Review article

Review: Nectar biology: From molecules to ecosystems

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ABSTRACT

Plants attract mutualistic animals by offering a reward of nectar. Specifically, floral nectar (FN) is produced to attract pollinators, whereas extrafloral nectar (EFN) mediates indirect defenses through the attraction of mutualist predatory insects to limit herbivory. Nearly 90% of all plant species, including 75% of domesticated crops, benefit from animal-mediated pollination, which is largely facilitated by FN. Moreover, EFN represents one of the few defense mechanisms for which stable effects on plant health and fitness have been demonstrated in multiple systems, and thus plays a crucial role in the resistance phenotype of plants producing it. In spite of its central role in plant-animal interactions, the molecular events involved in the development of both floral and extrafloral nectaries (the glands that produce nectar), as well as the synthesis and secretion of the nectar itself, have been poorly understood until recently. This review will cover major recent developments in the understanding of (1) nectar chemistry and its role in plant-mutualist interactions, (2) the structure and development of nectaries, (3) nectar production, and (4) its regulation by phytohormones.

1. A brief history of nectary research

Sugary secretions from plants were noted in antiquity, and while the term 'nectar' was used, most descriptions were vague, lacked functional understanding, and did not always differentiate the sugary substance from honey (Table 1, reviewed in Ref. [1]). It was not until 1735, when Linnaeus noted specialized tissues that secreted nectar and coined the term 'nectary,' that enthusiasm in nectar research began in earnest [2]. Through the 1820s and early 1830s there were competitions to decipher the function of nectar/ies, and in 1833 it was suggested that nectar may aid in the attraction of animals to facilitate fertilization [3]. In 1848, the topographical and functional distinction between floral and extrafloral nectaries was proposed [4], and by the second half of the 1800s, the role of nectar in the co-evolution of plants and animals was being discussed by Darwin and colleagues [5]. In the late 1800s and early 1900s scientists focused on nectary occurrence, structure, and taxonomic utility [6], with nectary ultrastructure and the complexity of nectar composition in relation to plant-animal interactions being given priority through the latter half of the 20th century (e.g., reviewed in Refs. [7–9]).

Towards the close of the 20th century the first manuscripts about genes involved in nectary development and function were being published (Table 2). For instance, *CRABS CLAW* (*CRC*) was the first gene reported to be involved in floral nectary development [10,11]. Similarly, the first reports on nectar proteins and the genes encoding

them came out [12,13]. By the 2000s advances in 'omics' approaches allowed the rapid study of many facets of nectar/y biology, including transcriptomics (e.g. [14–16]), proteomics (e.g. [17,18]), and metabolomics (e.g. [19–22]). As described later in this manuscript, these advents have led to key understandings about molecular mechanisms of nectary development and maturation, nectar production, the regulation of these processes by phytohormones, and the involvement of non-sugar metabolites in plant-animal interactions.

2. On the importance of nectaries and nectar

This review will focus on molecular aspects of nectary development, structure, and function, but it should be kept in mind that the study of nectary biology opens up the potential to understand the co-evolution of plants and animals [23], mediate agricultural gains [24], augment pollinator nutrition and health [25–27], and to model the impacts of nectar at the ecosystem level [28–30]. These studies could not come at a more pressing time as world population and food demand is increasing [31] and worldwide biodiversity is declining, including those of both plant and pollinator populations [32,33]. Animal-mediated pollination is required for, or at least augments, the production of 87 out of 115 (~76%) of the leading global food crops [34], including almonds, cherries, squash, soybeans, canola, sunflower, apples, cotton, coffee and citrus fruits [35]. The annual value of these animal-pollinated crops is estimated to be $29 billion in the U.S. alone

Table 1
Partial list of major events in the study of nectaries and nectars.

8th century BCE	Homer's Iliad refers to nectar as the 'drink of the gods'
4th century BCE	Aristotle recognizes the relationship between honey bees and plants
ca. 40 BCE	Marcus Terentius Varro recognizes sugar secretions by plants as honey [1].
1735	Floral nectaries are described and the term nectarium is coined by Carl Linneaus [2], but the function of nectar remains unclear.
1746	Linnaeus observes EFNs, though hypothesizes the nectar to be a waste product.
1822	Societé Linnéenne of Paris begins to host competitions to understand the function of nectaries [276].
1833	The relationship between nectar, animal attraction, and plant fertilization is recognized [3].
1870s	The 'abominable mystery' and the role of EFNs in defense are discussed by Delpino, de Saporta, Hooker, Belt, Darwin [5].
ca. 1900–1950	Nectary systematics and plant-pollinator interactions are more widely studied. Scientists begin studying ecological functions of EFNs [1]. Nectar proteins are first reported [277].
1955	First reports that pre-nectar is derived from phloem sap [58,278].
1961	Nectar sugars from 889 plant species reported [9].
1970s and 1980s	Fahn publishes two highly cited reviews and describes nectary vasculature in relation to function and nectar quality in great detail [7,205].
	Herbert and Irene Baker manuscripts highlight the complexity of nectar chemistry. They also create hexose- and sucrose-dominant nectar terminology [8]. The presence of secondary metabolites and microbes in nectars are first reported [64,279–283] and similar studies continue to present day [27,68,73,74,77,125–143,146–160].
1990s	Characterization of nectary anatomy across many families, including the Brassicaceae [199]. First identification, cloning, and characterization of nectar proteins [13]. Studies on nectar proteins in various species continue to present day [12,17,75,76,102,103,284–288].
1999	CRABS CLAW, a transcription factor, is required for nectary development [10].
2001	EFN secretion is inducible by both herbivory and jasmonic acid (JA) [289]. Later, JA is also shown to be involved in regulating FN secretion [245].
2004–2009	Molecular aspects of starch metabolism in floral nectaries is reported, as well as its regulation by the transcription factor NtMYB305 [224,228,230,246]. Studies on nectar microbes and their ecological impacts begin in earnest [140]. Arabidopsis nectary transcriptome analyzed. A surprising number of genes with enriched-expression in nectaries is uncovered [15].
2010–current	Multiple reports on the roles of individual genes in nectary development and function. For example, the sucrose uniporter SWEET9 is implicated as a key advent leading to Darwin's 'abominable mystery' [232]. Nectar secondary metabolites have demonstrable beneficial effects on pollinator health [26,27,136]. The capacity for nectar-producing plants to have a positive impact on the ecology of agro-ecosystems is reported [29,30].

[36] and account for one-third of total food production [34].

Many insect species, especially those in the orders Coleoptera, Diptera, Hymenoptera, and Lepidoptera, offer pollination services to plants [37,38], but birds [39], bats [40], and nonflying mammals [41] also rely on floral nectar (FN) as a food source. Of special concern is the domesticated honey bee (*Apis melifera*), which by far is the major pollinator of crops [42]. Unfortunately, populations of managed honey bee hives have experienced sharp declines in the past few decades [43,44]. In response to declining pollinator populations, nations have applied funds towards understanding factors impacting pollinator

Table 2
Partial list of cloned genes with known or implicated functions in nectaries and nectars.[a]

Species	Gene	Function and/or mutant phenotype
Arabidopsis	ARF6, ARF8	Transcription factors; double mutants lack nectaries [243]
	BOP1/BOP2	Transcription factors; double mutants lack nectaries [215]
	COI1, DAD1, AOS2	Jasmonate signaling and response; mutants do not produce nectar (Schmitt et al., in preparation)
	CRABS CLAW (CRC)	Transcription factor required for nectary development [10,210–212]
	CWINV4	Cell wall invertase required for nectar production; mutants do not produce nectar [229]
	GA2Ox6	GA inactivation; mutants produce 40% less nectar than wild-type [22]
	JMT1	Jasmonic acid carboxyl methyltransferase; has nectary-enriched expression, but the function specific to nectaries is unclear. May be involved in floral scent evolution or in mediating JA responses [290]
	MYB21	Transcription factor, ortholog of *NtMYB305*; mutants lack nectar and expression of SWEET9, as well as other genes required for nectar production (Schmitt et al., in preparation)
	MYB57	Transcription factor; mutants have smaller nectaries and produce ~50% less nectar than wild-type [20]
	PIN6	Transmembrane protein involved in auxin transport; mutants produce 60% less nectar and display decreased auxin response in lateral nectaries [20]
	Sesquiterpene synthase	Sesquiterpene synthase; mutants lack several floral volatiles found in wild-type [68]
	Sucrose phosphate synthase 1F/2F	Involved in sucrose synthesis in mature nectaries. Silenced lines do not produce nectar [232]
	SWEET9	Sucrose uniporter; mutants do not produce nectar [232]
Brassica spp.	BcNTR1	Jasmonic acid carboxyl methyltransferase; ortholog to Arabidopsis *JMT1* [290]
	BrSWEET9	Sucrose exporter; mutants do not make FN [232]
Jacaranda mimosifolia	JNP1	GDSL lipase that may have direct antimicrobial activity or influence nectar lipid composition [70]
Nicotiana	NtSWEET9	Sucrose exporter; silenced lines do not make FN [232]
	NtMYB305	Transcription factor; silenced lines lack nectar, have altered starch metabolism, and lack expression of nectarins [224,230,246]
	Nectarins I-V	Nectar proteins involved in limiting microbial growth [12,284–288]
	NtCOI1	JA receptor; mutants do not produce nectar and have altered starch utilization [16]
Petunia	Psy3, Psy4	RNases that may limit microbial growth [78,79]
	NEC1	Likely ortholog of SWEET9 [222]
Populus	PttSLAH3	SLAH3-type anion channel in extrafloral nectaries, permeable to both nitrate and chloride in vitro; implicated in nectar secretion [272]

[a] Nectarins from a number of other species have also been identified, but not cloned or fully characterized. These are not listed here.

health and measures that can be used to support their populations. For example, Canada developed the Canadian Pollination Initiative (C-ANPOLIN, http://www.uoguelph.ca/canpolin/) in 2009 with a budget of CAN$5 million. This effort served as models for similar programs in France and the United Kingdom [45]. Later, U.S. President Barack Obama created the Pollinator Health Task Force in 2014 to develop a plan to protect pollinating insects, which resulted in the *National Pollinator Research Action Plan* [46]. A major recommendation of this action plan was to focus on understanding pollinator nutritional needs and ensuring they have adequate access to proper sources of pollen and nectar.

Extrafloral nectar (EFN) is also of great ecological and agricultural importance [47], but this area of research is perhaps understudied. EFN has been reported in many crop plants that attract predatory insects [48–51], mainly ants and parasitoid wasps, to prevent herbivory (e.g., in peach, cacao, cashews, cotton, and bean) [52–54]. This tritrophic means of pest control is being explored in mixed planting systems [i.e. mixtures of plants that produce EFN with those that do not (e.g. integrated pest management, IPM)] as an alternative to chemical insecticides that often come with human and environmental health risks [47,55–57]. (A note to the reader: hereafter floral and extrafloral nectar are abbreviated as FN and EFN, respectively; whereas, 'floral nectary' and 'extrafloral nectary' are spelled out so as to differentiate between these glandular tissues and their secretions.)

While keeping an eye toward agronomic, historical, and ecological context, the subsequent parts of this manuscript will focus on recent major developments in the understanding of (1) nectar chemistry and its role in plant-mutualist interactions, (2) the structure and development of nectaries, (3) mechanisms of nectar production, and (4) its regulation by phytohormones.

3. Nectar chemistry and its roles in biotic interactions

3.1. Sugars

Nectar is the primary source of carbohydrates for pollinators and defensive mutualists. The main solutes found in most nectars are varying ratios of sucrose, glucose and fructose [58]. The sucrose-to-hexose ratio, which can range from nearly all sucrose to all hexose, and the total sugar concentration, which can range from as little as 8% (w/v) to as high as 80% [58], are fairly consistent within a given species and are important in plant-mutualist interactions [8]. For example, hummingbirds prefer dilute, sucrose-rich nectars, whereas short-tongued bees and flies favor more concentrated hexose-rich nectars [8]. Similarly, some ants rely on myrmecophytic plants that produce hexose-rich EFN because they cannot digest sucrose [50]. Rarer sugars, including arabinose, galactose, mannose, gentiobiose, lactose, maltose, melibiose, trehalose, melezitose, raffinose, and stachyose have also been identified in nectars of some flowers, which can be toxic to potential pollinators [8,58–60].

3.2. Non-sugar nectar metabolites

Nectar composition must lead to the attraction of mutualists while at the same time deterring exploitative visitors, such as nectar robbers (insects that remove nectar without pollinating the plant) and potentially harmful microbes [61,62]. Approximately 10% of nectar dry weight is represented by many classes of non-sugar metabolites [63], which have diverse functions. These classes of compounds include amino acids [64], vitamins [65], alkaloids [27,66], phenolics [67], terpenoids [27,68,69], lipids [19,20,70,71], metal ions [72], hormones [73], and proteins [74–80]. Like sugars, the composition and amounts of these compounds are highly variable between species and type of nectary. The biological functions of these compounds in nectars are not yet fully understood, but the diversity in nectar quality and quantity (which can range from less than one microliter to over several milliliters [81]), clearly impacts the specificity of plant-animal interactions [8]. Recent progress in this area of study is outlined below.

3.2.1. Amino acids

Amino acids, though much less concentrated than sugars, have been found in all nectars examined to date and are key sources of nitrogen for mutualists [82,83]. They have also been proposed to provide flavor to nectar [84], and new developments show that amino acids may service pollinators in more ways than just as building blocks for proteins. For example, proline accumulates at high concentrations (~2 mM) in the nectars of a number of angiosperms, such as soybean, ornamental tobacco, *Cucurbita pepo* L., and *Brassica napus*, among others [73,85,86]. Unsurprisingly, honey bees (*Apis mellifera*) prefer nectars rich in proline [85]. It was hypothesized that this preference is due to honey bees' ability to taste proline, as well as this amino acid's potential role in insect flight [85]. Indeed, proline was recently confirmed to be important for energy production in the flight muscles of some bees and wasps [87].

The ratio of carbohydrates-to-amino acids also appears to play a role in pollinator visitation. Honey bees were shown to exhibit a preference for essential amino acids and willingly gave up sugars to acquire these amino acids [88]. In the case of phenylalanine, honey bees were willing to give up 84 units of sucrose for 1 unit of amino acid [88]. Furthermore, honey bees showed a distaste for glycine, but adding 100 or more units of sucrose was able to offset the negative effects of 1 unit of amino acid [88]. This study is one of a few that has shown that plants can substitute the costly production of carbohydrates for amino acids and that the deterring effects of some amino acids may in turn be masked by higher carbohydrate concentrations. Clearly, the impacts of amino acids on nectar function are proving to be quite complicated and warrant further study. For example, the presence of non-standard, psychoactive amino acids in nectars, which may affect pollinator behavior, were recently reported [89]. A more in-depth look at amino acids in FN has been reviewed [89], but it is also important to note that amino acids in EFN also affect plant-mutualist interactions (e.g. [90]).

3.2.2. Nectar proteins (nectarins)

Both FN and EFN have been shown to contain distinct, consistent arrays of proteins (nectarins), which are known to both tailor nectar chemistry for their animal mutualists (e.g. [50,91]) and to prevent microbial growth [74–80]. An understanding of this latter function is essential because microbial infection of plants via the nectaries occurs in cotton, bean, squash, apple, pear, aucuba, banana, pineapple, hawthorn, and gourds [92–98]. For example, fireblight is caused by the colonization of FN by *Erwinia carotovora* and subsequent invasion of the floral vasculature through the nectary glands [99,100] and is one of the most disastrous diseases of apples and pears.

In support of the view that nectarins play a defensive role, five proteins identified in the FN of ornamental tobacco have been shown to be involved in a redox cycle leading to high concentrations of hydrogen peroxide, which limits microbial growth in nectar (Table 2, reviewed in [77]). Similarly, acacia EFN contains glucanases and chitinases that protect it from infestation by fungal pathogens [74]. Multiple other reports on nectarins have come out in recent years, but are not detailed here due to space constraints (e.g., [78,79,86,101–105]).

3.2.3. Lipids

Lipids, such as free fatty acids and oils, have been found in many nectars, including those of Arabidopsis and *Brassica rapa* [19,20,70,71]. Lipid concentrations can be so high as to give nectars a milky appearance, such as the nectar of *Jacaranda mimosifolia* [70]. Interestingly, non-esterified free fatty acids can be found in nectars at quite high concentrations (e.g. 0.6 mM in *Jacaranda*) [70].

Given the preponderance of lipids in nectars, it is very surprising that their biological functions have been largely ignored to date. It has been posited that nectar lipids may form a film on the surface of nectar

droplets to limit evaporation from nectar [106,107], but potential specific roles in plant-mutualist interactions have not been extensively explored. This may be due to the perception that pollinators primarily obtain lipids through pollen consumption [108]. Dietary lipids are essential for pollinator health. For example, these lipids provide honey bees with key precursors for a variety of physiological processes, such as molting hormone production [109]. More recently, a deficiency of linolenic acid (18:3) in pollen was linked to impaired learning in honey bees [110]. The assertion that lipids may serve as a nutritional resource or attractant for insect visitors is not new [71]. Honey bees prefer pollen rich in C:18 fatty acids [71], suggesting that they may prefer nectars with similar fatty acid profiles [70]. Further, some specialized bees visit flowers that offer oils through specialized organs known as elaiophores instead of, or in addition to, nectar and/or pollen [111,112].

Considering the above information, it is interesting that no studies to date have explored if pollinators exhibit a preference for specific lipid content or concentration in nectar. Since lipids are a rich energy source one could presume their presence in nectar is important for mutualist nutrition. A second more speculative view is that some nectar lipids may prevent microbial growth, as free fatty acids, such as the same types found in nectars, can have direct antimicrobial effects, possibly through disrupting membrane structure [113]. Clearly, the role of nectar lipids is a topic that requires further research.

3.2.4. Secondary metabolites

Similar to amino acids and proteins, secondary metabolites (SMs) add an additional level of functionality to the extensive network of compounds found in nectar. There is speculation that SMs in nectar may be a result of inevitable 'leaky' transport to the nectary via another tissue's defense systems [114,115], yet, nectar SMs have been shown to have functional roles in the enticement of pollinators [62,83,116,117] and deterrence of exploitative nectar robbers [61,114,118–123]. For example, nectaries can be involved in the evolution of floral scent (e.g. [68,117]), while at the same time secreting glycosides, alkaloids and phenolic compounds that appear to be toxic or unpalatable to nectar robbers (e.g. [61]). Recent developments in the functional understanding of several SMs in nectars are described below.

Volatile organic compounds (VOCs) have been found in nectars and may be involved in pollinator attraction. These VOCs may directly be synthesized by the nectary [68,117] or may dissipate into the nectar from nearby floral tissues [117,124]. A sesquiterpene synthase highly expressed in Arabidopsis nectaries (see Table 2) is required for the production of several floral volatiles, which may serve as attractants to floral visitors [68]. The authors suggested the terpenes emitted might provide a dual function as well, as terpenoids also can have antimicrobial activity [117,125,126].

Some nectar alkaloids, such as gelsemine in *Gelsemium sempervirens* nectar, can have greater deterrent effects on pollinators rather than nectar robbers [61]. Deterring pollinator visitation with high concentrations of nectar alkaloids may seem counterintuitive at first glance, but a more distasteful nectar leads to shorter flower visitation and reduced volumes of consumed nectar, potentially leading to higher rates of desired outcrossing [127]. For instance, in *Nicotiana attenuata,* nicotine, a known herbivore deterrent, increased the number of flowers visited and reduced volume of nectar removed from each flowers by hummingbird and moth pollinators [124]. Fewer exploitative ants were also noted by synthetic nectars supplemented with nicotine than ones without. The authors pointed out that the goal of nectar production is not to maximize consumption per flower, but rather to maximize pollen transfer to other plants, making the presence of these chemical deterrents evolutionarily favorable. Much as in the case of amino acids, honey bees will tolerate high nicotine levels in nectars if sugar concentrations are also high, which can actually benefit honey bee health [128,129]. Caffeine is another alkaloid identified in citrus and coffee nectars that benefit both pollinators and plants by increasing

foraging memory in honey bees [130] and increasing pollination efficiency [131].

Interestingly, SMs may also provide a mimicking strategy to attract pollinators. In both buckwheat (*Fagopyrum esculentum*) and Mexican sunflower (*Tithonia diversifolia*), the nectar is supplemented with three phenolics that mimic queen mandibular pheromone (QMP) of the honeybee [132]. In addition to QMP, two deterrent non-QMP phenolics, chlorogenic and isochlorogenic acid, with the former known to be toxic to honey bees, are also present in *T. diversifolia* nectar. However, the presence of QMP appears to mask the deterrent effects of chlorogenic and isochlorogenic acid on bee visitation [133].

Another potential reason for the persistence of SMs in nectar is to provide a direct health benefit to pollinators. The consumption of SMs has been shown to reduce the pathogen load in bumble bees and in some cases increase the survivorship of these bees [27,133–135]. A recent report demonstrated that the resistance of *Crithidia bombi*, a bumble bee parasite, varies between different SMs found in nectar [136]. *C. bombi* growth was reduced by naturally occurring concentrations of anabasine, eugenol, and thymol but remained unaffected by naturally occurring concentrations of gallic acid, caffeic acid, and chlorogenic acid. Although anabasine, eugenol and thymol reduced *C. bombi* growth, their effectiveness varied significantly across four different strains [136]. The varied effectiveness of the different SMs on parasitic growth may be a strong driver of infection rates in bees. Flowers that produce higher concentrations of SMs, such as thymol, may reduce infection and increase overall bee colony health. These types of studies have led to the idea that some pollinators may visit select flowers to self-medicate depending on the type of biotic stress they are undergoing [137,138].

The impacts of different combinations of sugars, lipids, amino acids, proteins, and SMs on pollinator visitation still warrants more detailed investigation. For instance, the presence of an individual compound in nectar may not always be conducive to improved pollinator attraction, but a synergistic (or antagonistic) effect may come into play when two or more compounds co-occur and serve relevant ecological functions. Although some studies on artificial nectars found that some SMs deter pollinators, bumblebees (*Bombus terrestris*) are unable to detect a variety of SMs that deter other pollinators when presented at natural concentrations [139]. This would suggest that some of these compounds may be able to persist, as pollinators may be unable to sense them at naturally occurring concentrations, particularly when sugar concentrations are high. Newly emerging techniques allowing for high throughput metabolomic analyses of nectars may allow for researchers to continue exploring the variety of nectar constituents and the implications for plant-mutualist and non-mutualist interactions.

3.2.5. Microbial communities in nectar

The relationship between nectar chemistry and ecological functionality is clearly complex when just considering the function of enticing or deterring different visitors. This binary relationship becomes even more complicated when we begin to consider a third consumer of nectar: microbes. Given its nutrient-rich nature, it is not surprising that bacteria, yeasts, and fungi are found in some nectars and can change their composition [74,140–143]. As discussed above, many nectars appear to actively limit microbial growth in order to prevent infection of floral tissues [77,144]. Another reason nectars may have antimicrobial activities is to avoid the consumption of sugars and other metabolites by microbes, which clearly could impact mutualist visitation. In light of this possibility, it is intriguing that yeasts and other microbes have been identified as widespread inhabitants of nectars. Although this is not an extensive review on the current status of research on nectarivorous microbes, we highlight a few recent findings in this exciting field of nectar research.

Yeasts and bacteria inhabit FNs from a wide variety of species, and these microbes are most likely transferred to inoculant nectar via pollinator visitors [140–143,145]. Yeast communities inhabit nectars at

high densities, but these communities generally have only a single or few species present [146]. A yeast nectar specialist, ascomyceteous *Metschnikowia reukaufii,* has emerged as the most abundant yeast found in nectars [140,147,148].

Perhaps most interesting is the fact that these microbial communities can alter the chemical composition of nectar, which in turn may influence pollinator visitation. Through their metabolic activities, microbial communities can shift a sucrose-dominant nectar to a hexose-dominant one [145,149–152], shift nectar pH [151,152] and consume amino acids [153]. Furthermore, both bacteria and yeast have been shown to interact with nectar SMs [154,155]. The absence of nicotine in the nectar of a genetically transformed *Nicotiana attenuata* plant significantly altered the nectar bacterial community when compared to wild-type *N. attenuata* nectar [154]. The authors hypothesize that differences in pyridine-alkaloid composition shape bacterial communities in nectar. These bacterial communities then affect the chemical composition of the nectar leading to alterations in nectar consumption and plant pollination [154]. A very recent report suggests the impacts of SMs on microbial growth and metabolism is both species- and compound-dependent [155]. This study also found that the ability of microbes to alter SM concentrations in nectar is also species- and compound-dependent, suggesting that some SMs do not serve as the primary mechanism of filtering microbial growth. Perhaps the most interesting finding was that the presence of *M. reukaufii* increased nectar consumption of catalpol-containing nectar by pollinators, which was otherwise a deterrent to some floral visitors [111].

How the above alterations in nectar chemistry by microbes impact the visitation of pollinators is still a very young and active area of research, but it would be logical to hypothesize that the metabolic activity of nectarivorous microbes may influence the quality of nectar available to pollinators. For instance, nectar microbes were found to significantly reduce the concentration of aspartic acid, glutamic acid and proline in the nectar of *Mimulus aurantiacus* [153]. As previously discussed, proline is an important attractant of some pollinators, so microbial consumption of amino acids could clearly negatively affect visitation and plant fitness. Nectar inhabiting microbes, such as *M. reukaufii*, can enhance male fitness [156] but can also reduce plant fecundity [157]. Honey bees avoided nectars inhabited by several different bacterial species, but not when inhabited by *M. reukaufii*, most likely due to the alteration of nectar chemistry by the bacterial inhabitants [152]. Microbial communities may also enhance the attractiveness of nectar by warming it through their metabolic activity [158], as warm nectar has been shown to be preferred by some bees [159,160]. Undoubtedly, the influence of nectivorous microbes on plant fecundity and the dynamic relationship between plants and their pollinators still requires further investigation. Future studies should aim to explore a wider variety of plant taxa with diverse pollinators to elucidate how the inoculation and interplay of nectarivorous microbes may influence pollinator preference and health, as well as overall plant fitness.

4. Nectary evolution, diversity, structure, and development

4.1. Evolution and diversity of nectaries

Nectaries are the glands responsible for synthesizing and secreting nectar and can appear nearly anywhere on a plant [161,162]. The only place where a nectary has yet to be found is in roots, although they do produce sugary exudates that impact biotic interactions in the rhizosphere [163]. Nectar production is not a monophyletic trait and the presence of nectaries has come and gone within individual plant families several times throughout plant evolution [164]. To date, 3941 species of plants have been reported to produce EFN, with 93% of them being eudicots [164]. A few species of bracken ferns (*Pteridium aquilium*) [165] and gymnosperms (*Ephedra, Welwitschia*) are also reported to produce EFN [166]. FN appears to be more common than

EFN in angiosperms, but there have been no calculations on worldwide patterns or frequencies. An estimated 87% of angiosperms benefit from animal-mediated pollination [45], however, some of these flowers do not produce nectar (e.g. deceptive orchids [167,168]). Conversely, some highly self-pollinating plants that do not require pollinators, like Arabidopsis, have maintained functional nectaries and proven useful for studying nectary development and function [169]. Similarly, wind-pollinated plants with relictual nectaries do exist [170]. It is unclear if these nectaries enhance outcrossing and genetic fitness, or if not enough time has passed for functional nectaries to be lost [169].

There are several hypotheses as to how nectaries arose, and since they likely evolved independently multiple times [161,162,171], several explanations may be needed [172]. One leading hypothesis is that hydathodes, which are secretory sites along angiosperm leaf margins and epidermis involved in alleviating positive xylem pressure (guttation) [173,174], may be the evolutionary precursors to some nectaries [161,162]. Lending support to this hypothesis, hydathode secretions do contain relatively dilute sugars, ions, and other metabolites, which may be attractive to insects [161,162]. In turn, the genetic programming needed to form a functional hydathode may have been co-opted to develop secretory glands elsewhere on plants. An alternative view is that some nectaries, particularly ones on flowers, may have evolved from other reproductive secretions, such as stigmatic exudates and gymnosperm pollination drops. Indeed, stigmatic exudates have been observed to serve as a nectar-like reward in some cases [162]. Intriguingly, direct links between stigmatic and nectary function, including an exchange of sugars via reabsorption and secretion, have been reported in *Streptosolen jamesonii* [175]. Gymnosperm pollination drops, which are present on female cones and function to receive pollen from male cones, have also been postulated to serve as an early form of nectar reward to animal visitors [176–178], though it is unclear how this trait could have been transmitted to distantly related angiosperms. Regardless of origin, plant-mutualist interactions have co-evolved through the action of nectaries in many ways [179–181].

The distinction between floral and extrafloral nectaries is mainly a topographical one. Since extrafloral nectaries can occur on vegetative structures located very close to flowers, such as cotton bracteal nectaries, several terms, like 'reproductive' and 'extra reproductive' nectaries, have been created for clarification [182]. Still, the terms 'floral nectary' and 'extrafloral nectary' are much more commonly used. Regarding specific locations on the plant, extrafloral nectaries can appear nearly anywhere on aerial tissues, but they are most commonly associated with stipules, petioles, and leaf blades [162]. Floral nectaries can also be located in a variety of floral tissues (receptacle, sepal, petal, stamen, filament, anther, ovary, style, stigma) (excellently reviewed in [171,182]).

4.2. Nectary structure

Nectaries can be classified into two general forms: structured and non-structured (Fig. 1) [182]. Non-structured nectaries are inconspicuous or 'gestaltless' and may only be detected by the presence of nectar [183–185]. These nectaries are rare, or perhaps underreported, due to the difficulty of finding them. As the name implies, these types of nectaries usually have no differentiated tissue that can be identified as a functional unit. One such recently reported example includes the extrafloral nectaries of *Brassica juncea* (Fig. 1F), which appear to be little more than irregular pocket-shaped openings on the surface of stems with no discernible direct link to the vasculature [185].

'Structured' nectaries appear at regular positions on predictable structures and are usually well differentiated from the surrounding tissue, two examples of which are shown in Fig. 1A–E. These nectaries typically have three distinct cell types: (1) epidermal, (2) vascular, and (3) parenchymal [186]. The epidermis of such nectaries may contain secretory trichomes, but, more commonly, the nectary epidermis is interspersed with modified stomates sometimes referred to as 'nectar-
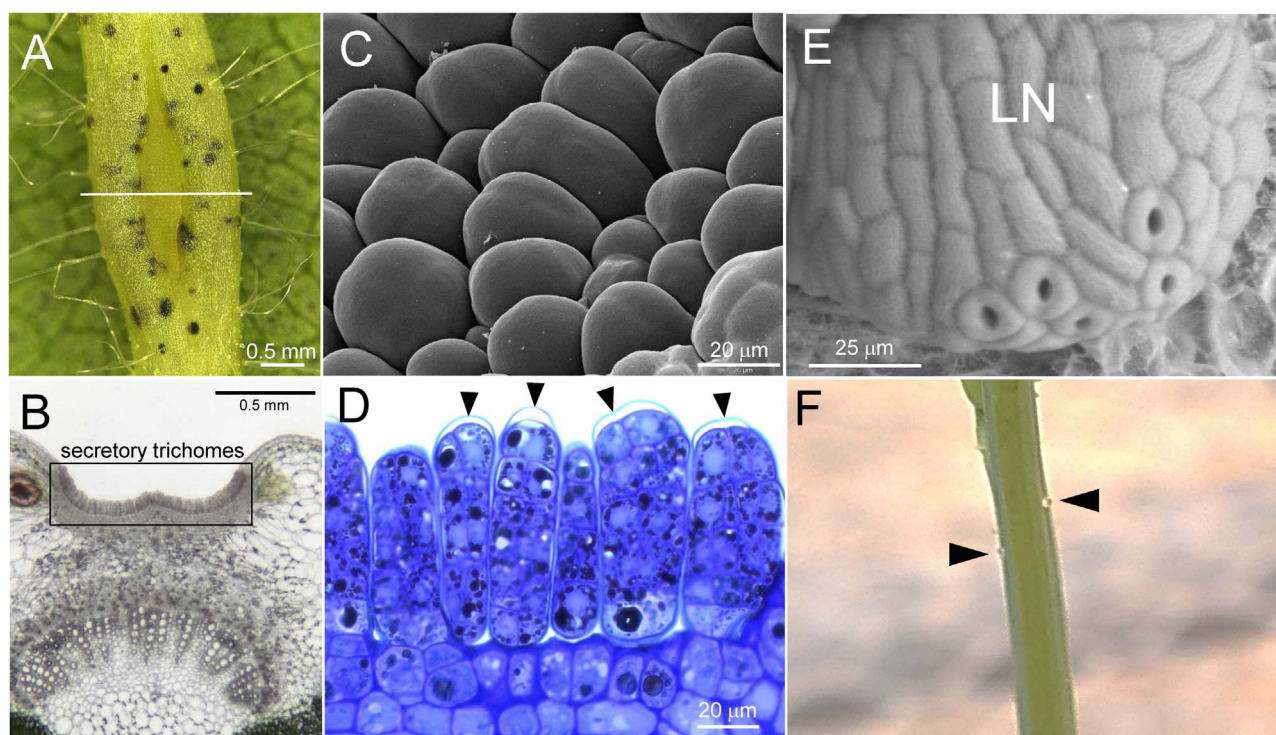
Fig. 1. Examples of 'structured' and 'non-structured' nectaries. (A–D) Cotton foliar extrafloral nectaries, which lie along the mid-vein of cotton leaves. (A & B) Images of nectaries showing invagination that contains a field of secretory trichomes. B is a cross-section of a foliar nectary along line shown in A. The box highlights the region of secretory trichomes, which are shown in more detail in C & D (photo credit for A-D: Elizabeth Chatt, Basil Nikolau, and Harry Horner). Arrowheads in Image D indicate the location of a cuticle covering secretory trichomes. (E) One-half of a bi-lobed Arabidopsis floral lateral nectary (LN), which has open stomates that serve as the presumed sites of nectar secretion. Note the wavy cuticle covering the surface of the nectary. (F) Some nectaries are 'non-structured,' containing no identifiable differentiated tissue, as is the case of the EFN (arrowheads) produced along the inflorescence stems of *Brassica juncea* [185].

ostomata' [187]. It was recently reported that nectar may even be produced by nectaries with both nectarostomata and secretory trichomes located adjacent to one another [188]. In the case of *Salvia farinacea*, the nectarostomata were found to be responsible for aqueous nectar secretion, whereas the trichomes appeared to secrete oils into the final 'mature' nectar [188]. It is also important to note that a thick cuticle covers the nectary epidermis in most species, including both those with stomates [A.R. Davis in 189,190] and secretory trichomes [191–194] (e.g. see Fig. 1E and D, respectively).

In nectaries that have them, stomates are presumed to be the pores from where nectar is secreted, and are usually reported as being 'modified' and permanently open [195–198]. For example, while nectars have been reported to accumulate high levels of abscisic acid [(+)ABA)] [73], the nectarostomata of *Vicia faba* are insensitive to (+)ABA levels up to 1.6 mM [197]. One explanation for why nectarostomata may be permanently open is that close contact is maintained between subepidermal and guard cells (i.e., there is a very small substomatal space), thereby restricting guard cell movement [196,197,199]. Interestingly, nectary stomates also tend to violate the one-cell spacing rule found within leaf epidermal cells (e.g. Fig. 1E), which states that two stomates never develop contiguously [200]. This one-cell spacing is thought to be crucial for proper foliar stomatal function due to the necessary rapid exchange of ions and water with surrounding tissues to regulate stoma aperture [200]. The genetic programming that allows the violation of this one-cell spacing in nectaries is not understood.

While control of stomatal aperture does not appear to be a common mechanism for regulating nectar secretion, nectary stomates are often occluded with insoluble osmiophilic material derived from cuticular channels [196,201–203]. Occluded stomas are usually noted in secretory or post-secretory, rather than in pre-secretory nectaries [196,201–203], and therefore may play a crucial role in limiting nectar secretion. It should also be noted that unintentional "apical openings"

(essentially ruptures) have been reported to appear on the surface of nectaries adjacent to nectarostomates [198], which could serve as routes of unregulated nectar secretion in some species.

The nectary parenchyma underlies the epidermis and is largely responsible for modifying 'pre-nectar' metabolites derived from phloem (more detail below in Section 6). These cells are often, but not always, innervated with vasculature [204,205], the type and amount of which have functional implications as to how much nectar is produced. The most common vascular tissue in nectaries is phloem, though sometimes both xylem and phloem will be present [204]. The nectar may contain up to 80% sugar if phloem makes up most of the vascular tissue [204], whereas sugar concentration may be as low as 8% if xylem predominates [58].

4.3. Molecular mechanisms of 'structured' nectary development

The most studied model of nectary development has been *Arabidopsis thaliana*. Floral development in Arabidopsis is well understood and has led to the ABC(E) model of development, which has been found to be applicable across evolutionarily divergent lineages in the angiosperms [10,206]. This model explains formation of the floral whorls by the concerted overlapping actions of various transcription factors (class A, B, C and E) in the floral meristem leading to establishment of a floral organ's identity. Amazingly, the Arabidopsis nectary itself is an ABC-independent floral structure and can form independently of the floral identity genes [11]. Flower development in Arabidopsis progresses from Stage 1 (flower buttress formation) to Stage 20 (seeds fall), with landmark events marking each stage. Floral nectary development in Arabidopsis begins at the base of the stamens in the third whorl around Stage 9, approximately 3.5 days before anthesis [207], as an outgrowth that is composed of several layers of cells. The lateral nectaries initiate before the median nectaries via coordinated cell divisions in two localized regions succeeded by divisions in an

Table 3
Major potential routes of nectar secretion.

Secretion type	Mechanism
Apoplastic	Pre-nectar metabolites derived from the phloem travel around nectary cells. Sink status may be maintained by cell wall invertase activity.
Granulocrine/merocrine	Nectar metabolites are packaged into vesicles and secreted via fusion with the plasma membrane.
Eccrine	Nectar metabolites are exported across the plasma membrane by pores and transporters. This mechanism is strongly supported by the finding that the plasma membrane localized sucrose uniporter SWEET9 is required for nectar production in multiple species.
Holocrine	Programmed cell death of nectary cells followed by rupture of cuticle.

expanded domain [11]. Studies on floral homeotic mutants with abnormal organ identities in each of the whorls [208,209] revealed that nectary development is closely associated with the floral region that gives rise to the third whorl, irrespective of the floral organ present at the whorl [11]. This suggests that stamen development is separable from nectary development, although a close relationship might exist. While a plethora of genes have been implicated in floral development, the players in nectary development are few and understudied, but discussed below.

CRABS CLAW (CRC) is a YABBY-family transcription factor required for floral nectary development in both rosids and asterids, two major phylogenetic lineages of eudicots [10,11,210–212]. CRC expression persists in nectaries even after development and during secretion, but since *crc* flowers lack nectaries, it is unclear what function it may play in regulating nectar production. It should be noted that constitutive expression of CRC is not sufficient to result in ectopic nectary development [10]. Through studies with mutants defective in ABC floral organ identity genes, it has been proposed that A and B class gene function (and not C class function) suppress CRC expression, while A and C function have a spatiotemporal effect on CRC mRNA accumulation [10]. Recently JAIBA, a class II homeodomain leucine zipper transcription factor, was reported to be important for male and female reproductive development and fruit formation, and also supposedly interacts with CRC in floral meristem deterministic processes [213]. Though no nectary or nectar phenotype was reported for the *jab* mutant, it would be interesting to examine if JAIBA has a role in nectary development, adding another molecular player to the nectary developmental program. While CRC is required for nectary development within two major clades of the eudicots, it is unclear the extent to which it, or its homologs, plays a role in floral or extrafloral nectary formation in other eudicot clades or monocots. Indeed, examinations of basal eudicots suggests CRC may not be required for nectary development outside of the rosids and asterids [212,214].

Another class of transcriptional co-activators, BLADE-ON-PETIOLE (BOP), is important for nectary development in Arabidopsis. BOP1 and BOP2 are required for proper nectary development since the flowers of *bop1/bop2* double mutants lack nectaries [215]. Similarly, auxin biosynthesis in flowers activates the expression of the transcription factors AUXIN RESPONSE FACTOR 6 (ARF6) and ARF8, which are involved in petal, stamen, and gynoecium development. *arf6/arf8* double mutants also fail to develop nectaries suggesting that auxin signaling is a prerequisite for the activation of nectary developmental pathways [216].

Future studies on nectary development should involve an understanding of the transcriptional network that mediates the initiation and development of nectaries. Surprisingly, the direct targets of CRC, BOP1/2, and ARF6/8 are still unknown. Studies with floral homeotic gene mutants in combination with CRC, ARFs, and BOPs can help uncover other genetic circuitry that might delineate nectary initiation and formation. ChIP-seq studies also hold tremendous potential to identify targets of the transcription factors in nectary-specific processes. Lastly, and notably, no genes have been implicated in extrafloral nectary development to date.

5. Mechanisms of nectar production

5.1. Overview

Both floral and extrafloral nectaries have evolved multiple times [164], so it is not surprising that the molecular mechanisms underlying nectar synthesis and secretion may be highly variable. Further, FN is produced in a fixed ontogenetic pattern (e.g. usually at anthesis and certain times of day); whereas EFN secretion is often rapidly inducible by herbivory and needs to be produced on demand [47]. Phloem sap can be thought of as 'pre-nectar' for most species, but it is clear that the chemical composition of nectar is usually quite different from that of phloem, as has been demonstrated for sugars and amino acids, in particular (e.g. [217]). This view does not necessarily discount the ability of some nectaries to perform *de novo* photosynthesis to produce sugars that will later be used to make nectar [218]. Either way, it then follows that most nectaries are true glandular tissues, which modify and store metabolites until secretion is induced by specific developmental and external cues. Potential mechanisms of FN secretion are described below and listed in Table 3, with EFN production discussed within the context of these models in Section 7. Lastly, some nectaries reabsorb nectar not collected by mutualists. The occurrence and prevalence of nectar reabsorption has been reviewed [219], but the molecular mechanisms for how this occurs have not been reported (see Section 5.3 for more detail).

5.2. Models of floral nectar secretion

5.2.1. Apoplastic

In one model of FN secretion, metabolites in phloem sap move apoplastically (around parenchymal cells) to the nectary surface (Table 3) [169,220], but this action would neither account for the distinct differences in chemical composition usually found between nectar and phloem sap, nor the fact that floral nectaries are known to often store large caches of starch prior to anthesis. Indeed, the presecretory floral nectaries (pre-anthesis) in many species store large amounts of starch in plastids, which is rapidly degraded just prior to anthesis and nectar secretion (e.g., schematic in Fig. 2A; [218,221–231]).

5.2.2. Merocrine/granulocrine secretion

While starch-derived sugars serve as a major source of nectar carbohydrate in many species, the specific mechanisms of how these sugars and other metabolites are transported and ultimately secreted is still somewhat in question. One model suggests that pre-nectar metabolites are transported symplastically (through cells) via plasmodesmata in the underlying nectary parenchyma until they reach secretory cells at or near the nectary surface [7,205,231]. It has been hypothesized that at this point nectar metabolites are packaged into endoplasmic reticulum (ER) and/or Golgi-derived vesicles and secreted via fusion with the plasma membrane (granulocrine or merocrine type secretion). This model is rooted in ultrastructural analyses that have repeatedly demonstrated the presence of extensive ER, Golgi, and vesicular networks in nectary secretory cells [7,205], but this hypothesis has not yet been experimentally tested.
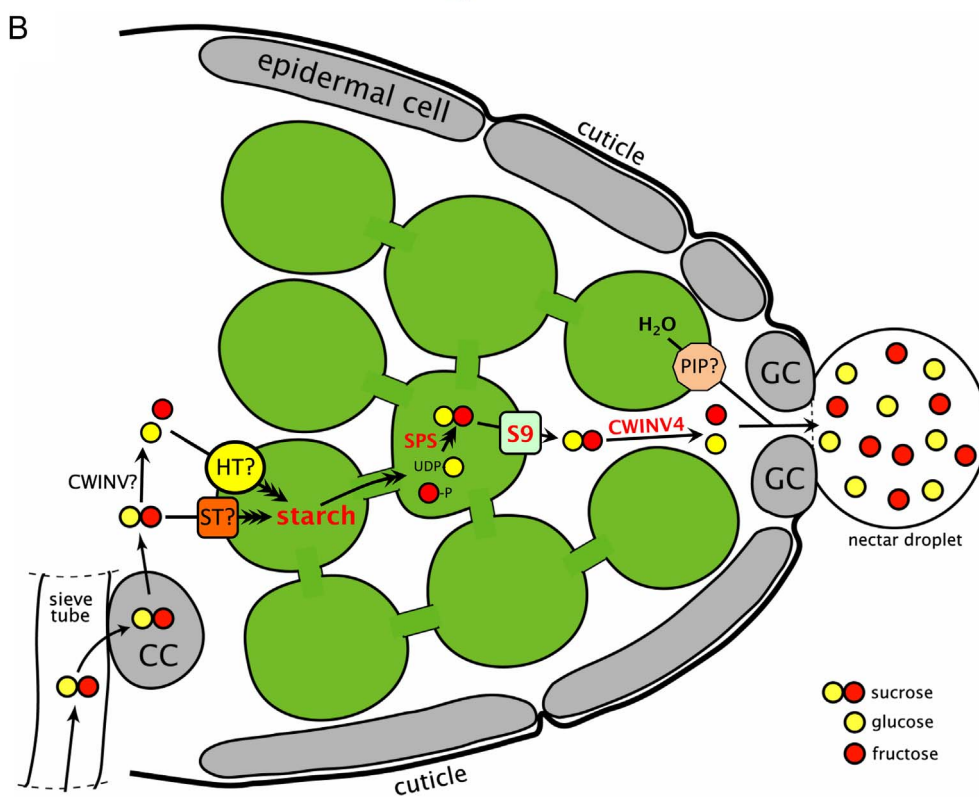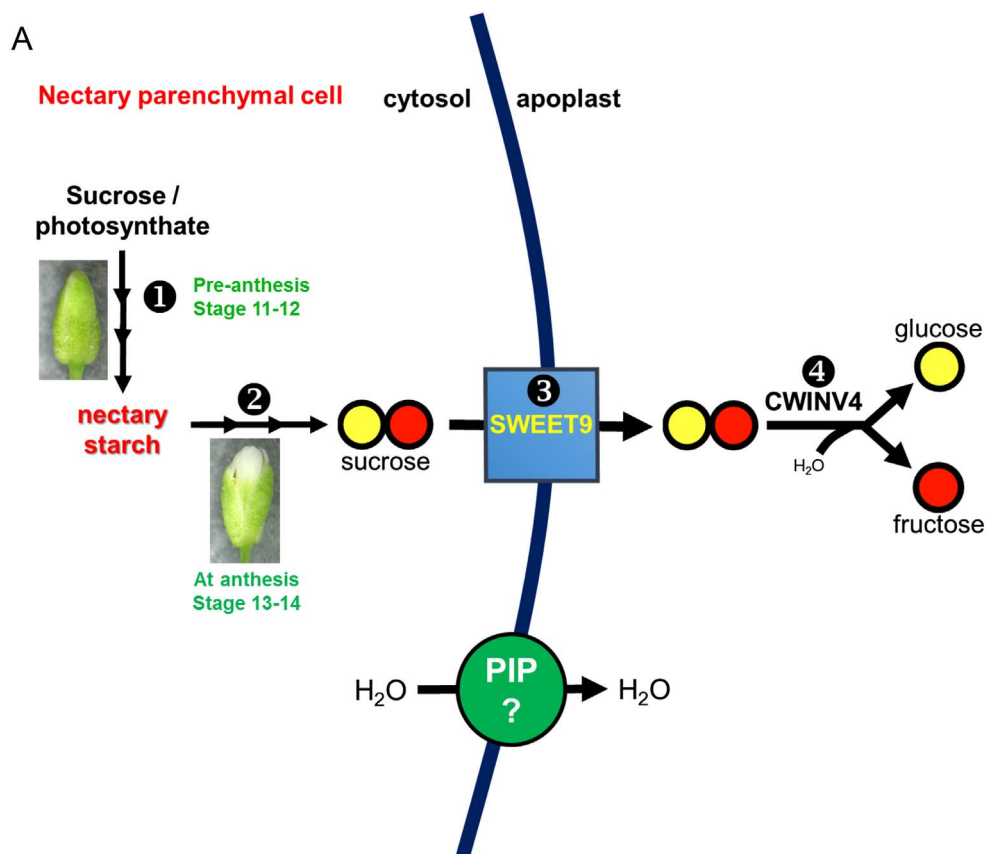
(caption on next page)

Fig. 2. Proposed mechanism for eccrine-based nectar production by a floral nectary.

(A) Sucrose synthesis, export, and extracellular hydrolysis from a single nectary parenchymal cell. (1) Floral nectaries from many families accumulate starch up until anthesis. While nectaries are largely sink tissues [224,227,228], it has been postulated that green nectaries may be capable of *de novo* photosynthesis [218]. (2) At anthesis, starch in the parenchyma is broken down and synthesized into sucrose. Arabidopsis nectaries silenced for sucrose biosynthesis do not produce nectar [232]. (3) Sucrose is subsequently exported out of the cell by the uniporter SWEET9. *sweet9* mutants do not produce nectar [232]. (4) Once outside of the cell, sucrose is hydrolyzed into glucose and fructose by the apoplastic CELL WALL INVERTASE4 (CWINV4), thereby maintaining a constant sucrose gradient and negative osmotic potential [229]. It is unclear if aquaporins (plasma membrane intrinsic proteins, PIPs) play an important role in the rapid movement of water across membranes in nectaries. Like *sweet9*, *cwinv4* flowers do not produce nectar.

(B) Schematic model for nectar secretion within the context of a floral nectary that has a proper parenchyma, epidermis, and stomates. Sucrose is transported to the nectary via the phloem. It is unclear if sucrose is directly imported into the parenchyma by a sucrose transporter (ST?) or immediately hydrolyzed into hexoses by an invertase (CWINV) and imported by a hexose transporter (HT?). Regardless, the subsequent sugars are deposited as starch up until anthesis. The starch degradation products UDP-glucose and fructose-6-phosphate are then converted into sucrose by the action of sucrose-phosphate synthase (SPS) and sucrose phosphatase. The subsequent export and extracellular hydrolysis of sucrose by SWEET9 (S9) and CWINV4 occurs as in image A, with final secretion occurring through open guard cells (GC). Steps known to be required for FN production are shown in red.

5.2.3. Eccrine secretion

Another model for nectar production is eccrine-based secretion, which relies on plasma membrane-localized pores and transporters to export nectar metabolites from parenchymal cells. The eccrine model of nectar secretion is perhaps the most supported by recent literature, at least in terms of the bulk flow of sugars. Specifically, evidence strongly suggests that nectary starch is degraded at anthesis and sucrose is subsequently re-synthesized from those sugars by sucrose phosphate synthases (SPS) and other enzymes [232]. These sucrose molecules are then exported into the apoplast by the plasma membrane-localized sucrose uniporter SWEET9 [232]. At the extracellular space CELL WALL INVERTASE4 (CWINV4) cleaves the sucrose into the hexose monomers fructose and glucose [229]. This invertase action has two effects: (1) it creates a constant driving force for sucrose export, and (2) it creates a negative water potential (one disaccharide molecule hydrolyzed into two monosaccharides) causing water to move towards the sugars, thereby forming nectar droplets [229]. Mutants lacking or silenced for *SPS*, *SWEET9* or *CWINV4* do not produce nectar, whereas over-expression of SWEET9 leads to a ~300% increase in total nectar [232]. Given the intensive metabolic demands of this secretory process, it is not surprising that nectary parenchymal cells are also known to contain high numbers of mitochondria [188,201,202].

An alternative way to evaluate the finding described above is that SWEET9 and CWINV4 are involved in maintaining nectary sink status (i.e. involved in sucrose import from the phloem rather than export), however, *sweet9* nectaries still appear to accumulate high levels of starch, suggesting it is involved in moving sucrose out of nectary cells. The apparent conservation of SWEET9 across species that make FN, and an analysis of the evolution of the SWEET family of transporters, suggest that the working model of nectar secretion shown in Fig. 2 was a key advent in the rapid radiation of flowering plants [232].

It should be noted that each of the three models for nectar secretion mentioned above (apoplastic, merocrine/granulocrine, and eccrine) are not necessarily mutually exclusive. Indeed, discerning between granu-locrine- and eccrine-type secretion is not easy, and both mechanisms have been suggested to occur in different species [202,203]. Complicating these analyses is the fact that simultaneous apoplastic flow of sugars supplied from sieve tubes, without prior storage in amyloplasts, cannot be excluded [231]. Thus, these mechanisms could be acting in tandem with one another, particularly with respect to the export of different metabolites from secretory cells. For example, most nectars studied to date contain arrays of specifically secreted defensive proteins as discussed in Section 3.2.2. The selective secretion of proteins from eukaryotic cells is almost exclusively dependent on vesicular-based trafficking [233]. Therefore, even if the bulk of nectar solutes are transported via eccrine secretion, merocrine-based processes are still likely important factors in producing 'mature' nectar.

5.2.4. Holocrine secretion

Lastly, an apparently rare type of nectar secretion, holocrine, involves the programmed cell death of nectary parenchymal cells followed by rupture of the plasma membrane and the overlying cuticle (reviewed in Ref. [191]). Examples of this type of secretion include the

floral nectaries of soybean [234] and one type of extrafloral nectary of poplar [169].

5.3. Nectar reabsorption

Nectar reabsorption is an understudied subfield of nectar biology and is a well-debated topic for its physiological and ecological under-pinnings. A study of alfalfa nectaries using ¹⁴C labelled sucrose was one of the first to suggest that nectar does get reabsorbed [235]. Since then, multiple studies have implicated nectar reabsorption as being required for resource recovery [231,236], which is logical since nectar is an energy-rich resource. Studies utilizing micro-autoradiography have demonstrated that sugars are reabsorbed by nectaries, even as they continue to produce nectar [175,236–239]. This observation suggests a dynamic equilibrium in the regulation of nectar composition. The reabsorption of nectar is also suggested to have ecological roles since it can allow modulation of nectar composition and concentration, thus allowing phenotypic plasticity according to pollinator visitations [219]. An intriguing study in *Grevillea robusta* led to the suggestion that nectar reabsorption takes place after the volume of nectar reaches a certain threshold [240]. While nectar reabsorption studies have centered on floral nectaries, a recent study utilizing confocal imaging of a fluor-escent dye suggests that reabsorption also occurs in unicellular and multicellular secretory trichomes of extrafloral nectaries [193].

The occurrence of nectar reabsorption is reasonably well documen-ted, but the cellular mechanisms through which this happens are not understood. Possible mechanisms could center around sugar sensing and signaling in the nectary cells [236], intertwined with sensing other environmental cues, which could then trigger nectar reabsorption via a currently unknown set of pores and transporters. Detailed molecular studies investigating trafficking pathways in nectary cells would yield interesting insights into the enigma of nectar reabsorption.

6. Regulation of nectar production

The onset of FN production needs to be carefully coordinated with petal opening, pollen shed, stigma receptivity, and pollinator activity. Similarly, in many cases EFN is only made when the plant is either under attack by herbivores or when it is colonized by predatory mutualists [47,84]. Then it is not surprising that phytohormones and downstream responses play significant roles in regulating the function of both floral and extrafloral nectaries. The relative impacts of jasmonic acid (JA), auxin (IAA), gibberellins, and a key transcription factor, *NtMYB305/AtMYB21*, on nectary function are described below.

6.1. Jasmonates and NtMYB305/AtMYB21

Jasmonoyl-L-isoleucine (JA-Ile), the active form of JA, signals through COI1-JAZ co-receptor complexes to control key aspects of plant defenses and development [241]. JA-Ile is best known for its role in plant responses to herbivory. Not surprisingly, both wounding and exogenous JA application induce EFN production [83]. Cell wall invertase activity, which is required for FN secretion in Arabidopsis

[229], is induced in response to JA-treatment in both *Ricinus communis* and *Acacia cornigera* [17,242].

Recent studies have also demonstrated that JA is involved in developmental and maturation processes, particularly in flowers (e.g. [216,243,244]). For example, JA levels peak in *Brassica napus* flowers just prior to anthesis, which is coincidental with the onset of nectar production [245]. Exogenous application of phenidone, a chemical inhibitor of JA synthesis, reduced nectar secretion, whereas exogenous JA treatment increased it [245]. Not surprisingly, tobacco flowers silenced for JA synthesis and response do not produce nectar and appear to have altered starch utilization [16,230]. We have also found that Arabidopsis JA synthesis mutants do not secrete nectar and this phenotype can be complemented by exogenous application of methyl jasmonate (MeJA) (Schmitt et al., in preparation).

Related to the points above, the JA-responsive transcription factor *NtMYB305* is required for nectary maturation and nectar secretion in tobacco [224,230,246]. Tobacco lines silenced for *NtMYB305* fail to accumulate starch and lose expression of several nectarins [224,230,246]. *AtMYB21* is the apparent Arabidopsis ortholog of *NtMYB305*, as *myb21-4* flowers also do not secrete nectar and lose expression of *SWEET9* and other genes required for nectar synthesis (Schmitt et al., in preparation).

6.2. Auxin (IAA)

Auxin (IAA) is a key hormone regulating nearly all aspects of plant development, as well as responses to changes in the biotic and abiotic environment (extensively reviewed, e.g., [247–252]). IAA activates transcriptional responses through binding to the TIR1 F-box receptor, which leads to ubiquitin-mediated degradation of AUX/IAA transcriptional repressors, thereby de-repressing Auxin Response Factors (ARFs) and activating auxin responsive genes [253,254].

Auxin was implicated in regulating nectar production as far back as the 1950s, with somewhat contradictory results depending on the species and concentration of exogenously applied hormone [255–258]. However, it is clear, at least in the case of some floral nectaries, that auxin is actively synthesized and metabolized in nectaries beginning at anthesis [259,260]. More recent reports indicate that a number of auxin-related genes display nectary-enriched expression profiles in the Brassicaceae [14,15]. For example, *PIN6*, which encodes an auxin transporter, is a nectary-enriched gene whose expression level is positively correlated to total nectar production in Arabidopsis and *B. rapa* [20]. Wild-type plants expressing the auxin-responsive *DR5:GFP* reporter display intense signal in lateral nectaries beginning at anthesis, whereas there is strongly decreased auxin response observed in *pin6* lateral nectaries [20]. Further, exogenous auxin treatment increased nectar production from 2-to-10 fold in both wild-type Arabidopsis and *B. napus*, but nectar in *pin6* mutants was not increased when treated with auxin [20]. Conversely, the auxin transport inhibitor NPA reduced nectar production in wild-type plants by more than two-fold, but had no significant effect on *pin6* nectaries [20]. Cumulatively, these results identify auxin as a key factor in nectary

function. It has been suggested that auxin induces the secretory process in nectaries rather than the transport of sugar to it [258], although the possibility of exogenous auxin altering subcellular sorting dynamics of PIN6, like has been shown other auxin transporters [258], needs to be explored. Further studies are also needed to understand how auxin signaling initiates in a floral anlagen (organ primordium) and progresses in the nectary pre- and post-secretion. This can be attained through a combination of imaging auxin biosensors and genetic approaches [261]. A potential role for auxin in regulating the activity of EFN has not yet been reported, but should be explored.

6.3. Gibberellins

Gibberellins (GAs) are well known for their roles in regulating stem elongation and seed germination [262]; however, little is understood of their involvement in nectary function. GA signaling occurs through binding to the GID1 receptor, which leads to the polyubiquitination and subsequent proteasomal degradation of DELLA transcriptional repressors (reviewed in Ref. [262]).

Recent results suggest that GA may negatively regulate nectar production. A gene involved in GA catabolism, *GA 2-OXIDASE6* (*GA2Ox6*, At1g02400), is highly expressed in the mature nectaries of Arabidopsis and *Brassica* spp., but at very low levels in immature nectaries and most other tissues. GA 2-oxidases catalyze the oxidation of C19 gibberellins leading to the inactivation of bioactive GAs [22]. Multiple mutants for *GA2Ox6* produce 40% less nectar than wild-type plants, whereas overexpression of GA2ox6 in nectaries increases nectar [22]. Consistent with these results, nectar output is restored to near wild-type levels in *ga2ox6* flowers treated with the GA synthesis inhibitor paclobutrazol. Lastly, *ga2ox6* flowers have a decrease in expression of genes involved in nectar production, including *CWINV4* and *PIN6*, as well as a significantly lowered nectary auxin response.

The results described above suggest that GA negatively regulates nectary function via control of the auxin response pathway, but this is contradictory with a prior report from the mid-1980′s that *Brassica napus* flowers treated with exogenous GA₃ displayed large increases in nectar production and pollinator visitation [263]. However, GAs have also been reported to inhibit nectary maturation [264], and the timing, concentration, location, and mode of application can muddle the interpretation of such studies. Clearly, more research on the impacts of gibberellins in nectary function is needed. Like auxin, a potential role of gibberellins in EFN production has not yet been reported.

6.4. Coordinated control of nectar secretion

There are a number of well-known interactions between IAA, JA and GA in plants, both in terms of homeostasis and downstream response. It is therefore difficult to study the role of these hormones in nectary function in isolation. A multitude of signaling factors thus needs to be considered when building a model for hormonal control of nectar production. In Arabidopsis flowers, for example, IAA acts through ARF6 and ARF8 to induce JA synthesis, leading to the
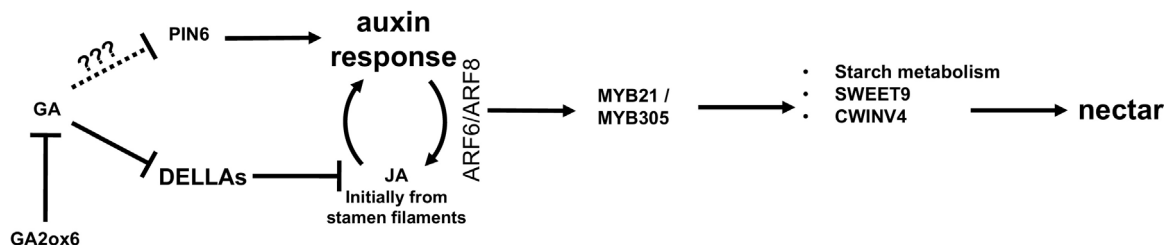
Fig. 3. Proposed model of floral nectary regulation. GA induces JA production in stamen filaments [265–267], which likely diffuses to anthers to induce dehiscence, as well as to nectaries to induce auxin production in a positive feedback loop [243]. In turn, IAA may induce ARF expression and lead to MYB21/MYB305 expression [243], which are required for the transcription of downstream players in nectar production, including SWEET9 and CWINV4 (Schmitt et al., in preparation). GA also inhibits PIN6 expression and auxin response in nectaries [22], possibly to modulate auxin and JA levels and ultimately nectar production.

expression of *MYB21* and *MYB24*, which together promote stamen and petal growth, floral maturation [243] and control the expression of genes required for nectar production (Schmitt et al., in preparation). Less clear is how GA fits into this pathway, as the flowers of GA deficient mutants have reduced levels of JA, lower expression of *MYB21/24* [265–267] and do not produce nectar (Carter, unpublished observation), yet the presumably elevated levels of GA in *ga2ox6* lead to lower levels of *PIN6*, *CWINV4*, and nectar production. Taken together, a rudimentary model of nectary regulation can be proposed as shown in Fig. 3.

Plants undergoing herbivory have systemic JA-dependent responses, such as EFN secretion, but the exact source of JA in flowers is unknown. One clue may lie in the finding that DAD1, a lipase required for linolenic acid liberation from membranes (the first step in the octadecanoic pathway) is solely expressed in elongating stamen filaments of Arabidopsis [244]. *dad1* flowers have short filaments, non-dehiscent anthers [244], and do not produce nectar (Schmitt et al., in preparation). We speculate that stamen filaments are the primary source of JA in flowers during the latter stages of maturation and that this JA diffuses upward to induce pollen shed and downward to induce nectar secretion. Such a model would actively coordinate nectar production with reproductive processes.

These cumulative findings suggest that the hormonal basis of floral nectary secretion is under the control of a complex signaling pathway involving transcriptional factors and levels of hormones that change as the nectary develops and matures. Understanding the process in its entirety would involve studying gene expression and key players in hormone biosynthesis and signaling pathways in a spatiotemporal manner throughout the flower. Moreover, interactions of IAA, JA and GA with the other hormones such as ethylene, ABA, brassinosteroids and salicylic acid will also need to be considered if a robust model of nectar production is to be proposed. It is likely that extrafloral nectaries may have a simpler mode of regulation, perhaps being solely dependent on endogenous JA levels.

7. Open questions and speculation about nectary biology

Many general aspects of nectary biology are deserving of much more research. These include features of nectary development, regulation, and modes of nectar production **across taxa**, as well as how non-sugar metabolites are produced and influence mutualist behavior and health, some specifics of which are discussed above. While not comprehensive, some additional specific needs, open questions, and speculation pertaining to each of these areas are highlighted below.

- The working model of eccrine-based FN secretion shown in Fig. 2 has strong support from currently available data, at least for floral nectaries, but significant open questions and problems with this model persist. If the nectaries of *sweet9* and *cwinv4* flowers still accumulate starch, **how are phloem metabolites, and in particular sucrose, transported into nectaries? And how is this sink status maintained?** To address this question, the expression profiles of nectaries during the starch-filling stage (pre-anthesis) will need to be examined to identify potential candidates.
- **Do aquaporins play a role in nectar secretion?** Nectar secretion is clearly an osmotically-dependent process. Aquaporins facilitate the rapid movement of water across membranes, but it unclear if they play a role in nectar secretion. There is evidence of nectary-enrichment in the expression of some aquaporins in Arabidopsis [15].
- Some nectaries are green and capable of performing photosynthesis *in vivo* [218]. Then **to what extent are green nectaries dependent on phloem for carbon?** Phloem feeding experiments with labelled markers may be helpful in teasing apart carbon coming from the phloem versus direct fixation by nectaries.
- **How is a sucrose-rich nectar produced within the context of the working model of eccrine-based secretion shown in** Fig. 2**?** Some nectars have high levels of sucrose and little or no hexoses (e.g. [268]), which necessitates the removal of CWINV4 from the eccrine-based secretory process. Then the major problem with the working model shown in Fig. 2 lies in the finding that SWEET9 appears to be a uniporter [232], which by definition would be dependent solely on concentration gradients to facilitate the movement of solutes across lipid bilayers [269,270]. It is possible that the secretory cells produce and maintain such a high concentration of sucrose that a constant driving force is maintained, but this would mean the cells would need to endure prolonged periods of cytosolic sucrose > 1 M (the equivalent of a 'typical' 35% sucrose-rich nectar (w/v)). It is possible that SWEET9 activity is somehow modulated, perhaps by post-translational modification or membrane potential, to favor sucrose export over import. It should be noted that the potential for sucrose reformation from hexoses post-exudation cannot be excluded, although a molecular mechanism for apoplastic sucrose synthesis has not been reported in plants.
- **How are non-sugar metabolites secreted into nectar?** The sole protein demonstrated to be directly involved in the transport of solutes into nectar is SWEET9, a sucrose uniporter [232]. But as nectar is much more than simple sugar water, how then are non-sugar solutes delivered into nectar? These include classes of compounds that tailor nectar to corresponding pollinators, prevent microbial growth, and limit nectar thievery as discussed previously. It is therefore imperative to examine mechanisms of how these non-sugar metabolites are synthesized and transported into nectar. Recent analyses of nectary transcriptomes could provide potential targets for future studies [14–16].
- **Do stomates play a role in the regulation of nectar secretion?** Stomates are the pores from where nectar is secreted in a majority of species. As such, one could certainly envision stomatal aperture as a means to control nectar release, as is used to manage gas exchange in leaves and some hydathode secretions [174]. These stomates are assumed to be 'modified' and permanently open, but it appears that this assertion has been experimentally tested in a single species, *Vicia faba* (e.g. with (+)ABA treatments and across development) [196–198]. It would therefore be worthwhile to expand such pioneering studies to other species, as well as to extrafloral nectaries.
- **How do trichome-based nectaries secrete nectar?** While a majority of nectaries have a proper parenchyma, epidermis, and stomates, some nectaries utilize patches of secretory trichomes to produce nectar [171]. Microscopic examinations of *Abutilon* nectaries suggest a symplastic route of 'pre-nectar' into their secretory trichomes [271], but it is unclear if an eccrine-based mode of secretion, similar to that proposed in Fig. 2, may be involved in sugar efflux at trichome apices. Importantly, an anion channel that is permeable to both nitrate and chloride *in vitro* has been implicated in EFN secretion by one type of extrafloral nectary with secretory trichomes in poplar [272]. The authors assert that this channel, which is highly expressed in bipolar secretory trichomes, may function in a similar manner to brush border cells in animals. The function of brush border cells depends on ion flow into the extracellular space, followed by osmosis, to initiate and maintain the secretion process. It is unclear if a homolog of SWEET9 or some other mechanism of sugar export would participate in this system.
- **To what extent are the mechanisms of nectar production conserved across species?** The current working model for nectar secretion shown in Fig. 2 primarily comes from studies on the floral nectaries of three eudicots: Arabidopsis, *Brassica rapa*, and tobacco. Therefore a phylogenetic evaluation of nectaries across species and nectary type (floral versus extrafloral, stomatal versus trichome-based) would be highly valuable. Of note, to date no reports exist on gene expression in the nectaries of any monocot, even though they can be prevalent in some lineages.
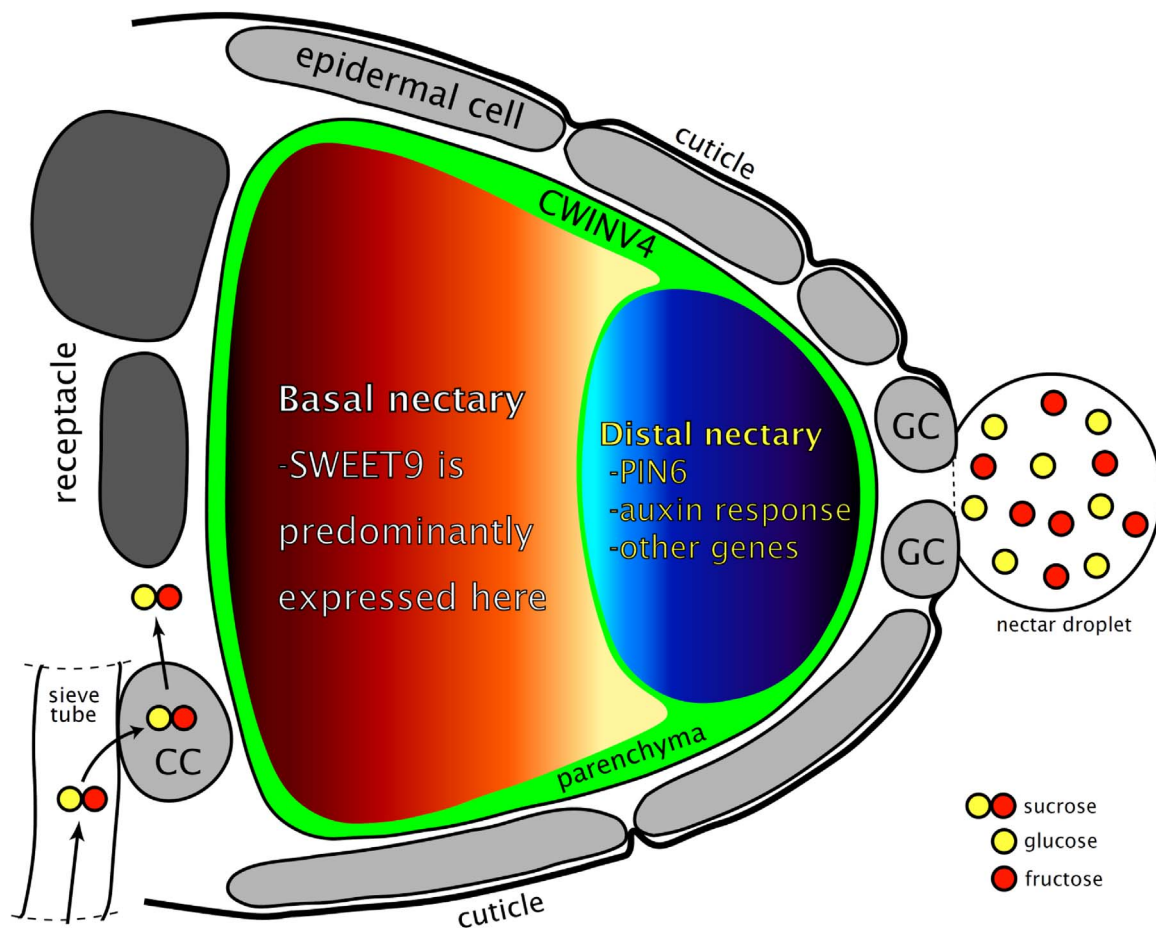
Fig. 4. The nectary parenchyma may have sub-domains. Arabidopsis CWINV4 is expressed throughout the mature nectary parenchyma [229], whereas SWEET9 is primarily found in the basal parenchyma closest to the phloem supply [232]. Conversely, PIN6, which is involved in auxin transport, is exclusively expressed in the distal region of the nectary nearest the stomates from where nectar is secreted [20]. PIN6 expression closely overlaps with the auxin response in mature, open flowers (Stage 15) in the distal nectary region, as monitored by DR5-based reporters [20].

- **What about mechanisms of EFN secretion?** As mentioned before, floral nectaries follow a set ontogenetic pattern of nectar secretion, whereas EFN is often inducible by herbivory, which is unpredictable. It is not surprising then that starch accumulation is not commonly reported in extrafloral nectaries, including those of bean (*Ricinus communis*) and Acacia [17,242]. If an extrafloral nectary from a given species does not store starch, it needs to rapidly induce sink status to obtain sugars from the phloem. Indeed, both cell wall invertase activity and subsequent nectar secretion are inducible in bean nectaries by herbivory and jasmonic acid [242]. Still, these nectars contain metabolite profiles and proteins that are distinct from those found in the phloem. Thus, it is still unclear if apoplastic, merocrine, or eccrine routes of nectar secretion are used to produce EFN.

- **Does the nectary parenchyma contain functional sub-domains?** While dozens of Arabidopsis genes display enriched expression in nectaries [15], it has recently become clear that there are temporal and spatial differences in the expression of these genes. For example, *CWINV4* is expressed evenly throughout the mature lateral nectary parenchyma [229], whereas *SWEET9* and *PIN6* display distinct differences (Fig. 4). *SWEET9* is primarily expressed in the basal region of the nectary closest to the phloem supply [273], but since *sweet9* nectaries still accumulate starch, it is unlikely that it is involved in maintaining sink status pre-anthesis (Stage 11–12). Conversely, *PIN6*, which is involved in auxin transport, is exclusively expressed in the distal region of the nectary nearest the stomates from where nectar is secreted [20]. Interestingly, the auxin response in mature, open flowers (Stage 14–15) is only observed in

the distal nectary region (at the nectary tip), which closely overlaps with *PIN6* expression [20,259]. It is currently unclear what roles these parenchymal subdomains may play in nectar production, but one could envision a scenario where basally-localized SWEET9 exports sucrose into the extracellular space, which in turn is hydrolyzed into hexoses by CWINV4 toward the distal end of the nectary, thereby creating a polar flow of sugars and water through the stomates.

- **How do lipids influence nectar function?** Recent studies have focused on the effects of proteins, amino acids and SMs on biotic interactions mediated by nectars. Lipids have been found in the nectar of a variety of plant species, some of which are known to be important for the health of pollinators. To date, almost no reports have examined how nectar lipids may influence pollinator preference or maintain the overall ecological function of nectar. Honey bees are believed to primarily obtain lipids through pollen, but it would not be surprising if nectar provided an additional source of lipids. Alternatively, free fatty acids, as observed in nectars, can have direct antimicrobial activities [113].

- **What are the synergistic, or antagonistic, effects of non-sugar compounds on pollinator visitation?** The majority of studies that have explored the function of non-sugar compounds in nectar have taken a reductionist approach by only examining one type of compound. In nature, these compounds occur as a purposeful mixture of solutes to attract mutualists and deter non-mutualists. Future studies should aim to elucidate how these compounds work in conjunction to provide the necessary ecological functions of nectar.

- **What subcellular dynamics are involved in nectar secretion?** Fixed and sectioned nectaries imaged by light and electron microscopy have provided significant insight into nectary ultrastructure and have suggested mechanisms of nectar production. However, the use of modern cell biology approaches to understand nectaries has been woefully inadequate. For example, as nectar production depends, at least in part, on vesicular-based secretory processes, membrane trafficking dynamics needs to be deciphered in spatio-temporal detail, particularly *in vivo*, to enable a better understanding of the events that lead up to nectar production.
- **What are the impacts of nutrient status and GxE interactions on nectar production?** It would be surprising if nutrient and water status did not have large impacts on nectar production in a given genotype. Similarly, gene-by-environment interactions would be expected to affect nectary function. These topics have received some attention (e.g. [274,275]), but certainly warrant further research on a broader scale, both in terms of species and environmental parameters studied, as well as the identification of specific loci responsible for any observed variation within populations.

8. Concluding remarks

While there have been remarkable advances in the understanding of nectaries and nectars, the field is still understudied and would benefit from the expanded use of interdisciplinary approaches that range from molecules to ecosystems. Groups have independently investigated aspects of nectary structure and development, mechanisms of nectar production, the molecular biology of nectaries, and nectar chemistry and its role in biotic interactions in select model systems. However, holistic and coordinated efforts to conduct comparative studies across species and nectary types have been lacking. Technological advances in 'omics' techniques will likely provide important candidates for future study, ranging from the identification of unique nectar solutes to target genes involved in their synthesis and secretion, as well as whether these mechanisms are conserved among different species and nectary types. But, as the primary function of nectar is to mediate the attraction of mutualists, such studies, for example, should also systematically evaluate the impacts of individual genes on nectar quantity and quality, and how this in turn affects plant-mutualist interactions and subsequent fitness. The field is also in need of much more encompassing studies on the molecular evolution of nectaries. Given the solid foundation laid by others, and the excess number of open questions, it is an exciting time to be a nectar biologist.

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