Figure 3-3 A&B).

The reproductive output of aphids reared on *hyd2* and *ste1-1* lines was also significantly reduced compared to controls (t_{14} = 6.85, P < 0.0001 and t_{16} = 6.40, P < 0.0001, respectively); in both cases it was reduced ~50% compared to controls (Figure 3-3 C&D). A repeated measures ANOVA showed that aphid daily reproduction on these

two mutants was also significantly different relative to controls (Table 3-4), and differences were significant for each day, except for day 11 for aphids feeding on *hyd2* (Figure 3-3 E&F).

Group II – *smt2* and *SMT2*-OE lines

These lines produce typical phytosterols but modified ratio of campesterol to *β*-sitosterol (Table 3-1). Adult body mass was significantly different on these lines ($F_{2,8}$ = 135.71, $P < 0.0001$). However, post hoc analyses showed that this was only the case for aphids reared on *smt2* plants, which were smaller compared to control lines (Figure 3-4 A); aphids on the *SMT2-OE* lines were not different compared to aphids on control lines.

We observed significant differences in reproductive output of aphids on these three lines ($F_{2,22}$ = 12.63, P = 0.0003); aphids on *smt2* produced fewer offspring (~70%) compared to the control lines (Col-0), while aphids on SMT2-OE lines produced the same number of offspring compared to control lines (Figure 3-4 B). Repeated measures ANOVA revealed a significant difference in aphid daily reproduction (Table 3-4), but post hoc tests showed this was only the case for aphids on the *smt2* lines (Figure 3-4 C). No daily differences in reproduction were observed (Table 3-4).

Group III – *HMG1*-OE and *psat1-2* lines

These two lines produce typical sterols, but at higher levels compared to control plants (Table 3-1). However, growth and reproduction of these two lines is similar to wild-type plants (Bouvier-Navé et al 2010, Lange et al 2015). Aphid body mass for aphids

reared on *HMG1*-OE ($t_4 = 0.80$, $P = 0.2338$) and *psat1-2* ($t_4 = 0.5$, $P = 0.6783$) was similar to aphids reared on control lines (Figure 3-5 A&B). Likewise, aphid total reproduction on *HMG1*-OE ($t_{17} = 0.17$, $P = 0.5671$) and *psat1-2* ($t_{14} = 0.01$, $P = 0.4963$) lines was similar compared to aphids from the control plants (Figure 3-5 C&D). Finally, we observed no differences in daily reproduction for aphids on either line, including within different days (Table 3-4; Figures 3-5 E&F).

Group IV – *cas1-1*

This plant line produces typical sterols, but also accumulate 2,3-oxidosqualene. The body mass of aphids reared on *cas1-1* lines was significantly reduced (by approximately 40%) compared to aphids reared on C24 wild-type plants ($t_4 = 38.50$, $P < 0.0001$; Figure 3-6 A). Differences in size on these two treatments, at days 7 and 15, are shown in Figure 3-6 D.

Total reproduction for aphids on *cas1-1* lines was also significantly reduced (~50%) compared to aphids on control lines ($t_{14} = 9.42$, $P < 0.0001$; Figure 3-6 B). Finally, repeated measures ANOVA showed that daily reproduction was significantly reduced compared to aphids on control lines (Table 3-4), For aphids on the wild-type C24 plants, reproduction was relatively constant over days 8-13 (Figure 3-6 C). In contrast, daily reproduction for aphids on *cas1-1* plants increased from day 8 to day 11, and then decreased at day 13. Post hoc tests showed significant differences in reproduction for all but one of the days it was compared (Table 3-4, Figure 3-6 C).

3.3.2 Aphid sterol/steroid profile

We also measured the sterol/steroid profile of aphids from a single representative of each of the four Arabidopsis groups. Sterol profiles for aphids from Groups I-III, plus Col-0 (the background for these three groups), are shown in Table 3-2. Sterol profiles for aphids from Group IV (and its control line – C24) are shown in Table 3-3. For each group 30 aphids were pooled into a single sample. Two values are reported for each table: (1) sterol amount, expressed as ug sterol / mg lyophilized aphid tissue, and (2) percent of the total sterol profile.

Total sterol amounts were highest for aphids reared on control plants (Col-0 and C24), and the *psat1-2* plants (which have normal sterol profiles, but greater absolute amounts). They were lowest for aphids reared on the *hyd2* (which accumulates $\Delta^{8,14}$ sterols) and *smt2* (which has much more campesterol compared to sitosterol) lines. Sterol amounts for aphids reared on *cas1-1* lines (accumulate 2,3-oxidosqualene) were intermediate.

In terms of aphid sterol profile, three major sterols (*β*-sitosterol, cholesterol and stigmasterol) were identified in aphids from all the lines. *β*-sitosterol was the dominant sterol recorded; as a percent of the total sterol profile it ranged from 50-66%. Cholesterol levels ranged from 8-16%, while stigmasterol ranged from 6-12%.

3.4 Discussion

Studies exploring sterol suitability to insects have been mainly conducted on chewing insects, and most of these studies employed artificial diets to address the

importance of the amount, ratio and types of sterols to insects (Behmer & David Nes 2003). Relative to grasshoppers and caterpillars, systematic sterol studies on aphids are lacking, possibly due to the challenges of using artificial diets to mimic phloem, especially provide the high hydrostatic pressure that is normally provided by the phloem sieve element (Dinant et al 2010). Additionally, the solubility of sterols in phloem might be increased by interacting with other molecules (Behmer et al 2013). Therefore, relative to plant-reared aphids, aphids reared on artificial diets often exhibit reduced fitness (Douglas 2003, Emden 2009). In this study, we employed genetically modified plants producing altered sterol profiles, and investigated aphid performance on these plants. The lines we chose were targeted in plant sterol biosynthetic pathways, and all the plant sterol profiles were characterized and published. Our results demonstrated that the growth and reproduction of *Myzus persicae* can be negatively affected after feeding on plants producing atypical sterols. Surprisingly, the increased ratio of campesterol to β-sitosterol in GM plants also negatively affected aphid growth and reproduction. The plants that only produce excess suitable sterols, however, have no impact on aphids. Aphids that exhibited reduced performance also showed altered sterol profiles, indicating the sterol modification in plants played a key role in affecting aphids.

Aphids on plants producing atypical sterols suffered reduced body mass and reproduction, which were observed on all three lines of Group I and IV plants. The main difference between these two groups of plants was the type of atypical sterols they produced. For most insects, $\Delta^{8,14}$ and Δ^{7} sterols (produced by *hyd2* and *ste1-1* (Group I)) are known to be unsuitable. However, the suitability of 2,3-oxidosqualene (accumulated

in *cas1-1* (Group IV)) for insects is currently unknown. However, aphids on both *hyd2* and *cas1-1* exhibited reduced suitable sterols (cholesterol and *β*-sitosterol). Insufficient suitable sterols may affect certain physiological processes: First, the structural integrity of cellular membranes and the formation of lipid rafts requires a certain level of cholesterol (Simons & Ikonen 1997). The insufficient cholesterol could lead to reduced rigidity and increased permeability of the membranes, and also affect membrane proteins that are modulated by cholesterol (Grouleff et al 2015, Simons & Toomre 2000). Consequentially, aphids might have to spare a substantial amount of energy to maintain ion homeostasis and other membrane-associated activities disrupted by the "leaky" membrane, which perhaps explains the arrested growth as reflected by reduced body mass. Second, decreased cholesterol could affect the synthesis of ecdysteroids (Gilbert et al 2002). With the pulses of ecdysteroids controlling each molting process, insects with insufficient cholesterol might take a longer time to accumulate enough ecdysteroids to initiate the molting (Christiaens et al 2010, Lafont et al 2012). Although we did not record the duration of each instar directly, the delayed reproduction peak indicates a delayed development to adults when aphids fed on *hyd2*, *ste1-1* and *cas1-1*. Third, sterols have been found enriched in early embryos of many insect species including aphids, indicating critical roles of sterols in developing embryos (Bouvaine et al 2012, Guan et al 2013, Jouni et al 2002). Perhaps the failure in allocating high levels of sterols to early embryos led to the decreased reproduction of aphids.

It is interesting to note that neither the $\Delta^{8,14}$ sterols nor 2,3-oxidosqualene were recovered from aphids, in spite of their prominent presence in *hyd2* and *cas1-1*,

respectively. Because insects are unable to metabolize these sterols, another possibility is these unsuitable sterols were not ingested by aphids. We cannot neglect the fact that the sterol profile of *hyd2* and *cas1-1* was only measured in leaf tissue. Aphids, however, obtain nutrients from the phloem sap, in which $\Delta^{8,14}$ sterols and 2,3-oxidosqualene might be absent. In fact, several studies have analyzed sterol profiles of phloem and observed sterol profiles different from those obtained from leaf tissues. In contrast to the dominant presence of *β*-sitosterol and campesterol in the leaves, cholesterol and *β*-sitosterol are the major phloem sterols in the following four species: fava bean (*Vicia fava*), green bean (*Phaseolus vulgaris*), Chinese cabbage (*Brassica rapa*), and tobacco (*Nicotiana tabacum*) (Behmer et al 2011, Behmer et al 2013, Bouvaine et al 2012). Data in chapter 2 revealed different sterol profiles between phloem and leaves from Arabidopsis. Similarly, the major sterols in the phloem of Arabidopsis is different from leaves, and the *HYD1*-silenced plants only showed accumulation of Δ^8 sterols in the leaves, but not the phloem. It is likely that plants producing atypical sterols in this study also have distinct sterol profiles in the phloem. Measuring the phloem sterol profiles from these lines will help understand which sterols are present in phloem, and how it reflects the sterol profile of aphids.

Somewhat surprisingly we saw reduced performance on lines where there was a high ratio of campesterol to *β*-sitosterol. Campesterol is generally considered a suitable sterol, particular for caterpillars and beetles. However, a few studies have found that flies (*Culex pipens* and *Musca domestica*) and bees (*Apis mellifera*) exhibit reduced fitness when feeding on diets containing campesterol, indicating that for these insects the C24-dealkylation of campesterol is less efficient than *β*-sitosterol (Behmer & David Nes 2003).

The suitability of campesterol to aphids has not yet been studied. However, compared to the insect species mentioned above, aphids are usually known as having low capacity for C24-dealkylation. Only a limited amount of *β*-sitosterol and no stigmasterol can be metabolized into cholesterol by aphids (Bouvaine et al 2012, Campbell & Nes 1983). Whether campesterol is inferior to cholesterol or *β*-sitosterol needs to be investigated. Interestingly, similar to aphids on *hyd2* and *cas1-1*, aphids on *smt2* exhibited drastically reduced cholesterol and *β*-sitosterol. Again, the reduced suitable sterol content could be the key factor for the decreased body mass and reproduction of aphids on *smt2*. As for campesterol, it is possible that this sterol is absent or present at low levels in the phloem. Among the four plant species that have sterol profiles of phloem analyzed, campesterol was not detectable in the phloem sap of fava bean, green bean and Chinese cabbage. Although it was detected in tobacco, the level exhibited in phloem was much lower than in leaf tissue (Behmer et al 2011, Behmer et al 2013, Bouvaine et al 2012).

Aphids did not show any gain or loss in their performance after feeding on plants (*HMG1*OE and *psat1-1*) that over-produced suitable sterols. This finding is consistent with previous findings on grasshoppers (*Schistocerca americana*) that when diets contain only suitable sterols there is no difference in performance, as long as the minimum threshold is exceeded (Behmer & Elias 1999). Studies on *Drosophila* have revealed that insects have the capability of regulating the cellular sterol concentrations (Carvalho et al 2010). When excess sterols are available, they are generally converted into sterol esters or removed via cellular efflux by insects (Jouni et al 2002, Sieber & Thummel 2012). One of the most intensively studied regulatory mechanisms is through hormone receptor 96 (HR96), which

is a nuclear receptor that was found in both mammals and many insects. HR96 can sense the changes in sterol levels and then regulate downstream genes involved in sterol absorption, esterification and efflux (Bujold et al 2010, Horner et al 2009). Aphids, however, lack HR96 (Bonneton & Laudet 2012). In our study, aphid performance was not affected by surplus sterols, indicating aphids may also regulate their sterol levels. A different mechanism through Niemann-Pick type C proteins (NPC1 and NPC2) might be involved in this process. One of the two NPC1 homologs, NPC1a, also responds to sterols and it was identified in aphids (Zheng et al 2018). Further functional studies are needed to investigate whether NPC1a is the key player in sterol regulation of aphids.

In conclusion, our study has demonstrated that aphid performance can be affected by feeding on lines producing certain sterol profiles. The lines that severely retarded the aphid growth and reproduction have the potential to be utilized to control insect herbivore pests. This novel approach is environmentally friendly, and the effects on the nontarget species (such as human and other vertebrates) are minimal, because the limitation in sterol resource and the constraints in sterol metabolism are unique to insects. To better understand the mechanisms of how aphids were affected by some of the lines we studied, it is worthwhile to analyze the phloem sterol profiles in the future and compare them with the aphid sterol profiles. Additionally, comparative studies, investigating the performance of chewing insects and sucking insects on sterol-modified lines, their sterol profiles, and the relationship with the tissue consumed, will shed new lights on sterol physiology and ecology of insects, and provide potential for broader applications of sterol-modified plants in pest management.

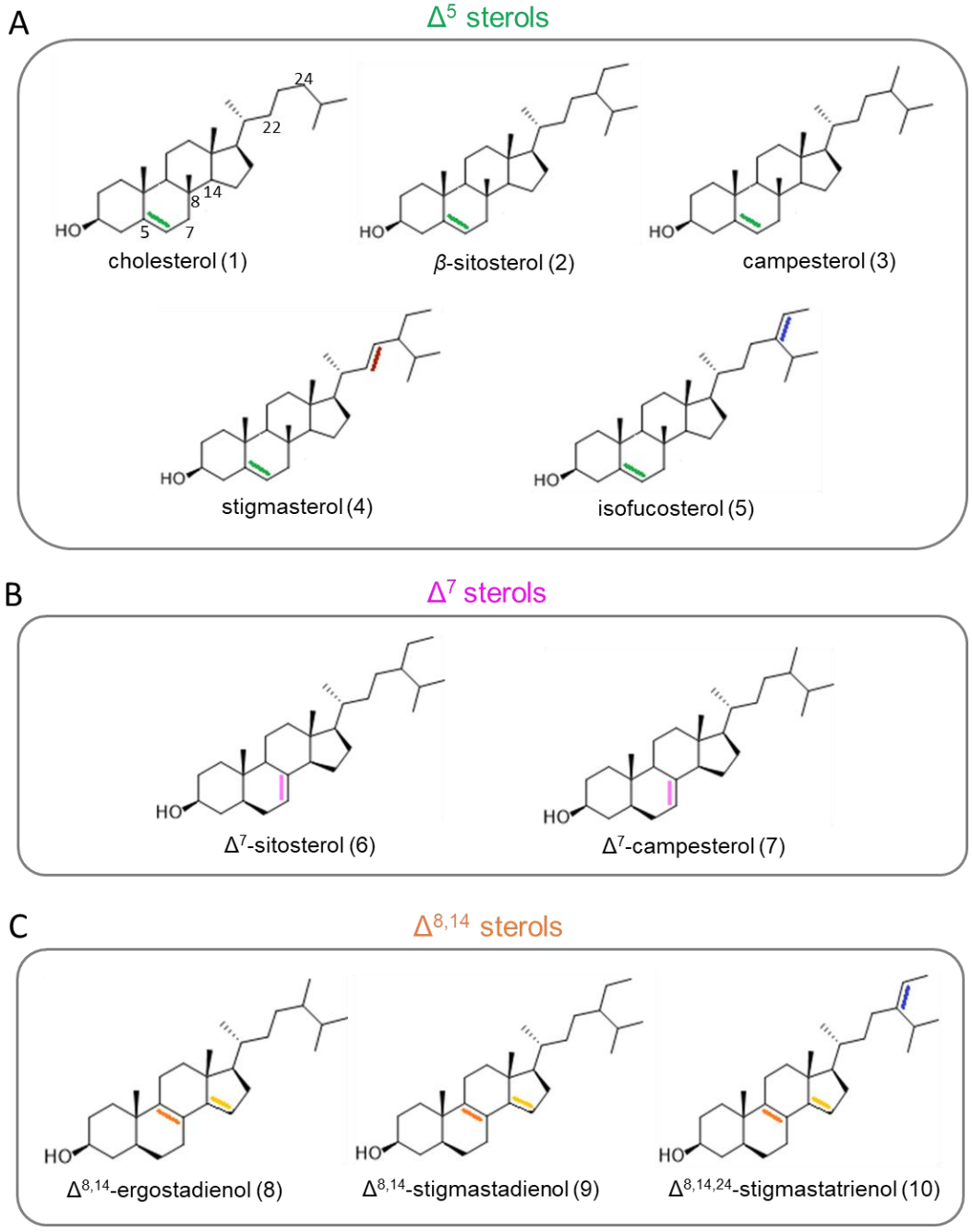

Figure 3-1 Sterol structures of interest.
A, B, C represent Δ^5, Δ^7, $\Delta^{8,14}$ sterols, respectively. The numbers in the structure of cholesterol indicate the critical carbon positions in all the sterols mentioned in this study.

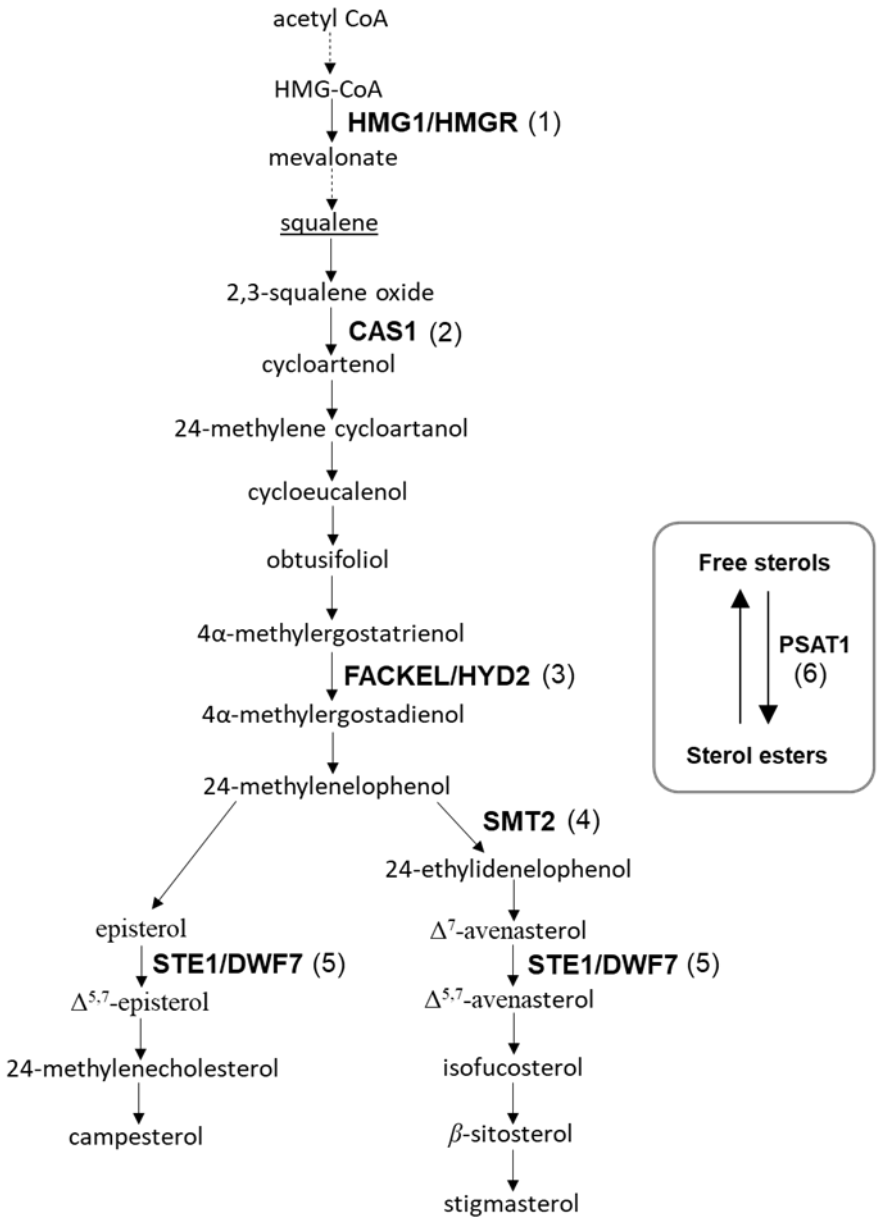

Figure 3-2 Simplified sterol biosynthetic pathway and the esterification of free sterols. Seven lines used in this study were targeted in six enzymatic steps: (1) HMG1/HMGR (3-hydroxy-3-methylglutaryl coenzyme A reductase) overexpressor, (2) CAS1 (cycloartenol synthase 1) mutant, (3) FACKEL/HYD2 (C-14 reductase) mutant, (4) SMT2 (sterol-C24-methyltransferase 2) mutant and overexpressor, (5) STE1/DWF7 (Δ^7-sterol-C5-desaturase) mutant, (6) PSAT1 (phospholipid:sterol acyltransferase 1) mutant. Solid arrows stand for single step, dash arrows stand for multiple steps involved.

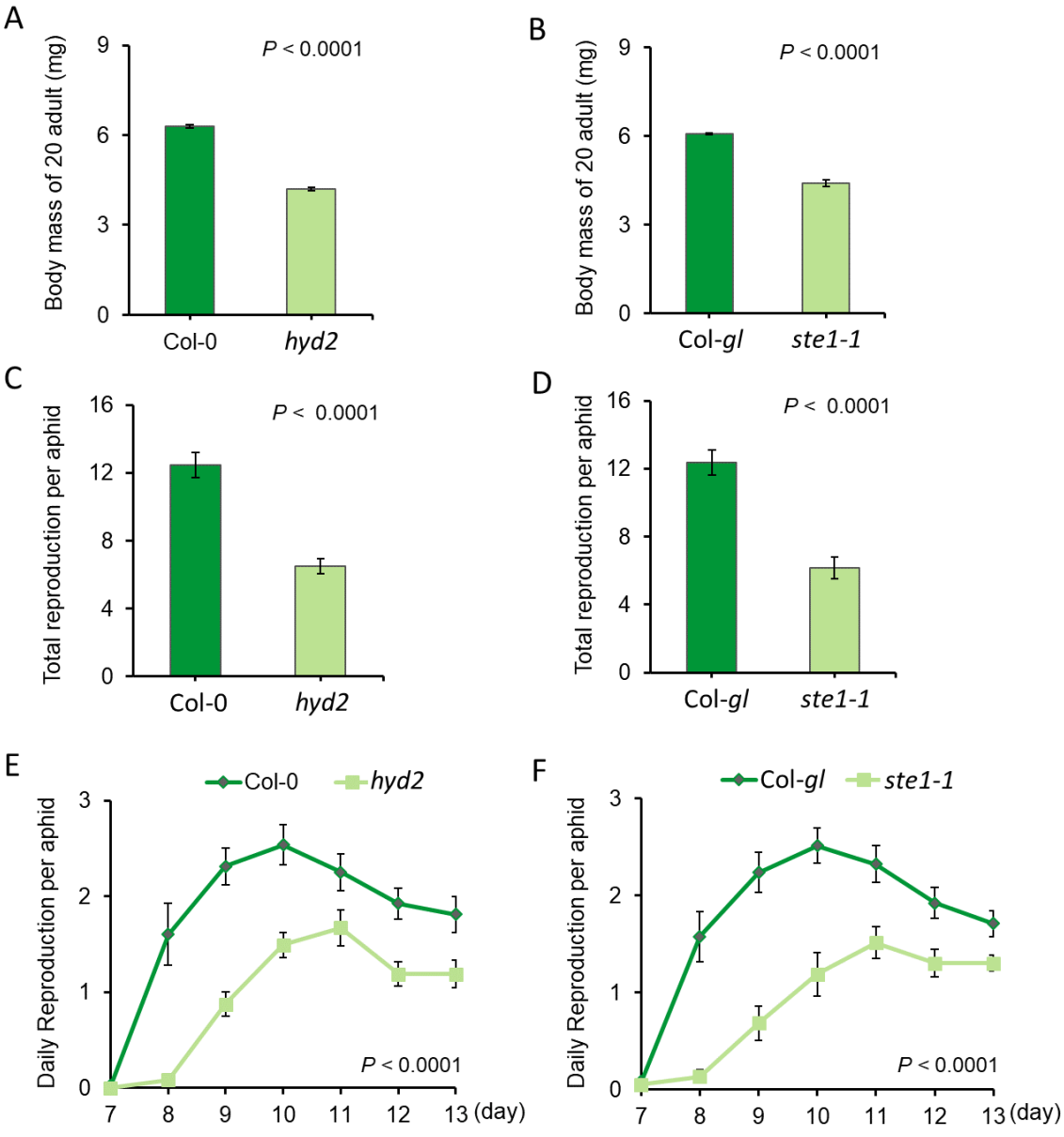

Figure 3-3 Decreased growth and reproduction of aphids on *hyd2* and *ste1-1* (Group I). Aphids on both *hyd2* and *ste1-1* displayed significantly reduced body mass (**A** and **B**) and decreased total reduction (**C** and **D**) comparing to their control, respectively. The daily reproduction of aphids on *hyd2* and *ste1-1* were reduced, and the reproductive peak reached in day 11 instead of day 10 (**E** and **F**). See Table 3-4 for the statistical analysis of daily reproduction.

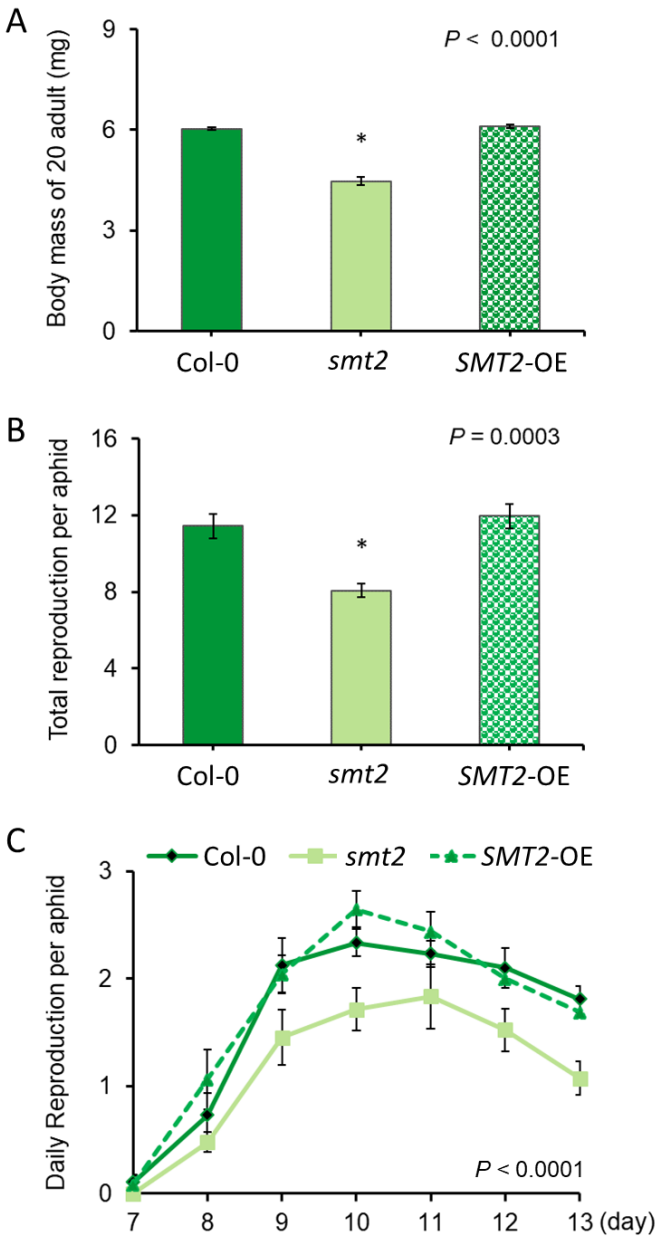

Figure 3-4 Decreased growth and reproduction of aphids on *smt2*, but not on *SMT2*-OE (Group II).

The body mass (**A**) of aphids was significantly reduced after rearing on *smt2* comparing to aphids on Col-0, while no difference was observed for aphids on *SMT2*-OE. Total reproduction (**B**) and daily reproduction (**C**) of aphids was significantly decreased on *smt2*, but not on *SMT2*-OE. See Table 3-4 for the statistical analysis of daily reproduction.

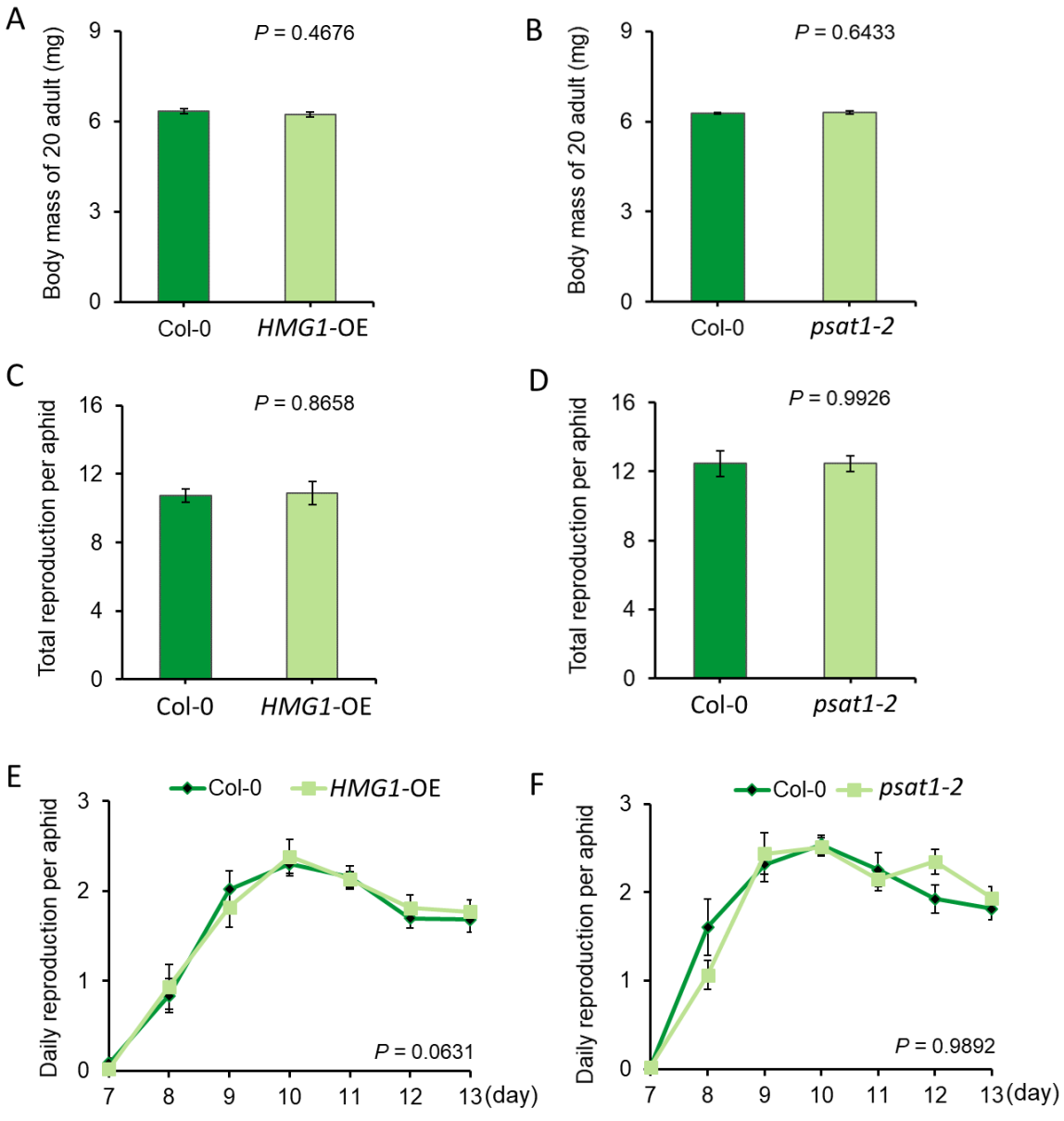

Figure 3-5 Aphids growth and reproduction were not affected on *HMG1*-OE or *psat1-2* (Group Ⅲ).

No difference in body mass was observed for aphids on *HMG1*-OE or *psat1-2* comparing to those on Col-0 (**A** and **B**). The total reproduction (**C** and **D**) and daily reproduction (**E** and **F**) did not show difference after aphids feeding on *HMG1*-OE or *psat1-2*. See Table 3-4 for the statistical analysis of daily reproduction.

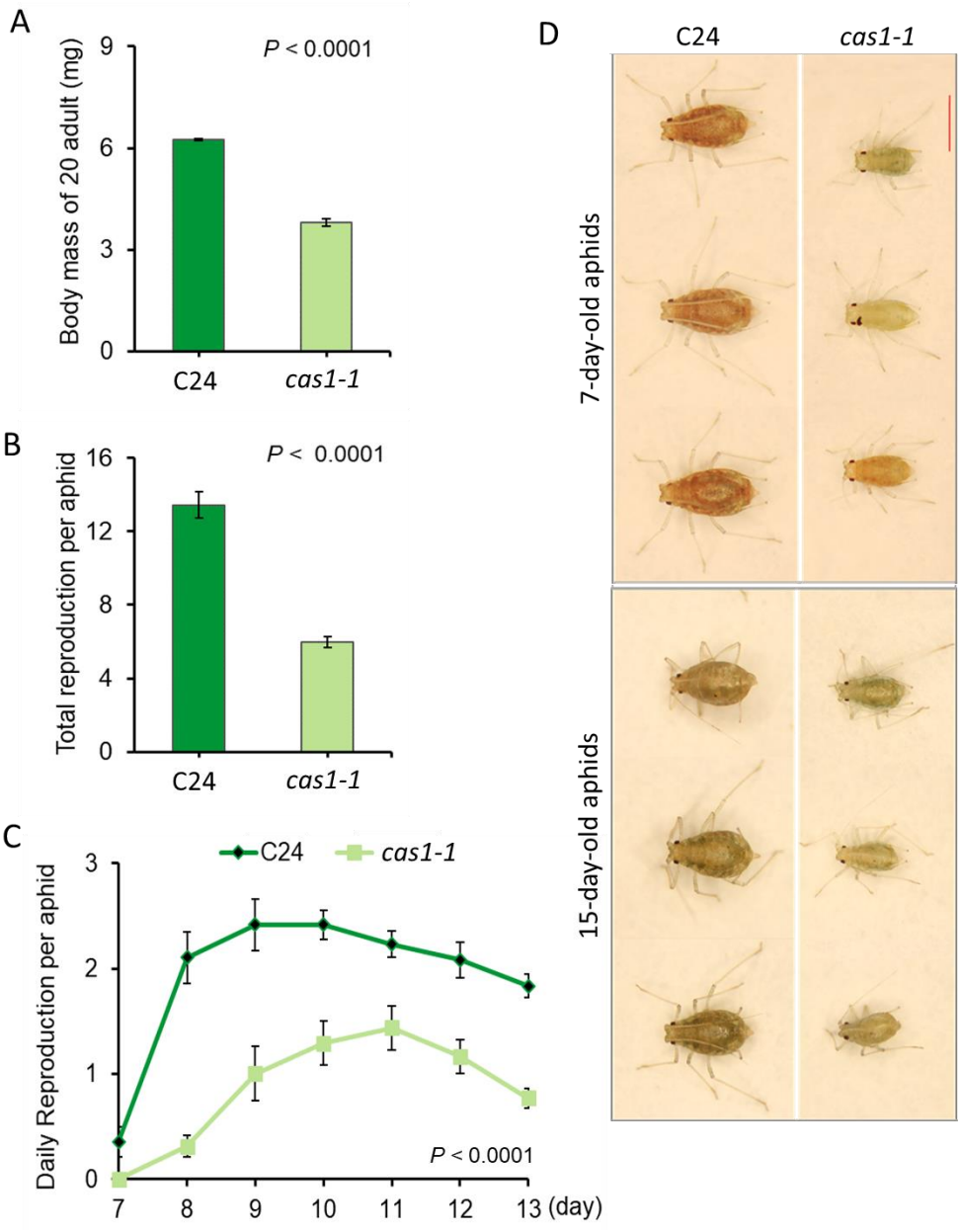

Figure 3-6 Decreased growth and reproduction of aphids on *cas1-1* (Group IV). Aphid body mass (**A**), total reproduction (**B**) and daily reproduction (**C**) were significantly reduced on *cas1-1* comparing to the wild-type C24. (**D**) The representative aphids on *cas1-1* were smaller than those on C24 in both day 7 and day 15. Scale bar represents 1mm. See Table 3-4 for the statistical analysis of daily reproduction.

Table 3-1 Sterol profiles of different Arabidopsis lines used in this study

Sterol (structure in Fig. 1)	wild-type[a]	*HMG1*-OE	*hyd2*	*smt2*	*SMT2*-OE	*ste1-1*
β-Sitosterol (2)	65.1	**31.0**	14.5	**21.0**	**75.0**	24.7
Campesterol (3)	14.7	8.0	1.0	**44.0**	**4.0**	4.3
Stigmasterol (4)	5.0	4.0	nd	2.0	6.0	2.5
Isofucosterol (5)	3.6	**37.0**	nd	4.0	3.0	3.1
Cholesterol (1)	2.8	2.0	1.8	2.0	0.5	0.1
Δ^7-Sitosterol (6)	nd	–	–	–	–	**58.5**
Δ^7-Campesterol (7)	nd	–	–	–	–	**4.4**
$\Delta^{8,14}$-Ergostadienol (8)	nd	–	**5.4**	–	–	–
$\Delta^{8,14}$-Stigmastadienol (9)	nd	–	**63.0**	–	–	–
$\Delta^{8,14,24}$-Stigmastatrienol (10)	nd	–	**11.4**	–	–	–
Relative total sterols[b]	1	**3.5**	0.9	1.5	1.3	0.9

Values presented in the table are percentage of each sterol in total sterols.
The numbers followed sterol types in parenthesis refer to the sterols in Fig. 1.
[a]The average values of different wild-types from independent studies were calculated.
[b]The total sterol amount of each transgenic line was normalized with the total sterol of its corresponding wild-type.
nd, not detected.
–, not measured or undetectable in the given line.

Table 3-2 Sterol/steroid profiles of aphids on *hyd2* (Group I), *smt2* (Group II), *psat1-2* (Group III)

	Col-0	*hyd2*	*smt2*	*psat1*-2
β-Sitosterol	52 (66)	25 (53)	20 (50)	40 (58)
Stigmasterol	12 (16)	11 (24)	7 (16)	12 (18)
Cholesterol	14 (18)	9 (19)	8 (20)	16 (24)
Unknown steroid	–	2 (4)	–	–
Total sterols/steroids	78 (100)	47 (100)	35 (100)	68 (100)

Values in the table represent the μg sterol/steroid detected in per mg lyophilized aphid tissue; values in the parentheses represent the percentage of each sterol/steroid in total sterols/steroids.

Table 3-3 Sterol/steroid profiles of aphids on *cas1-1* (Group IV)

	C24	*cas1-1*
β-Sitosterol	43 (66)	38 (64)
Stigmasterol	12 (18)	6 (10)
Cholesterol	10 (15)	8 (14)
Unknown steroid	—	7 (12)
Total sterols/steroids	65 (100)	59 (100)

Values in the table represent the μg sterol/steroid detected in per mg lyophilized aphid tissue; values in the parentheses represent the percentage of each sterol/steroid in total sterols/steroids.

Unknown steroids in table 3-2 and table 3-3 are different steroids.

Table 3-4 Statistics analysis of aphid daily reproduction

Effect	*hyd2*	*ste1-1*	*smt2* & *SMT2*-OE	*HMG1*-OE	*psat1*-2	*cas1-1*
Line	$\mathbf{F_{1,111} = 105.57}$	$\mathbf{F_{1,111} = 101.76}$	$\mathbf{F_{2,160} = 18.20}$	$F_{1,111} = 3.53$	$F_{1,111} = 0.00$	$\mathbf{F_{1,111} = 134.02}$
Day	$\mathbf{F_{6,111} = 41.41}$	$\mathbf{F_{6,111} = 22.26}$	$\mathbf{F_{6,160} = 58.05}$	$\mathbf{F_{6,111} = 34.81}$	$\mathbf{F_{6,111} = 56.33}$	$\mathbf{F_{6,111} = 23.39}$
Line*day	$\mathbf{F_{6,111} = 5.73}$	$\mathbf{F_{6,111} = 7.36}$	$F_{12,160} = 0.79$	$\mathbf{F_{6,111} = 2.92}$	$F_{6,111} = 1.60$	$\mathbf{F_{6,111} = 3.53}$
Day 7	$t_{14} = 1.00$	$t_{16} = 0.53$	$F_{2,22} = 0.67$	$t_{17} = 0.40$	$t_{14} = 0.00$	$\mathbf{t_{14} = 2.55}$
Day 8	$\mathbf{t_{14} = 4.69}$	$\mathbf{t_{16} = 5.22}$	$F_{2,22} = 1.84$	$t_{17} = 0.32$	$t_{14} = 1.50$	$\mathbf{t_{14} = 6.73}$
Day 9	$\mathbf{t_{14} = 6.32}$	$\mathbf{t_{16} = 6.09}$	$F_{2,22} = 2.39$	$t_{17} = 0.67$	$t_{14} = 0.42$	$\mathbf{t_{14} = 3.98}$
Day 10	$\mathbf{t_{14} = 6.05}$	$\mathbf{t_{16} = 4.92}$	$F_{2,22} = 8.05$	$t_{17} = 0.35$	$t_{14} = 0.16$	$\mathbf{t_{14} = 4.51}$
Day 11	$t_{14} = 2.19$	$\mathbf{t_{16} = 3.19}$	$F_{2,22} = 2.16$	$t_{17} = 0.14$	$t_{14} = 0.47$	$\mathbf{t_{14} = 3.25}$
Day 12	$\mathbf{t_{14} = 3.61}$	$\mathbf{t_{16} = 2.93}$	$F_{2,22} = 2.26$	$t_{17} = 0.62$	$t_{14} = 1.99$	$\mathbf{t_{14} = 3.92}$
Day 13	$\mathbf{t_{14} = 3.22}$	$\mathbf{t_{16} = 2.73}$	$F_{2,22} = 7.52$	$t_{17} = 0.45$	$t_{14} = 0.67$	$\mathbf{t_{14} = 7.20}$

Bold numbers indicate the significant difference.

CHAPTER IV

CONCLUSION

Insect performance can be affected by insufficient suitable sterols in the diet, or the presence of unsuitable sterols above certain levels (Behmer & David Nes 2003). My studies in this dissertation showed that the constraints of insect sterol metabolism can be utilized for pest control, by using plants with modified sterol profiles. My results demonstrated that plants producing unsuitable sterols (Δ^8 sterols) can be generated by silencing the *HYD1* gene without sacrificing plant growth. Both chewing insects *P. xylostella* and sucking insects *M. persicae* fed on *HYD1*-silenced Arabidopsis lines with modified sterol profiles showed reduced growth, delayed development and decreased reproduction. In addition, using the established Arabidopsis lines with various modified sterol profiles, I investigated the performance of aphids on these lines and discovered: aphids reared on plants accumulating atypical sterols (*hyd2*, *ste1-1*, and *cas1-1*) exhibited retarded growth and reproduction. While increased suitable sterols in *HMG1*-OE and *psat1-2* and has no effect on aphids, the increased ratios of campesterol to *β*-sitosterol (*smt2*), however, negatively affected aphid performance. I measured sterol profiles DBM and GPA and demonstrated that feeding on plants with modified sterol profiles resulted in insects with lower fitness.

My findings in *P. xylostella* that unsuitable sterols tend to accumulate in insect bodies are consistent with previous studies on other similar caterpillars, such as *Helicoverpa zea* and *Manduca sexta* (MacDonald et al 1990, Nes et al 1997, Svoboda et al 1995). The Δ^8 sterols accumulated in *P. xylostella* are likely the key factor to the reduced

performance of this insect. In the discussion of Chapter II, the potential damage of Δ^8 sterols to the midgut membranes was discussed. In grasshoppers (*Schistocerca americana*), insect midguts exhibited much higher proportion of unmetabolized sterols than other tissues (Behmer et al 1999b). This might not be surprising due to the fact that the midgut is a very active tissue with rich membrane structures, and the consistent regeneration of the midgut membranes means Δ^8 sterols can be easily integrated into the membrane structure. Whether the midgut is the tissue with the most accumulated Δ^8 sterols remains to be discovered. To further test this hypothesis in the future, it is necessary to perform tissue-specific sterol analysis of insects in the future, combining with the transcriptome study of *P. xylostella* reared on sterol-modified plants or synthetic diets containing Δ^8 sterols. It will be interesting to investigate the potential expression change in genes associated with membrane transporters, insect immune responsive genes, sterol regulatory factors, etc. Furthermore, a tissue-specific transcriptome analysis (particularly in midgut, oocyte, brain, etc.) of insects reared on different sterols could be valuable to fill the knowledge gap.

The findings that different sterol profiles were recovered from chewing and sucking insects is interesting. Although both insects were severely affected by silenced plants bearing Δ^8 sterols, the mechanism appears to be very different. Our two independent aphid studies in Chapter II and III have consistently shown that unsuitable sterols were not present in aphids, and all the ill-performed aphids exhibited reduced amount of sterols in the body, indicating that the insufficient suitable sterols plays a key role in retarding aphid growth and reproduction. A study on insects feeding on late goldenrods (*Solidago*

altissima) found that, although the dominant sterol in this plant is spinasterol (a Δ^7 sterol), very few Δ^7 sterols were recovered from aphids (*Uroleucon luteolum*) feeding on it. On the contrary, the leaf beetle feeding on this plant (*Trirhabda virgate*) showed a dominant presence of Δ^7 sterols in the body (Janson et al 2009). The ecological aspects, in terms of different sterol profiles of insects sharing the same host, was intensively discussed in the late goldenrod study. My findings, furthermore, revealed the different sterol profiles of aphids is likely due to a different sterol profile in the phloem. In our sterol analysis of phloem, I found the sterol profile of aphids appears to influenced by the phloem sterols instead of sterol profiles of the leaf tissue. In fact, previous studies in tobacco, cabbage and two bean plants has also revealed phloem tends to have different major sterols from the leaf tissue - cholesterol and β-sitosterol, rather than the three typical phytosterols (Behmer et al 2011, Behmer et al 2013, Bouvaine et al 2012). For aphids that feed on phloem sap, this can be advantageous to have food resources with higher proportions of cholesterol ready to use instead of other sterols which have to be metabolized first. Especially as stated previously, aphids showed low capacity to dealkylate phytosterols, and performed best on cholesterol rather than other phytosterols (Bouvaine et al 2012, Campbell & Nes 1983). My findings in Arabidopsis were similar to the four plants species studied (*V. fava*, *P. vulgaris* cabbage, *B. rapa*, *N. tabacum*) (Behmer et al 2011, Behmer et al 2013, Bouvaine et al 2012). In addition, a novel finding in this dissertation is that the unsuitable sterols accumulated in the leaves did not present in the phloem. Whether this finding is universal in all or most plant species remains to be discovered.

One of the future directions of this study is to characterize the phloem sterol profiles of other plant species, such as spinach, alfalfa, which produce Δ^7 sterols as major sterols in the leaves. It will be an exciting discovery if these unsuitable sterols are also absent from the phloem like what I found in Arabidopsis.

This study showed the potential of using plants with modified sterols to control insects. Compared to pesticide application, the strategy of using sterol-modified plants is environmentally friendly, and insect-specific. As I mentioned in previous chapters, the mechanism of unsuitable sterols on caterpillars might associate with cell membrane structure, particularly in the midgut. As the major barrier between the outer and inner world of insect body, the multi-functional gut epithelial is a great target for intervention (Huang et al 2015, Linser & Dinglasan 2014). This provides a potential in developing plants with synergistical effects by combining unsuitable sterol production with other midgut-targeting methods. One example is silencing *HYD1* gene in Bt crops, which is discussed in Chapter II. One advantage of using plants with multiple modes of action against insects is to reduce the possibility of insects developing resistance against the plants. In the future, to better exploit sterol-modified plants as a novel strategy for controlling insect herbivorous pests, four new things can be learnt from my study: (1) RNAi can be a useful tool to generate plants with different levels of sterol modification. In this process, the impacts on plants can be minimized through screening the lines with optimal silencing levels which produce sufficient sterols for plants. (2) This study using established lines provides some ideas for other genes as candidates to be silenced, such as *HYD2*, *STE1* and *CAS1*. (3) This study shed new light on differences between chewing

insects and sucking insects in the types of sterols they obtained from the same host plant and differences in insect sterol metabolism. It is necessary to address tissue-specificities. (4) It is worthwhile to extend these findings on Arabidopsis to other plants, especially crop plants such as tobacco, cotton, etc., to apply this strategy more broadly in pest management.

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