A Sucrose Transporter and Proper Hormone Response are Essential for Nectary Function in the Brassicaceae

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I would like to acknowledge and thank my advisor, Dr. Clay Carter, for all of his help and support throughout my undergraduate and graduate career.

Thank you, Clay.

Dedication

I would like to dedicate this thesis to my friends and family.

Abstract

Nectar is a reward presented by flowers to attract pollinators to facilitate fertilization. While much is known about the chemical make-up of nectar, little is known about the mechanisms of production and secretion of this pollinator attractant. *SWEET9*, a nectary enriched gene, was demonstrated to be vital for nectar production in two Brassicaceae species, *Arabidopsis thaliana* and *Brassica rapa* as determined by *SWEET9*pro::*GUS* histochemical staining and RT-PCR,. The Arabidopsis mutant *atsweet9-3*, produced no nectar and three independent mutants in *B. rapa*, (*brsweet9-1*, *-2*, and *-3*) similarly produced no nectar. All four mutants had normal nectary morphology. Transporter assays of SWEET9 expressed in *Xenopus* oocytes displayed sucrose uniport activity, suggesting a direct role in sugar export.

To determine a potential mechanism for the regulation of *SWEET9* expression, the plant hormone jasmonic acid (JA) was investigated because it was previously implicated in nectary function. Indeed, JA synthesis (*aos-2* and *dad1*) and response (*myb21-4*) mutants displayed an absence of floral nectar, in addition to male-sterility. When treated with exogenous MeJA, *aos-2* and *dad1* mutants regained their nectar production and fertility, while the *myb21-4* transcription factor mutant was insensitive to treatment. Significantly, *SWEET9* expression was strongly decreased in the JA response mutant *myb21-4,* in addition to several other genes known to be important in nectary function. For example, all three JA mutants studied displayed decreased expression of *PIN6*, a nectary enriched gene required for proper auxin homeostasis in the nectaries of Arabidopsis. Additionally auxin response was lost in the JA synthesis mutant *aos-2*, suggesting an important hormonal crosstalk between JA and auxin.

To further investigate the link between JA and the auxin response in nectaries, mutants with altered endogenous auxin levels were created. Mutants with decreased nectary auxin produced 50% less nectar than wild-type plants and had reduced auxin response. Cumulatively, these results identify SWEET9 as a sucrose transporter required for nectar production and that JA plays a major role in the regulation of nectary-specific genes and other hormonal pathways important for nectar production.

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CHAPTER 1: Introduction

Introduction

Pollinator visitation is vital for the reproductive success of many flowering plants. Due to a plant's inability to move to find a mate, they often (up to 88% of all floweiring plants) rely on pollinator visitation for reproduction (Hoffman et al., 2003; Ollerton et al., 2011). This makes the attraction of these pollinators imperative to a plant's reproductive fitness, and a major factor in this attraction is nectar (Simpson and Neff, 1983). Floral nectar is an aqueous food source that is presented by many angiosperms as a reward to pollinators for visitation. However, nectar is not only "sugar water" as typically believed, but is a highly complex solution and often diverse between species (Baker and Baker, 1975), suggesting an intricate plant-pollinator co-evolution has occurred for at least some species (Pauw et al., 2009).

Nectaries and Nectar

Nectar

Nectar can vary in its carbohydrate concentration, from 8-80% (w/w) depending on the species (Baker and Baker, 1983). Sucrose is the dominant sugar in some nectars (sucrose-rich) such as in Lamiaceae, alternatively nectar may be hexose-rich as in the Brassicaceae, where the dominant sugars are a combination glucose and fructose (Baker and Baker, 1982a; Davis et al., 1998). These variations in nectar sugar type and concentration can have an influence on pollinator preference, with sucrose-rich nectars typically visited by humming birds, butterflies and long-tongued bees, whereas hexose rich nectars are visited by flies and short-tongued bees (Baker and Baker, 1983).

Floral nectar contains many different solutes in addition to sugar and water. Nectars can contain varying amounts of alkaloids (Adler et al., 2006; Baker and Baker, 1975), amino acids (Baker and Baker, 1983; Carter et al., 2006), flavonoids (Truchado et al., 2009), lipids (Baker and Baker, 1975; Kram et al., 2008), minerals (Varassin et al., 2001), nectar proteins (or nectarins) (Carter and Thornburg, 2000; 2004), organic acids (Baker and Baker, 1975), phenolics (Baker and Baker, 1983), and terpenoids (Raguso, 2004). These solutes serve a wide array of purposes for the plant aside from attraction of preferred pollinators such as an increased desirability and nutritional level of the nectar, or the deteration of unwanted pollinators as well. For example, honeybees have shown preference to artificial nectars that are rich in the amino acid proline (Carter et al., 2006). The constituents may also prevent microbial growth and pathogen protection. For example, proteins isolated from leek floral nectars prevented pathogen growth and infection (Peumans et al., 1997). In 2007, Carter et al. demonstrated that the hydrogen peroxide produced in nectar was toxic to various plant pathogens. Another function of nectar solutes is to ward off potential nectar robbers or ineffective pollinators (Adler, 2000; Baker and Baker, 1983). In 1977, Crane demonstrated that the nectar of *Tilia* flowers, if fed to honeybees, killed the bees. Later it was determined that the sugar mannose was the solute responsible for the lethality to bees (Crane, 1978). In 2005, Adler and Irwin, studied the presence of alkaloids in nectars and found that presence of alkaloids had little impact on female reproductive success in *Gelsemium sempervirens* compared to male reproductive success suggesting a potential greater cost than benefit for the plant.

Nectaries

Nectar is synthesized in, and secreted from, small organs called nectaries. There are two types of nectaries, floral and extrafloral. Extrafloral (or extranuptial) nectaries are located away from the flowers and not involved in pollination (Bently et al., 1977). Nectar secreted from these extrafloral nectaries typically attracts ants, which defend the plant from herbivorous insects (O'Dowd, 1979). While extrafloral nectaries are an interesting aspect of plant biology, they are not the true focus of this study. Floral nectaries are typically a small mass of cells composed of three main cell types, (i) epidermal, (ii) parenchyma, and (iii) vascular (Figure 1). Epidermal tissue, the outer most layer contains modified stomata, which are permanently opened, these modified stomata are referred to as "stomates" (Davis and Gunning, 1992). The bulk of the nectary is composed of parenchyma cells and are the location of nectar synthesis. Lastly, the vascular tissue, containing both xylem and phloem or only phloem, services the nectary parenchyma with phloem sap containing the disaccharide sucrose and other metabolites (Baum et al., 2001); however, the nectaries of some species contain little or no vasculature, such as the Brassicaceae.

Nectar Secretion

Phloem is the suggested source of most nectar sugars, this sap may be referred to as pre-nectar (Pacini and Nepi, 2007). Pre-nectar must first be unloaded from the phloem into the nectar parenchyma cells (Figure 1). Once in the parenchyma, pre-nectar may follow

two potential routes through the parenchyma cells, (i) the symplastic route, where pre-nectar moves between cells through plasmodesmata, or (ii) the apoplastic route, where the pre-nectar migrates via the intercellular space. Two potential methods of secretion of sugars out of the parenchyma have been suggested as well. In eccrine secretion, the sugars are directly transported out of the cells across the cell membrane via hypothetical sugar transporters. In granulocrine secretion, vesicles are loaded with sugars. These vesicles, derived from the endoplasmic reticulum or Golgi, fuse with the cell membrane, releasing sugars from the cell via exocytosis. The sugars then travel out of the nectaries via the permanently opened stomates.

As mentioned previously, Brassicaceae nectar is predominantly made up of glucose and fructose, while phloem sap is sucrose rich. This contrast between nectar and phloem sugars suggests a modification of phloem sugar into nectar sugars. Indeed, nectaries have been shown to have a build up of starch granules prior to anthesis and floral opening (secretion typically begins at anthesis), which are then broken down at anthesis to provide sugars for nectar synthesis (Nepi et al., 1996; Ren et a., 2007). One gene that may be significant for the production of starch in nectaries is *CELL WALL INVERTASE4* (*AtCWINV4*). The protein CWINV4 cleaves sucrose into its two hexose monomers glucose and fructose, and it is the glucose that is converted into starch molecules. Mutations to *cwinv4* lead to a lack of nectar secretion and decreased starch accumulation in the nectary tissue (Ruhlmann et al., 2010). CWINV4 may also play a second role in the secretory process, where it may cleave sucrose exported by eccrine or

granulocrine secretion into hexoses, thereby maintaining a sucrose gradient as well as osmotic potential (water flow out of nectary parenchyma cells).

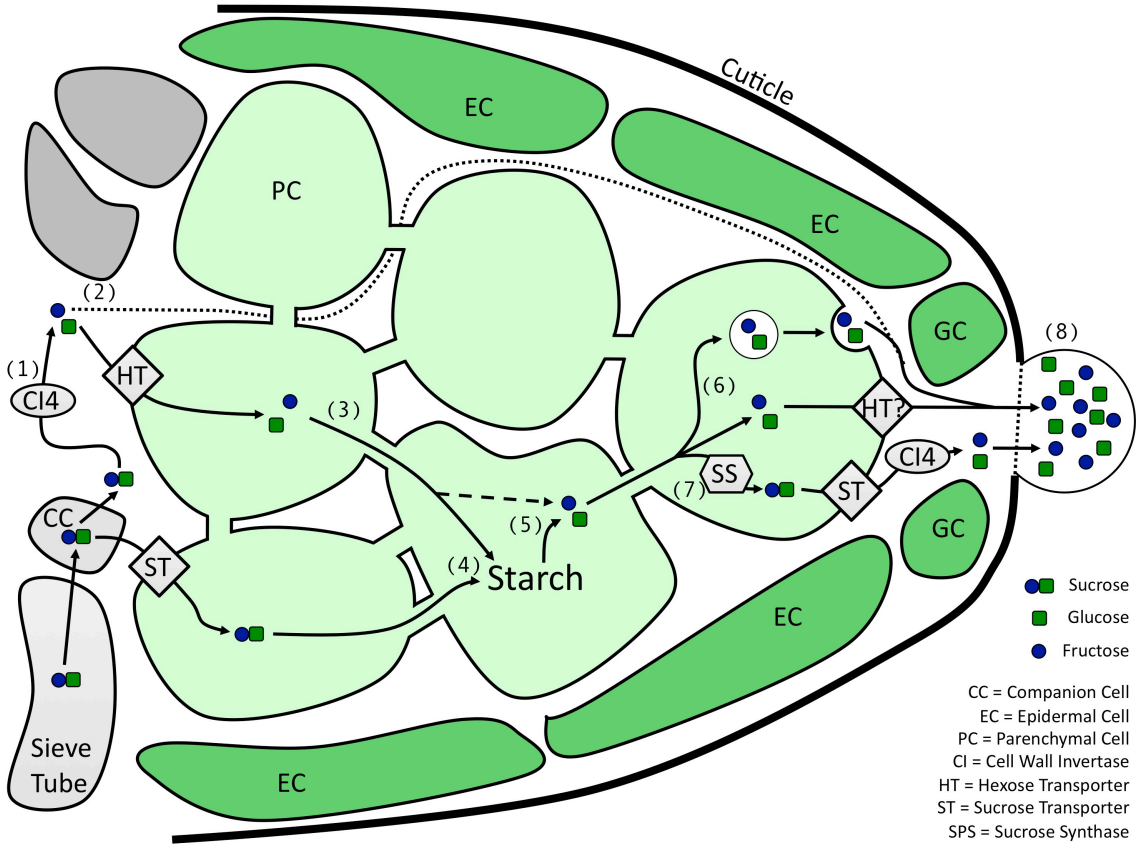

Figure 1: Nectary ultrastucture and proposed model of nectar synthesis. Nectaries are serviced with sucrose in the form of phloem sap. (1) Once in the nectary, the sucrose can be hydrolyzed into hexose sugars (glucose and fructose) by the extracellular invertase, CELL WALL INVERTASE 4 (CWINV4). (2) The hexose sugars may travel via the nectary apoplast moving between cells and out of the permanently open stomata, or (3) the sugars may be transported into the parenchyma cells and travel symplastically via the plasmodesmata. (4) After entering the parenchyma cells, the hexose sugars may be synthesized into starch, a molecule used in long-term storage of sugars. (5) Prior to anthesis and nectar secretion, the starch is broken down, where the sugars may (6) be packaged into vesicles derived from the endoplasmic reticulum or golgi apparatus, and these vesicles fuse with the plasma membrane releasing their contents, or the hexose sugars may be exported via a hexose transporter. (7) Conversely the parenchymal hexose sugars may be resynthesized by a sucrose synthase back into sucrose, where an unknown sucrose transporter transports the sucrose and these sugars are then hydrolyzed by CWINV4. (8) The hydrolysis of sucrose and transport of hexose sugars into the extracellular space creates a high solute environment that drives water and sugar out of the nectary stomates, which forms the hexose rich nectar (Figure adapted from Kram and Carter, 2009).

Model Systems for This Study

Two model species from the Brassicaceae family used in this study were *Arabidopsis thaliana* and *Brassica rapa*. This family includes the agricultural crops broccoli, cabbage, cauliflower, turnip, and canola. Arabidopsis and *B. rapa* flowers have four total nectaries, two lateral nectaries and two median. Lateral nectaries are located at the base of the short stamen and median nectaries are located at the base of the long stamen (Figure 2). The differentiation between these lateral and median nectaries stems from variations in size, nectary ultrasturcture, and activity. Lateral nectaries are larger compared to median nectaries, they are serviced by more vascular sieve tube elements and secrete approximately 96-100% of the total floral nectar produced (Davis et al., 1996; Davis et al., 1998). It is suggested that the increase of nectar output of lateral nectaries is due to the heavy service of phloem tubes to the lateral nectaries. However, the substantial amount of vasculature does not extend directly into the parenchyma, instead the vasculature is separated from the nectary by one or more layers of cells, referred to as the nonglandular or subglandular parenchyma that the sap must travel through to reach the nectary parenchyma (Durkee, 1983) The nectary vasculature of the Brassicaceae is supplying the nectary only with phloem sap since it lacks xylem (Baum et al., 2001; Davis et al., 1986).

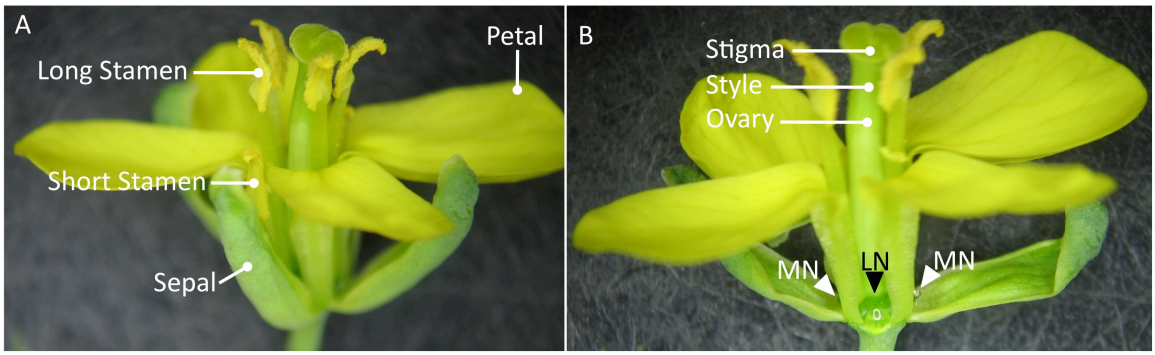

Figure 2: Floral anatomy of *Brassica rapa*. (**A**) Whole flower anatomy of *B. rapa* flower. (**B**) *B. rapa* flower with one sepal and one short stamen removed to expose lateral and median nectaries (LN: lateral nectary, MN: median nectary), the second lateral nectary on the opposing side of the flower is not visible.

Arabidopsis is highly self-pollinating; however, field studies have shown it is visited by pollinators, and not solely reliant on self-fertilization. It is suggested that this small amount visitation may lead to a small but important level of out crossing within the studied populations (Hoffmann et al., 2003). Arabidopsis has a fully sequenced genome and a large suite of mutants mapped to specific genes available through stock centers. Further, Arabidopsis has a very short life cycle, is compact, and is very easy to transform. Microarray chips for Arabidopsis have been available since ca. 2000, which allowed Kram et al. (2009), to conduct global transcriptome profiling of gene expression in nectaries at different stages of development and against control tissues from the rest of the plant, providing valuable data related to any nectary-specific genes that may be involved in nectar secretion. Genes highlighted in this microarray varied in function ranging from predicted sugar transporters and metabolic genes, as well as transcription factors, hormonal regulators and genes related to lipid metabolism (Kram et al., 2009). A draw back to the use of Arabidopsis as a model organism of nectar secretion is that, even

though the nectaries are fully functional (producing and secreting nectar), the nectaries are quite small and produce very little nectar.

B. rapa shares many similar traits to Arabidopsis, including floral structure and ontogeny of nectar production. Between the two species there is 85% similarity in their genomes. This similarity gives rise to the potential of orthologous genes to be determined, and Hampton et al. (2010), were able to use expressed sequence tags to determine differential gene expression in nectaries and correlate over 4,000 orthologous genes to Arabidopsis. *B. rapa* follows the same four-nectary morphology, and again the lateral nectaries produce the majority of the floral nectar. *B. rapa* does however have limited genetic resources available, and transgenic lines are much more difficult to produce.

Nectary Development in The Brassicaceae

Nectary development is a research area of great importance, but has been little studied. Due to floral nectaries' location among the floral organs, nectaries have been implicated to be under control of the so-called ABC homeotic genes. ABC homeotic genes regulate the development of various floral tissues, and it is the various combinations of genes expressed that lead to the development of different floral tissues (Bowman et al., 1992; Weigel and Meyerowitz, 1994). In Arabidopsis, single, double or triple mutations of ABC genes did not lead to the absence of nectaries, however some mutants experienced malformed nectaries (Baum et al., 2001). *CRABS CLAW*, a gene encoding a transcription factor required for nectary development, when constitutively expressed did not rescue the

malformed nectaries of the ABC mutants, suggesting multiple important factors mediate nectary development (Bowman and Smyth, 1999; Baum et al., 2001). The transcription factors *BLADE-ON-PETIOLE1* (*BOP1*) and *BOP2* have also been implicated as transcription factors required for the development of nectaries as well, but their exact role is unknown (McKim et al., 2008).

Sugar Metabolism & Transporters Potentially Involved In Nectar Production

Both the eccrine and granulocrine models of nectar secretion are dependent on putative sugar transporters. Due to nectar's high level of carbohydrate (Baker and Baker, 1983), it is understandable that sugar metabolism and transport proteins may play a significant role in nectar production. The microarray study conducted by Kram et al. (2009) confirmed this suggestion with a suite of sugar transporters, invertases and sucrose phosphate synthases being upregulated in nectary tissue. Aspects of these genes are covered in later chapters

Hormonal Regulation of Nectar Production

The plant hormones jasmonic acid and auxin have been previously implicated in proper nectary function and development, and their respective influences are discussed below.

Jasmonic Acid

The plant hormone jasmonic acid (JA) has been associated with inducible plant defense, growth and development. Interestingly, JA regulates floral reproductive organ development, specifically anther and pollen development (Ishiguro et al., 2001; Laudert et al., 1996). Many mutants deficient in JA biosynthesis or response display male-sterility. This phenotype may occur via three potential characteristics, (i) insufficient filament elongation, (ii) improper pollen maturation, or (iii) defective anther dehiscence (Ishiguro et al., 2001; Laudert et al., 1996; Reeves et al., 2012). In JA biosynthesis mutants, this male-sterility can be rescued by exogenous JA treatments. However, in JA response mutants such as *myb21* and *coi1*, treatments with JA does not rescue sterility (Mandaoker et al., 2006, Reeves et al., 2012).

Interestingly, JA has also been found to modulate extrafloral nectar secretion. Exogenous treatment with JA on *Marcaranga tanarius* leads to the secretion of extrafloral nectar (Heil et al., 2001). Furthermore, leaf wounding, which induces JA biosynthesis, can lead to the secretion of this extrafloral nectar in a wide array of species (Heil et al., 2004; Ness, 2003; Mondor et al., 2003). JA also impacts floral nectar in *Brassica napus*. Treatment of flowers with JA leads to an increased level of nectar production (Radhika et al., 2010). Radhika et al., 2010 also showed that endogenous levels of JA peak in flowers just prior to nectar secretion. In many male-sterile plants, without JA defects (a phenotype associated with JA deficiencies), nectar secretion is altered as well (Baum et al., 2001). It is suggested that there may be a close correlation between anther dehiscence and nectar production due to the importance of pollinator attraction and visitation occurring when the anthers are fully developed and are

presenting pollen. JA has also been implicated in cross talk with the plant hormone auxin. For example *AUXIN RESPONSE FACTOR6* (*ARF6*) and *ARF8*, are both induced by auxin, and initiate JA biosynthesis that leads to the expression of the transcription factor *MYB21* (Reeves et al., 2012). Additional aspects of JA biosynthesis and response are outlined in Chapter 3.

Auxin

The phytohormone auxin [also known as indoleacetic acid (IAA)] has been determined to be a key hormone in nectar production and secretion. Arabidopsis plants containing the auxin responsive promoter DR5 fused to the reporter gene *GUS*, display reporter activity lightly in the nectaries of young flowers, but activity increases as development progresses and pollination occurs (Aloni et al., 2006). Microarray data profiling nectary tissue determined many auxin related genes having nectary-enriched expression (Kram et al., 2009). *PIN6,* of the auxin efflux carrier family, was one of the enriched genes. Significantly, *PIN6* expression positively correlated with nectar amount (Kram et al., 2009; Bender et al., 2013). Also, exogenous treatments with IAA on Arabidopsis flowers lead to a 10-fold increase in nectar sugar, while treatments with the auxin transport inhibitor NPA lead to a reduction in nectar secretion (Bender et al., 2013). These data implicated auxin as a key regulatory factor of nectar secretion.

Study Impact

As mentioned previously, nectar has a large role in plant-pollinator relationships. Many studies have been conducted linking nectar carbohydrate content with pollinator preference and visitation. Baker and Baker (1982a; b) examined nectar sugar compositions i.e. sucrose-rich or hexose-rich and the pollinators that visit those different compositions. They showed that certain pollinators prefer certain nectar sugar ratios. Along with nectar carbohydrate content, nectar volume has been studied by both Baker and Baker (1983), and Cruden et al., (1983). Cruden et al. (1983) determined that larger pollinators such as bats and birds prefer flowers with larger nectar volumes. However Baker and Baker (1983) determined that these larger nectar volumes did not necessarily lead to higher nectar sugar amounts, and that the larger nectar volumes had a reduced concentration of carbohydrates. Similarly to the decreased sugar concentration in larger volume nectars, amino acid concentration was also lower in these large volume nectars. It was hypothesized that the nectar does not need to be high in protein because the bats and birds can get their protein elsewhere in their diet (Baker and Baker, 1983).

The interaction between the plants and their corresponding pollinators has a large impact on agriculture as well. Klein et al. (2007) reported that approximately one-third of food crops are animal pollinated. Also three quarters of the leading global crops are reliant on pollinators and that one half of those crops are dependent insects for pollination, including the honeybee (Klein et al., 2007). The importance of insect pollinated crops, particularly honeybee-pollinated crops has greatly increased with Colony Collapse Disorder (CCD). The honeybee (*Apis mellifera* L.) is one of the most

economically important and valuable pollinators (Watanabe, 1994) and CCD has lead to a rapid decline in the honeybee population (vanEngelsdorp et al., 2009). Understanding the mechanism in which these nectars are produced and secreted could lead to manipulating nectar constituents and producing a more nutritious and desirable nectar, therefore a better food source for pollinators like the honeybee. However, when generating transgenic plants one must be concerned with potential issues. Pollinators and their flowers have evolved to form a strong interaction and the alteration of nectar composition may yield poor results. Junior et al. (2008), conducted a study examining alterations in nectar constituents, and determined that alteration of solutes in nectar can affect both pollinator and nectar robber visitations to the flower. Meaning that even that once manipulated, studies must be conducted to determine the desirability of the floral nectar to both pollinators and nectar robbers.

The focus of this study is to determine potential roles of genetic and hormonal factors in nectar production of Arabidopsis. Specifically, *AtSWEET9*, a potential sugar transporter determined to be highly expressed in both the lateral and median nectary tissues (Kram et al., 2009) was studied to determine its molecular function and influence on nectary function. Furthermore, the impacts of the plant hormone jasmonic acid on nectary function and the regulation of nectary specific genes, including SWEET9, were studied. Lastly, this study examined and built on the already characterized effects of auxin on nectar production (Bender et al., 2013). The data generated from this study may provide great insight into the roles genetic and hormonal factors in nectar production and secretion.

CHAPTER 2: *SWEET9* Is A Sucrose Transporter Required For Nectar Secretion In The Brassicaceae

Introduction

Floral nectar is presented to pollinators as an incentive for visitation. This visitation leads to out-crossing, greater genetic diversity and increased fecundity (Cruden, 1972; Mooney and McGraw, 2007; Hoffmann et al., 2003). The visitation rate of pollinators has been shown to be associated with nectar constituents (Baker and Baker, 1973). While plant-pollinator interactions are an important biological relationship, little is known about the molecular mechanisms of nectary function or development. To date, only four genes have been shown to directly affect nectary development: *BLADE-ON-PETIOLE 1 (BOP1), BOP2* (McKim et al., 2008), *CRABS CLAW (CRC)* (Bowman and Smyth, 1999) and *PIN6* (Bender et al., 2013). *CELL WALL INVERTASE 4* (*CWINV4*) (Ruhlmann et al., 2010) and *PIN6* (Bender et al., 2013) have been also been partially characterized and modulate nectar production in the model plant *Arabidopsis thaliana*.

Nectaries and Nectar

Floral nectar is secreted by small organs called nectaries, which in the Brassicaceae (including *Brassica rapa* and *A. thaliana*) are located at the base of the flower underneath the sepals. However this location and structure is not true of all floral nectaries, as floral nectaries may appear in various portions of the flower and may take on many shapes, ranging from one continuous ring of tissue surrounding the style base to flat or cup shaped nectaries. In the Brassicaceae there are two types of floral nectaries, lateral and median (Figure 3). These nectaries differ in both morphology and activity. Lateral nectaries are larger organs, supplied by more extensive vasculature (i.e. sieve tube

elements), and produce 96-100% of the total nectar (Davis et al., 1998). In many plant families, including the Brassicaceae, nectar is secreted through the stomatal openings located in the epidermal tissue of the nectaries (Baum et al., 2001; Davis et al., 1986).

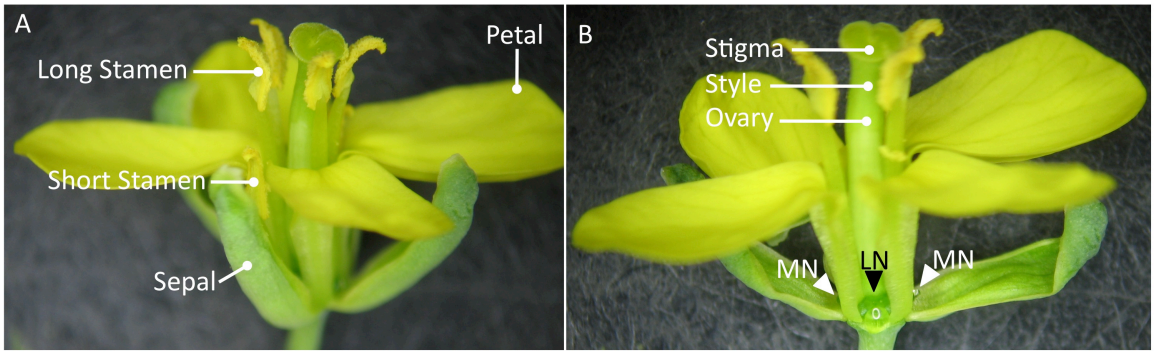

Figure 1: Floral anatomy of a *Brassica rapa* flower. (**A**) Whole flower anatomy of *B. rapa*. (**B**) *B. rapa* flower with one sepal and short stamen removed from the flower to expose the median and lateral nectaries (LN: lateral nectary, MN: median nectary), the second lateral nectary is on the opposing side of the flower and not visible.

Nectar contains many different solutes, which can serve in different functions. In addition to the typical carbohydrates associated with nectar, alkaloids (Adler et al., 2006; Baker and Baker, 1975), amino acids (Baker and Baker, 1983; Carter et al., 2006), lipids (Baker and Baker, 1975; Kram et al., 2008), proteins (Carter and Thornburg, 2000; 2004), phenolics (Baker and Baker, 1983), and terpenoids (Raguso, 2004) have been found in various floral nectars. These compounds not only act as attractants for specific pollinators (Baker and Baker, 1973), but can also inhibit microbial growth (Carter et al., 2007), prevent pathogen infection (Sasu et al., 2010) and ward off nectar robbers (Adler, 2000). While there can be many constituents in nectar, sugars can still make up the majority of nectar solute content, anywhere from 8-80% (w/w) (Baker and Baker, 1983), and are a very important attractant in nectar. In both Arabidopsis and *B. rapa* the nectar produced is high in hexose sugar (glucose and fructose), and low in sucrose (Davis et al., 1998).

While Brassicaceae nectar is hexose rich, other nectars may be sucrose rich, and these variations in sugar distribution can impact a pollinator's preference. Certain pollinators prefer hexose rich nectars, such as short-tongued bees and flies, while butterflies and long-tongued bees tend to visit flowers with sucrose rich nectars (Baker and Baker, 1983).

Mechanisms of Nectar Production

Nectar production in Arabidopsis is thought to begin with sucrose and other metabolites (pre-nectar) being transported via sieve tubes of the phloem to the nectary. Sucrose unloaded from the phloem into the nectaries may travel via two mechanisms through the nectary: apoplasitcally (around cells) or symplastically (through cells). Symplastic movement involves sugars being transported into the nectary parenchyma cells and moving cell to cell via plasmodesmata, where the sugars are converted into starch. Just prior to anthesis and nectar secretion, the starch is broken down and may be resynthesized as sucrose or left as hexose monomers. When the sugars have reached the end of the parenchymal cells (distal nectary), they may be exported via granulocrine or eccrine secretion. In granulocrine secretion sugars are packaged in endoplasmic reticulum or Golgi derived vesicles that fuse with the plasma membrane, releasing their contents into the apoplastic space. In eccrine secretion, sugars may be transported from the cell by either hexose or sucrose transporters at the plasma membrane. The secreted sugars create a high solute environment that drives water out of the nectary via osmosis. A visual model for nectar production is provided in Figure 1 of Chapter 1 on page 6.

The SWEET Transporter Family of Proteins

A class of transmembrane proteins named SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTERS (SWEET) has been recently identified as sugar transporters in plants (Chen et al., 2010). Multiple sequence alignment of *SWEETs* generated four clades (Chen et al., 2010). There are seventeen SWEETs in Arabidopsis; however, the function of only four AtSWEETs have been previously described. AtSWEET1, a clade I SWEET, functions as a glucose uniporter in Arabidopsis (Chen et al., 2010). AtSWEET8, of clade II, is an efflux transporter of glucose involved in pollen nutrition (Guan et al., 2008). Lastly, AtSWEET 11 and 12, clade III SWEETs, are involved in loading sucrose to the phloem at the plasma membrane (Chen et al., 2012). To date, both of the clade IV Arabidopsis SWEETs (AtSWEET16 and 17), have no molecular data on their functions.

Interestingly, a clade III *SWEET*, *AtSWEET9* (AT2G39060), displayed nectary-enriched expression via microarray analysis (Kram et al., 2009). An ortholog of *AtSWEET9* in *Petunia hybrida*, *PhNEC1,* was previously shown to have high expression in the nectary tissue. When *PhNEC1* was overexpressed, plants displayed a decreased level of starch accumulation located in the nectaries and therefore was implicated in nectar sugar transport or metabolism (Ge at al., 2000). In *Brassica rapa*, oilseed rape, another *SWEET9* ortholog was previously identified but a function has not been yet described (Hampton et al., 2010). Given *SWEET9*'s nectary-enriched expression and its putative sugar transport capabilities, it was hypothesized that it plays a role in de novo

nectar production. This chapter will focus on the characterization of *SWEET9* in *Arabidopsis thaliana* and *Brassica rapa*.

Results

SWEET9 Exhibits Nectary-Enriched Expression

AtSWEET9 was previously determined to be highly expressed in nectaries by microarray and RT-PCR analysis (Kram et al., 2009). This expression pattern was verified by cloning the 1.4kb AtSWEET9 promoter into the plant transformation vector pORE-R2, which contains the GUS histochemical reporter gene, to generate pPMK22. Plants transformed with pPMK22 showed strong GUS staining in both median and lateral nectaries (Figure 2a). RT-PCR was subsequently used to confirm the nectary-specific expression pattern of a putative ortholog in *B. rapa*, BrSWEET9 (Figure 2b).

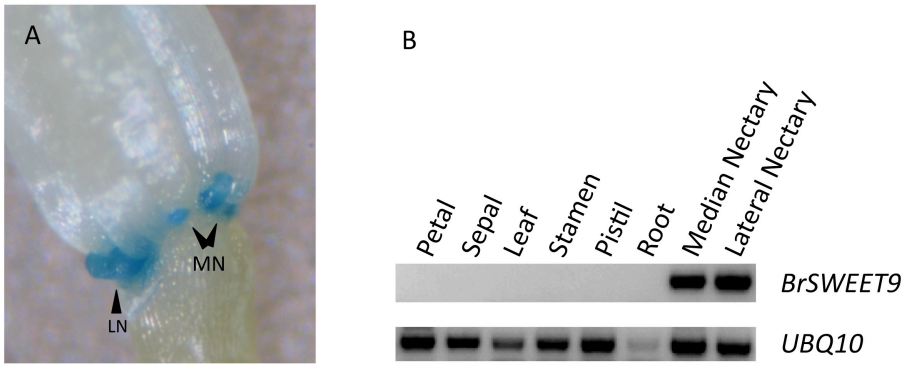

Figure 2: *SWEET9* has enriched expression in nectary tissue in both Arabidopsis and *Brassica rapa*. (**A**) GUS histochemical stain of the transgenic plant line pPMK22, which caries the *AtSWEET9* promoter fused to the GUS reporter gene. GUS activity was found only in the nectary tissue (LN: lateral nectary, MN: median nectary). (Sepals have been removed to expose nectaries). (**B**) RT-PCR screen of *BrSWEET9* in different *B. rapa* tissues. *BrSWEET9* is expressed heavily in the nectary tissues. *UBIQUITIN* (*UBQ10*) was used as an internal control.

SWEET9 is Required for Nectar Production in Arabidopsis and Brassica rapa

To determine the biological function of *SWEET9,* apparently single copy genes in the Brassicaceae, mutants were identified in both Arabidopsis and *B. rapa*. A homozygous T-DNA insertional mutant in Arabidopsis, *atsweet9-3* (SALK_202913C), was confirmed via PCR. The T-DNA insert is located in the fourth exon, position +779bp after the start codon. RT-PCR determined that no transcript was produced in the whole flowers of *atsweet9-3* (Figure 3a). *atsweet9-3* flowers produced no visible nectar droplet, unlike those of wild-type, while maintaining normal nectary morphology (Figure 3b and c).

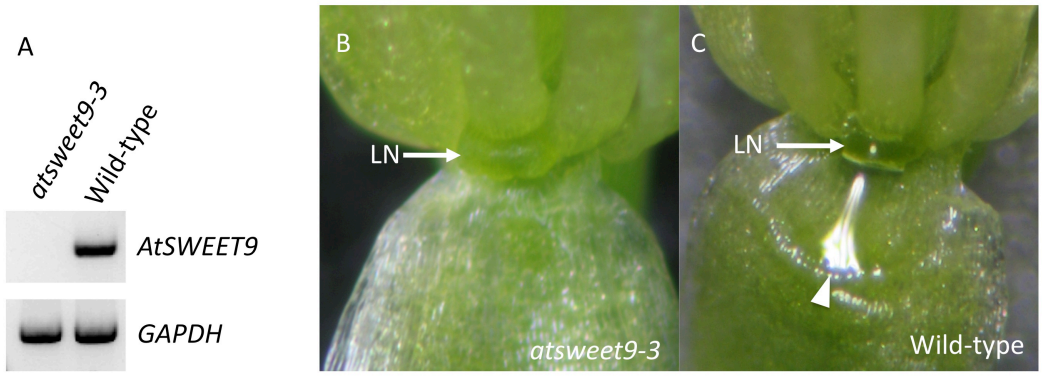

Figure 3: *SWEET9* is required for nectar secretion. (**A**) No *AtSWEET9* transcript was expressed in the T-DNA mutant *atsweet9-3*. *GAPDH* used as an internal standard. (**B**) Lack of nectar droplet in *atsweet9-3*. (**C**) Presence of nectar droplet in wild-type Arabidopsis. White triangle indicates nectar droplet location. (**B** and **C**) Sepals have been pulled back to expose nectary and nectar droplet (LN: lateral nectary).

In *B. rapa* three independent TILLING mutants were characterized. TILLING is a process through which point mutations are introduced throughout the genome via chemical treatment, followed by screening for mutations in the particular gene of interest. *brsweet9-1* contains a point mutation at position +407 from the start codon, generating a premature stop codon in the open reading frame. *brsweet9-2 and 9-3* contain point mutations that disrupt the splice site locations, *brsweet9-2* at position +524 and *brsweet9-3* at position +827 after the start codon of the unspliced pre-mRNA. RT-PCR was used to

examine transcript presence and length in the splice site mutants. *brsweet9-2* produced no detectable transcript in floral tissue (Figure 4a), whereas *brsweet9-3* had decreased expression of the *BrSWEET9* transcript. It should be noted that while *brsweet9-3* had decreased expression of *BrSWEET9*, there was also a larger product produced by RT-PCR, suggesting mis-spliced mRNA (Figure 4a). Direct sequencing and BLAST searches of the *sweet9-3* RT-PCR product demonstrated that both bands were amplified from *BrSWEET9* transcripts and that the larger band contained the unspliced third intron. Significantly, all three *brsweet9* mutants failed to produce any nectar; however, nectary morphology in the *brsweet9* mutants was consistent with those of wild-type plants (Figure 4b-d). Cumulatively, it was concluded that *SWEET9* is required for nectar secretion in both Arabidopsis and *B. rapa*.

Figure 4: *BrSWEET9* is essential for nectar production in *Brassica rapa*. (**A**) RT-PCR expression data in *B. rapa* splice site TILLING mutants. *brsweet9-2* had no visible transcript, while *brsweet9-3* showed decreased expression compared to wild type, and *brsweet9-3* had the presence of larger than wild type band. *UBIQUITIN* (*UBQ10*) was used an internal control. (**B** to **D**) Absence of nectar droplets in *B. rapa* TILLING mutants (**E**) Nectar droplet presence in R-o-18 (wild-type). White triangles indicate nectar droplet location. (**B** to **E**) One sepal and one short stamen have been removed to expose the lateral nectary (LN: lateral nectary).

SWEET9 Exhibits Sucrose Transporter Activity

To determine if SWEET9 functions as a potential sugar transporter, similar to SWEET11 and 12, transport assays using *Xenopus* oocytes were conducted. Uptake was measured by quantifying the amount of ^{14}C-labeled sucrose transported into the oocytes. Oocytes were injected with: full-length transcript cRNA (AtSWEET9 and BrSWEET9), a truncated cRNA fragment (AtSWEET9m and BrSWEET9m as negative controls) or water. Significant differences were observed between the whole and truncated cRNA injected oocytes, for both the Arabidopsis and *B. rapa SWEET9's*, $p < 0.01$ and $p < 0.05$ respectively (Figure 5a). Similar to the uptake assay, sucrose efflux was measured as well. *Xenopus* oocytes were again injected with either whole or truncated cRNA, but also injected with ^{14}C-labeled sucrose. Movement of sucrose out of the cell was measured over time, and again, the whole transcript injected oocytes had greater sucrose efflux activity (Figure 5b). Cumulatively, these data indicate that SWEET9 has sucrose uniport activity.

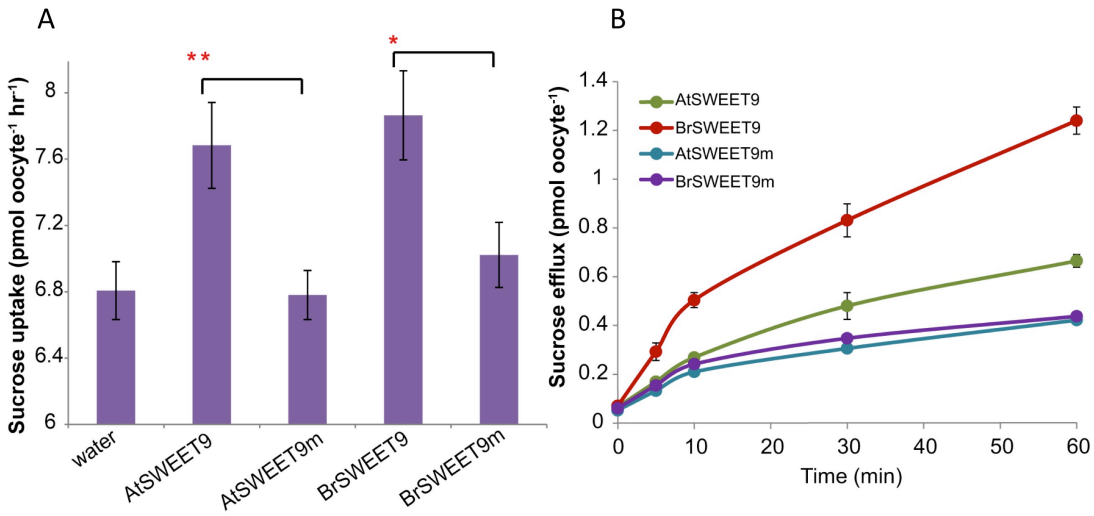

Figure 5: Sucrose transporter activity of SWEET9 in *Xenopus* oocytes. (**A**) Uptake of ^{14}C-labeled sucrose in *Xenopus* oocytes. Significant difference was observed in uptake activity between whole cRNA (AtSWEET9 and BrSWEET9) and truncated cRNA (AtSWEET9m and BrSWEET9m). *P < 0.05 and **P < 0.01 (**B**) Efflux of sucrose out of oocytes expressing whole or truncated SWEET9 having been injected with ^{14}C-labeled sucrose.

Discussion

Nectaries act as sinks for carbohydrates in flowering plants; these sinks are serviced with sugars via the phloem sap, a sucrose rich solution. This sugar may be directly secreted or first stored as starch, then broken down, modified and secreted out of the nectary. The general mechanism of nectar production and secretion (Figure 6) starts with the nectaries receiving sucrose from the phloem cells (Deeken et al., 2002). Sucrose is transported into nectary parenchyma cells via phloem and companion cells, which may then be converted into glucose and fructose by the extracellular invertase CWINV4 (Ruhlmann et al., 2010). Ruhlmann et al. (2010) characterized this nectary-enriched gene *CWINV4*, which hydrolyses sucrose into the hexose sugars glucose and fructose, and suggested that this invertase activity is responsible maintaining the sink status of nectaries. Like *sweet9* plants, *cwinv4* mutants produce no nectar. Once the sucrose has been converted to hexose sugars, it can be stored as starch prior to anthesis (Fahn 1979a, b, 1988; Ge et al. 2000; Ren et al. 2007). Then, just before anthesis, the starch granules are broken down and it is this break down of starch that is suggested to give rise to the sugar in floral nectar (Zhu et al., 1997; Peng et al., 2004; Ren et al., 2007). Next, it is proposed that sugars are moved to the apoplast via two potential mechanisms; (i) eccrine secretion, where either hexose or sucrose sugars are transported across the plasma membrane via sugar transporters or (ii) granulocrine secretion, whereby vesicles containing the hexose sugars fuse with the plasma membrane releasing their contents (Wist and Davis 2006; 2008). Finally, the new

apoplastic sugars and water flow out of the nectaries via the stomatal openings of the nectary (Baum et al., 2001; Davis et al., 1986).

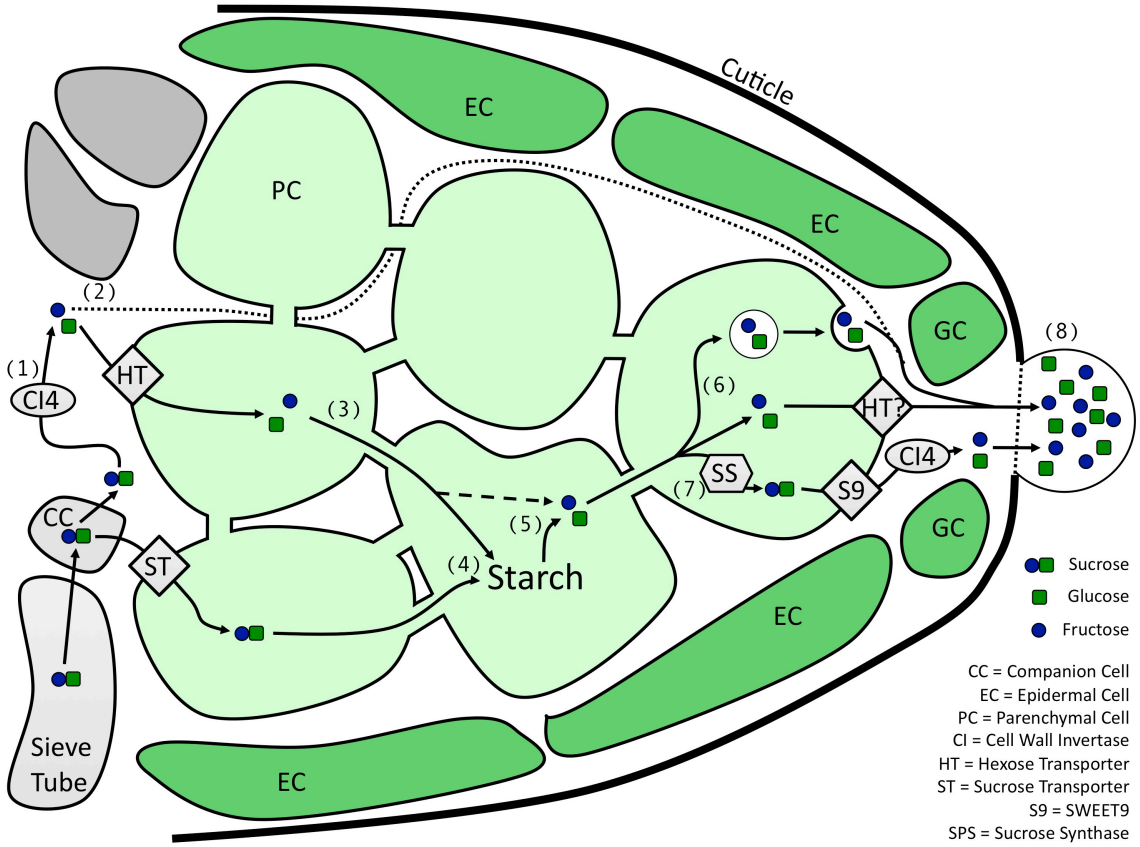

Figure 6: Revised model of nectar synthesis and secretion pathways in the Brassicaceae, involving SWEET9. Sieve tubes of the phloem provide nectaries with pre-nectar i.e. sucrose and other metabolites. (1) Sucrose may be hydrolyzed into glucose and fructose by the invertase, CELL WALL INVERTASE4 (CWINV4) (Ruhlmann et al., 2010). (2) The hexose carbohydrates are either transported apoplastically via extracellular spaces towards the nectary stomates. (3) Alternatively to the apoplastic movement through the nectaries, the then hexose sugars may be transported into the parenchyma via a hexose transporter where they can travel symplastically from cell to cell via the plasmodesmata. (4) As a method of long-term carbohydrate storage, pre-nectar sugars are stored as starch. (5) Just prior to anthesis starch is broken down into its hexose sugars. (6) These sugars may be packaged into vesicles, which fuse with the plasma membrane releasing their contents into the apoplast (granulocrine). Conversely to pre-nectar being transported via granulocrine secretion, hexose sugars may be transported via hexose transporters (eccrine), or (7) hexose sugars may be resynthesized into sucrose where they are transported via SWEET9 and hydrolyzed by CWINV4 back into glucose and fructose. (8) A build up of hexose sugars generates a high solute environment forcing sugars and water out of the permanently open stomates, generating the hexose-rich nectar. Adapted from Kram and Carter 2009.

SWEET9 displayed high expression in nectaries by microarray (Kram et al., 2009), RT-PCR, and reporter gene analysis (Figure 2). The nectary-enriched expression of *SWEET9* suggested its involvement in nectar production or nectary development in the Brassicaceae. Four independent mutants of *SWEET9* from two different species showed no production of floral nectar and had no observable morphological differences in nectary structure or size compared to wild type (Figures 3 and 4). These results implicate SWEET9 as a factor in floral nectar secretion, but not nectary development. SWEET9 also displayed sucrose transporter activity, capable of both uptake and efflux of ^{14}C-labeled sucrose, a chacteristic of uniport trasnporters (Figure 5). In addition to identifying SWEET9 as a sucrose transporter, expression of known plasma membrane located sucrose transporters, *AtSWEET11* and *12*, under the control of the *AtSWEET9* promoter in *atsweet9* mutants and restored nectar production (Lin et al., submitted).

sweet9 mutants had very similar phenotypes to *cwinv4* mutants. *cwinv4* mutants misaccumulated starch in the receptacle and down in to the pedicel. *sweet9* mutants displayed the same mislocalized starch accumulation (Lin et al., submitted). Furthermore Lin et al. (submitted) have shown that histochemical GUS staining in the nectaries is greatest in the proximal parenchyma (less at nectary tips). Ruhlmann et al. (2010) suggested that the mechanism of *CWINV4* in nectar production might be two-fold: (1) CWINV4 drives the movement of sucrose into the nectaries from source tissues, which is then deposited as starch in immature nectaries; and (2) upon maturation nectary starch is degraded, resynthesized into sucrose, exported by a sucrose transporter, and hydrolyzed by CWINV4 in the extracellular space. The break down of sucrose into its hexose

monomers by CWINV4 would create a high solute environment, which draws water out of the nectary. Following this model for CWINV4 activity, it is proposed that SWEET9 acts as a main sucrose transporter in this secretory pathway (Figure 6). We have shown that *SWEET9* follows a similar nectary specific expression pattern as *CWINV4* (Figure 2). Null mutants of *SWEET9* produce no nectar (Figure 3b and 4b-c), and they display similar starch staining patterns (Lin et al., submitted). However, SWEET9 does not exhibit invertase activity, instead it is shown to have sucrose transport activity (Figure 5).

Taken together, these results implicate SWEET9 as a main sucrose transporter involved in active nectar secretion and support the eccrine model of nectar production in the Brassicaceae. Future studies on the secretion of nectar will involve the examination of potential hexose transporters involved in the export of sugars out of the parenchyma. Additionally nectary-enriched, sucrose phosphate synthases will be investigated due to their role in synthesizing sucrose, which may then be transported out of the parenchyma by SWEET9.

Materials and Methods

Plant Growth Conditions

All plants were grown on a peat-based medium with vermiculite and perlite (Pro-Mix BX; Premier Horticulture) in individual pots. The plants were housed in a Percival AR66LX growth chamber with 16h day/8h night, photosynthetic flux of 150µmol m^{-2} sec^{-1} and at 23˚ C.

T-DNA Mutants

Arabidopsis plants, both wild type (Col-0) and *atsweet9-3* (SALK_202913C), were acquired from the Arabidopsis Biological Resource Center at The Ohio State University. Genomic DNA was isolated from whole leaf tissue, and served as template to be amplified with the *AtSWEET9* gene specific primers "*atsweet9-3*", which flank the T-DNA insertion site and the T-DNA specific primer "LBb1.3" to screen for the T-DNA insert according to established protocols (http://signal.salk.edu/tdnaprimers.2.html).

To determine *AtSWEET9* expression in Arabidopsis T-DNA mutants, RNA was isolated from whole flower tissue using Agilent Technologies' Absolutely RNA Miniprep Kit (Catalog #400800). 500µg of RNA was converted to cDNA using Applied Biosystems' High Capacity Reverse Transcription Kit (Catalog #4368814). cDNA transcripts were amplified with the primer pair: "*AtSWEET9* RT-F and *AtSWEET9* RT-R," and ran on a 1% agarose gel to detect the presence of the *AtSWEET9* transcript. *GAPDH* was amplified as well and used as an internal standard using the primer pair "*AtGAPDH* RT-F and *AtGAPDH* RT-R."

SWEET9:GUS Construct (pPMK22)

The 1.4 kb promoter region of *AtSWEET9* was amplified using New England Biolabs Phusion High-Fidelity PCR Kit (Catalog #E0553S) with the "*AtSWEET9* Promoter F-XhoI and *AtSWEET9* Promoter R-BamHI" primer pair. The purified PCR product was digested with the restriction enzymes XhoI and BamHI, and subsequently ligated into the promoterless vector pORE_R2, containing the GUS reporter and Kanamycin resistance

gene. This vector was given the name pPMK22. *Agrobacterium tumefaciens* (GV3101) cells were transformed to carry the pPMK22 vector and used to transform Arabidopsis using the floral-dip method described by Clough and Bent (1998). Transformed seedlings were selected on one half Muarshige and Skoog medium plates with 75µg/ml kanamycin.

GUS Histochemical Staining

Flowers were stained following the GUS staining protocol described by Jefferson et al., 1987.

TILLING Mutants

TILLING mutants were acquired from RevGen UK (John Innes Center, Norwich, UK; http://revgenuk.jic.ac.uk), and each TILLING mutant had a G to A point mutation. The *brsweet9-1* (JI33127-B) had a point mutation at position +407 after the start codon of the open reading from. This mutation generated a premature stop codon. Both *brsweet9-2* and *brsweet9-3* generated splice site mutations, at positions +524 (intron two) and +827 (intron three) of the unspliced pre-mRNA.

Each TILLING homozygous mutant plant was backcrossed to the background line, R-o-18, heterozygous plants were allowed to self-pollinate and the subsequent generation had DNA isolated and amplified using the primer pair: "BrSWEET9 TILLING SEQ F and BrTILLING SEQ R." PCR products were then sequenced via Sanger sequencing to determine homozygous mutants.

RNA was isolated from homozygous mutant whole flowers using Agilent Technologies' Absolutely RNA Miniprep Kit (Catalog #400800). 500µg of RNA was

converted to cDNA using Applied Biosystems' High Capacity Reverse Transcription Kit (Catalog #4368814). cDNA transcripts were amplified with the primers: "nodulin MtN3 Brassica F" and "BrSWEET9 TILLING SEQ R," and ran on a 1% agarose gel to detect the presence of the *BrSWEET9* transcript. *UBQ10* was amplified as well and used as an internal standard using the primer pair "*BrUBQ10* RT F and *BrUBQ10* RT R."

Transporter Assay

This method was followed by the protocol described by Chen et al., 2012. The cRNA injection, oocyte isolation, cRNA synthesis, linearization of the pOO2 vector, ^{14}C-labeled sugar uptake and efflux were carried out as described in Chen et al., 2010. For negative controls, oocytes were either injected with 50nL of RNAse free water, or truncated cRNA transcripts of *SWEET9*. In the efflux assays, 3 days after injection of cRNA, oocytes were injected with 50nL of 10mM ^{14}C-labeled sucrose. Sucrose transport was then measured.

Acknowledgements

I would like to thank Ms. I Lin and Dr. Wolf Frommer of the Department of Plant Biology at Carnegie Institution for Science in Stanford, CA, for performing the sugar transport assays in *Xenopus* oocytes.

Table 1: Oligonucleotides used in this study.

Oligo Name	**Sequence**	**Purpose**
atsweet9-3 F	TGCCGACCAAAATAAAAAGTG	*sweet9-3* genotyping
atsweet9-3 R	ACTTGGCAGATATCCACGTTG	*sweet9-3* genotyping
LBb 1.3	ATTTTGCCGATTTCGGAAC	T-DNA left boarder genotyping
AtSWEET9 Promoter F-XhoI	TTTGGATCCCTTCACTTTTCTCTCTTT	Cloning of *SWEET9* promoter into pORE_R2
AtSWEET9 Promoter R-BamHI	TTTCTCGAGAAAGCTTGTGTCTTGTTC	Cloning of *SWEET9* promoter into pORE_R2
AtSWEET9 RT F	TCTCGCAGTCTTTGCTTCTCCCTT	*AtSWEET9* RT-PCR
AtSWEET9 RT R	GTTTGCCCTTCACTTCATTGGCCT	*AtSWEET9* RT-PCR
AtGAPDH RT F	TTCGGTGAGAAGCCAGTCACTGTT	*AtGAPDH* RT-PCR
AtGAPDH RT R	AAACATTGGAGCGTCTTTGCTGGG	*AtGAPDH* RT-PCR
BRSWEET9 TILLING SEQ F	TGCTTGCAGGCAACATTGTGTCTT	*BrSWEET9* genotyping
BRSWEET9 TILLING SEQ R	CGGCCACGATCGCAACTTCG	*BrSWEET9* genotyping/*BrSWEET9* RT-PCR
nodulin MtN3 Brassica F	TCTTGTCTCCAGTGCCAACGTTCT	*BrSWEET9* RT-PCR
BrUBQ10 RT F	TTGAGGTGGAAAGCTCTGACACGA	*BrUBQ10* RT-PCR
BrUBQ10 RT R	AATCGGCCAATGTACGACCATCCT	*BrUBQ10* RT-PCR

CHAPTER 3: The Hormonal Regulation Of Nectar Secretion In Arabidopsis

Introduction

Nectar is an aqueous food reward presented by flowering plants to aid in the attraction of pollinators. The attraction of pollinators is very important to plant fitness by increasing out-crossing, fecundity, and genetic diversity. A plant's ability to attract pollinators can be directly influenced by its nectar quality. Plant-pollinator interactions play a very significant role in evolution, ecology and agriculture; therefore it is surprising that there is such a lack of molecular data related to the production or secretion of this attractant and reward, nectar. For example, there have only been four genes previously determined to directly alter nectary development, *BLADE-ON-PETIOLE1* (*BOP1*), *BOP2* (McKim et al., 2008) *CRABS CLAW* (*CRC*) (Bowman and Smyth, 1999) and *PIN6* (Bender et al., 2013). Similarly, only three genes have been characterized to effect nectar production in *Arabidopsis thaliana*, *CELL WALL INVERTASE 4* (*CWINV4*) (Ruhlmann et al., 2010), *PIN6* (Bender et al., 2013) and *SWEET9* (Lin et al., submitted).

Nectar and Nectaries

Carbohydrate rich nectar is produced by nectaries, small organs located at the base of the flower behind the sepals. In Arabidopsis and *Brassica rapa*, the flowers contain two pairs of different nectaries called, "lateral" and "median." These organs differ in both morphology and nectar output. The lateral organs are larger, serviced by more sieve tube elements, and produce 96-100% of total floral nectar (Davis et al., 1998). Nectar is

secreted from permanently open stomata called “stomates” located in the epidermis tissue on the nectary tips (Baum et al., 2001; Davis et al., 1986).

Floral nectar in Arabidopsis is high in hexose sugars, glucose and fructose, and low in the disaccharide sucrose (Davis et al., 1998). Nectar, while potentially rich in carbohydrates, up [to 80% (w/w)], can be made up of many different constituents. Floral nectars have been known to contain a diverse array of solutes: alkaloids (Adler et al., 2006; Baker and Baker, 1975), amino acids (Baker and Baker, 1983; Carter et al., 2006), lipids (Baker and Baker, 1975; Kram et al., 2008), proteins (Carter and Thornburg 2000; 2004), phenolics (Baker and Baker, 1983), and terpenoids (Raguso, 2004). These solutes serve a variety of functions aside from attraction of pollinators (Baker and Baker, 1973), such as inhibition of microbial growth (Carter et al., 2007), prevention of pathogen infection (Sasu et al., 2010) and the warding off of nectar robbers (Adler, 2000).

Jasmonic Acid in Nectar Production

Jasmonic acid (JA) is a multifunctional plant hormone involved in a variety of plant processes: anther dehiscence, fruit ripening, root growth, as well as plant resistance to insect herbivory and pathogens and most relevant to this study, nectar secretion (Bennett and Wallsgrove, 1994; Feys et al., 1994; Corti Monzon et al., 2012; Radhika et al., 2010). Both herbivory and exogenous JA application can induce nectar production from extrafloral nectaries (Heil et al., 2001). More recently, Radhika et al. (2010) determined JA levels in *B. napus* flowers peak just prior to nectar secretion, and exogenous application of JA increased nectar production rates as well.

JA Biosynthesis

The synthesis of JA in flowers is located within the stamen filament (adjacent to both nectaries and anthers) and begins with the conversion of chloroplast phospholipids into linolenic acid (LA) by phospholipases, such as DELAYED ANTHER DEHISCENCE 1 (DAD1) (Ishiguro et al., 2001). LA is then further converted in the chloroplast to 12-oxo-phytodienoic acid (OPDA) via three enzymes: LIPOXYGENASE (LOX), ALLENE OXIDE SYNTHASE (AOS) and ALLENE OXIDE CYCLASE (AOC). OPDA is then reduced by 12-OXO-PHYTODIENOIC ACID REDUCTASE (OPR) whose product is β-oxidized three times to generate JA (Stintzi and Browse, 2000). JA can be further modified via methylation to generate the volatile methyl jasmonate (MeJA), which is also a common floral scent produced by plants such as jasmine.

Jasominc Acid Biosynthesis Mutants

DAD1 (*DEFECTIVE IN ANTHER DEHISCENCE 1*) is a phospholipase, which cleaves phospholipids into linolenic acid (LA), the first step of JA biosynthesis (Ishiguro et al., 2001). *dad1* mutants have underdeveloped pollen and unopened anthers, although treatment with MeJA can lead to full pollen maturation and anther dehiscence (Ishiguro et al., 2001). Ishiguro et al., 2001 proposed that JA alters water transport in the vasculature in the anthers, due to the vacuoles of the anther still containing water. And that it is this disruption of water flow that leads to the lack of anther maturation (Ishiguro et al., 2001).

AOS (*ALLENE OXIDE SYNTHASE*) is a cytochrome P450 enzyme (CYP74A) necessary for the production of endogenous JA (Laudert et al., 1996). Mutants of this single copy gene phenocopy *dad1* mutants i.e. have under developed pollen and unopened anthers (Park et al., 2002). Treatment with the *AOS* substrate precursor LA in the *aos* mutant did not rescue the phenotype, but the downstream products OPDA or MeJA were able to restore anther dehiscence and pollen maturation (Park et al., 2002).

JA Response

Jasmonyl-L-isoleucine (JA-Ile), the active form of JA, signals through COI1-JAZ co-receptor complexes. JAZ proteins repress transcriptional activators in the absence of JA. When JA-Ile enters the nucleus its binds to COI1, leading to ubiquitin mediated degradation of JAZ repressors, thereby allowing transcriptional activation of JA-response genes. For example, MYB21, an R2R3 transcription factor (Kranz et al., 1998) has been shown to be involved in JA responses by being induced by JA in Arabidopsis (Mandaokar et al., 2006). MYB21 has been characterized to regulate anther filament elongation as mutants are unable self-pollinate with themselves due to their open anthers' inability reach the stigma (Shin et al., 2002; Mandaoker et al., 2006). Thus *myb21* partially phenocopies *aos* and *dad1* mutants. However, unlike the JA synthesis mutants mentioned above, these phenotypes are not rescued with JA or MeJA treatments.

While JA synthesis and response genes have been greatly implicated in floral development, specifically stamen and pollen development, little work has been done

examining their impact on the process of nectar synthesis or secretion, a process that is temporally linked with anther dehiscence and pollination (Baum et al., 2001).

Auxin in Nectar Production

Bender et al. (2013) investigated the influence of the plant hormone auxin [indoleacetic acid (IAA)] on nectar production in the Brassicaceae. Treatment with the synthetic auxin, NAA, led to a ten-fold increase in nectar sugar in *B. rapa*. While treatments with the auxin transport inhibitor NPA led to a two-fold decrease in nectar. Auxin levels increase in nectaries throughout development and continue to be present in the nectaries throughout pollination and embryo maturation (Aloni et al., 2006). These data lead to the question of how the alteration of endogenous auxin mediates nectar production**.**

The current study was undertaken to examine how nectar production is regulated, specifically in regard to the expression of genes known to be involved nectary function (*SWEET9*, *CWINV4*, *PIN6*). Given that JA can induce nectar production in both floral and extrafloral nectaries, it was hypothesized that JA is required for nectar production through the expression of *SWEET9*, *CWINV4* and *PIN6*. Further, it was also hypothesized that these responses are manifested through a JA-responsive transcription factor, MYB21. Lastly the potential crosstalk between JA and auxin was examined, as well as how the alteration of endogenous auxin impacts nectar secretion in Arabidopsis.

Results

JA is Required for Nectar Secretion in Arabidopsis

JA was previously suggested to be involved in nectar production (Heil et al., 2001; Radhika et al., 2010) To determine a definitive role of JA in nectar production, mutants of two JA biosynthesis genes, *AOS* and *DAD1* and the JA-responsive transcription factor *MYB21*, were identified in Arabidopsis. The *aos* mutant (*aos-2*, SALK_017756) contains a T-DNA insertion in exon 1. *DAD1* is a single exon gene and the T-DNA insert of the *dad1* mutant (SALK_138439) is located in that one exon. The transcription factor *myb21* mutant (*myb21-4*) was previously described (Reeves et al., 2012), as having a single point mutation at position 344 after the start codon in the mRNA sequence; Trp^{116} is converted to a premature stop codon, generating a truncated coding region. All three Arabidopsis mutants produced no nectar while maintaining proper nectary morphology (Figure 1a-c). The mutants also exhibited male sterility (data not shown), which was a previously known phenotype to all three mutants (Ishiguro et al., 2001; Park et al., 2002; Mandaoker et al., 2006). Significantly, exogenous treatment with the methylated volatile form of JA, MeJA, was able to restore nectar production in both synthesis mutants (Figure 1e and f) and rescue the plant's fertility (data not shown). The treatment, however, did not rescue nectar production in the response mutant, *myb21-4* (Figure 1g). Wild-type plants showed no apparent change in nectar volume in response to the MeJA treatment (Figure 1d and h). Thus, it was concluded that JA is required for nectar secretion in Arabidopsis.

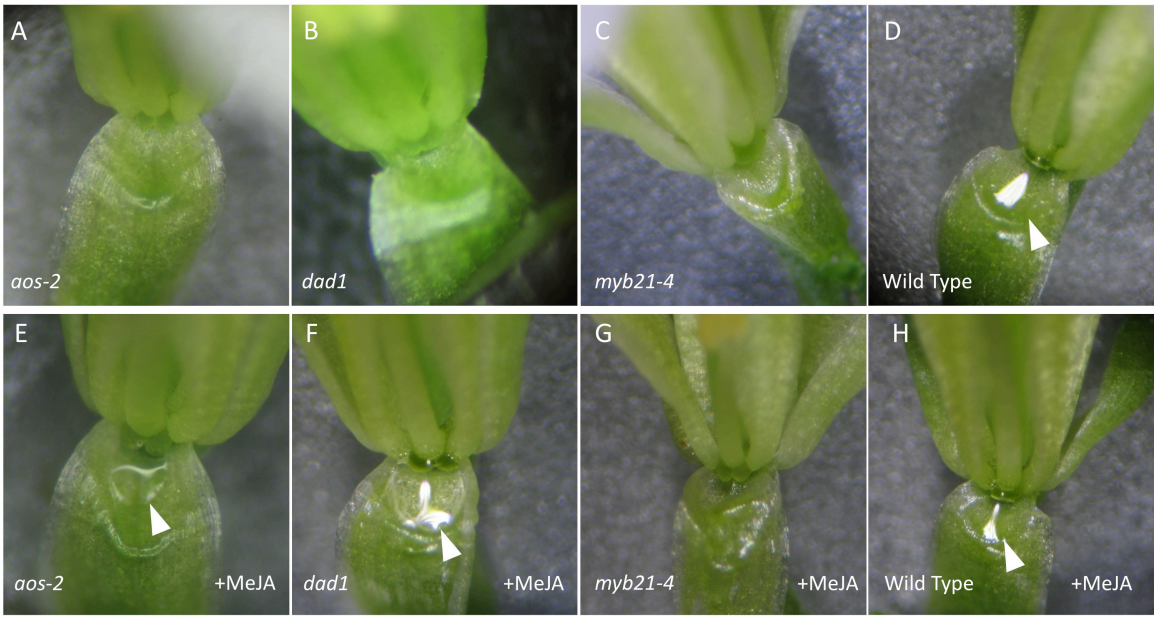

Figure 1: Examination of nectar production in JA synthesis and response mutants, both untreated and treated with exogenous MeJA. (**A** to **D**) The display of nectary and nectar phenotypes in untreated flowers. (**A** to **C**) JA synthesis and response mutants lack nectar droplets while maintaining proper nectary morphology. (**D**) Wild-type flower with a nectar droplet. (**E** to **H**) Flowers treated with MeJA and flower's response related to nectar secretion. (**E** and **F**) Both of the synthesis mutants, *aos-2* and *dad1* responded to MeJA treatment by producing nectar. (**G**) *myb21* mutants did not respond to MeJA treatment. (**H**) Nectar droplet in wild-type flowers treated with MeJA. (**A** to **H**) Sepals have been pulled back to expose nectary and nectar droplet. White triangles indicate nectar droplet location.

Given the altered nectar secretion patterns observed in the JA mutants, the expression of previously characterized genes involved in nectar production was examined. Figure 2a shows the transcript levels of *AtSWEET9*, *AtCWINV4*, and *AtPIN6* in each of the JA mutants. *AtSWEET9*, a sucrose transporter (Lin et al., submitted), had no detectable transcripts in *myb21-4* JA response mutant, while appearing to be normally expressed in the JA synthesis mutants (*aos-2* and *dad1*). *AtCWINV4*, a nectary specific invertase (Ruhlmann et al., 2010), also had strongly decreased transcript levels in *myb21-4*, and retained expression in the JA synthesis mutants. *AtPIN6*, a nectary-enriched auxin efflux

carrier (Bender et al., 2013), displayed decreased expression in all three JA synthesis and response mutants when compared to wild-type.

The reduction of *PIN6* expression in all three mutants suggested a strong link between JA and auxin in proper nectary function. To determine the regulation of *PIN6* in these JA deficient mutants, exogenous treatments with JA were used to examine gene expression of *PIN6*. *aos-2* and wild-type flowers were treated with MeJA and then *PIN6* expression was measured via RT-PCR. The treatment was able to rescue the expression of *PIN6* in the JA synthesis mutant *aos-2* (Figure 2b), similar to the JA treatments effect on nectar production, suggesting JA regulates the expression of *PIN6*.

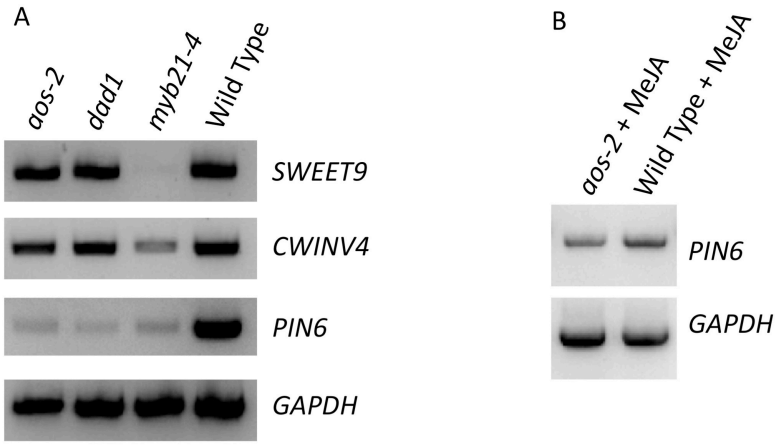

Figure 2: The expression patterns of nectary-specific genes (*SWEET9*, *CWINV4*, *PIN6*) in JA mutants (*aos-2*, *dad1*, *myb21-4*) and wild-type. (**A**) Synthesis mutants *aos-2* and *dad1* displayed similar profiles of examined genes. Wild-type expression of *SWEET9* and *CWINV4* was observed, while *PIN6* expression was decreased. The response mutant, *myb21-4*, displayed little to no expression of *SWEET9* and strongly decreased expression of *CWINV4* and *PIN6*. *GAPDH* was used as an internal positive control. (**B**) Gene expression of *PIN6* from cDNA generated from RNA isolated from whole flowers treated with exogenous MeJA. *PIN6* expression is restored in the biosynthesis mutant *aos-2* to near wild-type levels upon treatment. *GAPDH* was used as an internal control.

Auxin Response is Lost in the JA Biosynthesis Mutant aos-2

Bender et al. (2013) displayed the importance of auxin in nectar secretion. Based on these data of the importance of auxin in nectar secretion it was hypothesized that JA may be involved in a hormonal crosstalk with auxin and therefore an altered auxin response would be observed in the JA mutants. The auxin response was examined in JA mutants via the DR5::GUS reporter gene. The DR5 promoter fused to the GUS reporter gene displays GUS activity in the presence of auxin activity/response. Wild-type plants containing the auxin responsive DR5::GUS reporter gene displayed strong staining in both the median and lateral nectaries (Figure 3). However, in the *aos-2* mutant background containing DR5::GUS, the GUS staining was absent, suggesting a lack of auxin activity/response and that auxin activity/response in the nectaries is regulated by JA. *dad1* mutants carrying the DR5::GUS phenocopied the *aos-2* plants with no auxin activity (not shown).

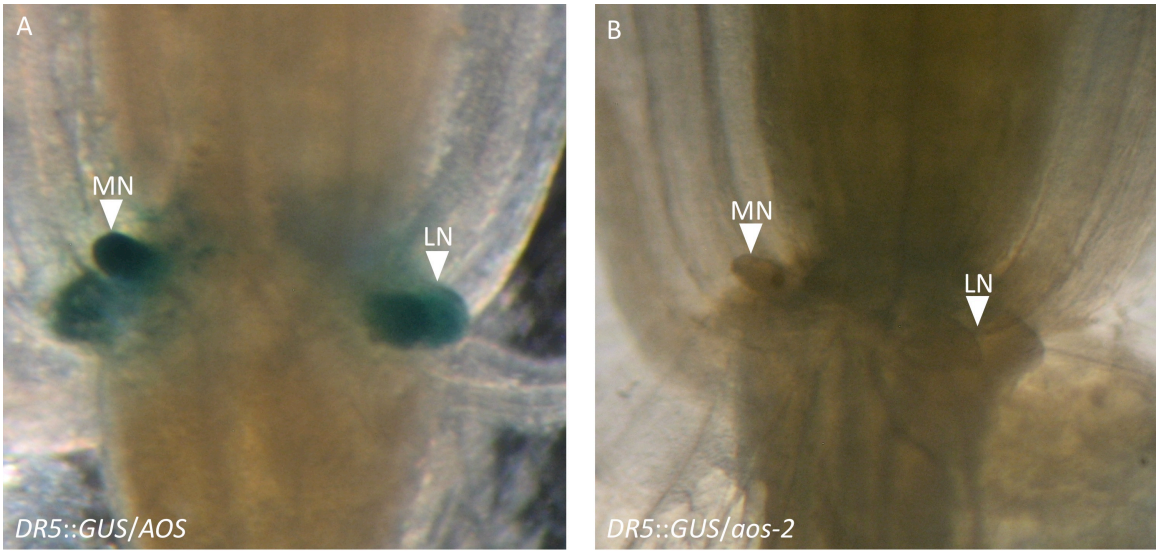

Figure 3: Auxin response is lost in the nectaries of the *aos-2* mutant. (**A**) GUS staining of wild-type Arabidopsis containing the DR5::GUS reporter gene, displayed strong staining within both the lateral and median nectaries. (**B**) GUS staining in the *aos-2* mutant containing the DR5::GUS reporter gene, lacked staining. (**A** and **B**) Sepals have been pulled back or removed to expose the nectaries (LN: lateral nectary, MN: median nectary).

Alterations of Endogenous Auxin Effects Nectar Secretion

Previous studies on the involvement of auxin in nectary function reported a positive correlation between exogenous auxin treatments and nectar sugar amount. Therefore the mediation of endogenous auxin was hypothesized to alter nectar amounts in Arabidopsis. Thus wild-type Arabidopsis plants were transformed with vectors containing a strong nectary specific promoter (*SWEET9pro*) driving expression of the auxin modulating genes *iaaL* and *iaaM*, and called PMK20 and 21 respectively. *iaaL* and *iaaM* have been implicated in pathogen invasion of plants. *iaaL* conjugates a lysine to IAA, which inactivates the auxin leading to a significant decrease in bioactive auxin (Romano et al., 1991). *iaaM* generates an increase in endogenous auxin by promoting the production of IAA **(**Klee et al., 1987). Sugar assays measuring the presence of glucose in the nectar were conducted and the plant line PMK20, containing the *iaaL* transgene, had 48.7±16% nectar compared to wild-type. The PMK21 plant line had a slight but statistically insignificant increase in nectar sugar amounts compared to wild-type (Figure 4a).

Auxin activity was also examined in the PMK20 and 21 plants using the DR5::GUS reporter gene system. GUS staining in the flowers of both wild-type and PMK21 plants produced staining in the nectaries, however, PMK20 flowers had no auxin response in the nectaries (Figure 4b). Thus it was concluded that the presence of the i*aaL* gene in the PMK20 plants altered the auxin response to endogenous auxin within the nectaries.

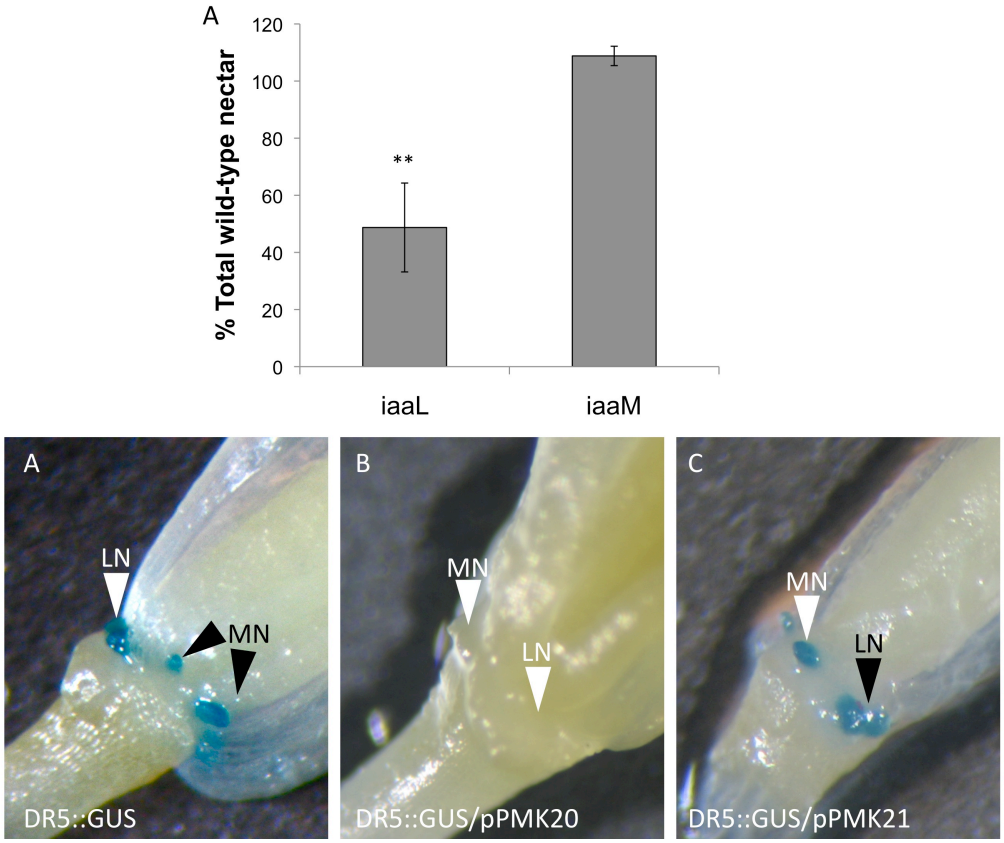

Figure 4: Nectar sugar amounts and auxin response in auxin PMK20, 21 and wild-type plants. (**A**) Nectar sugar amounts of PMK20 and 21 flowers. PMK20 displayed 50% nectar sugar compared to wild-type, ** $p < 0.01$. (**B**) Auxin-responsive GUS staining in the wild-type background containing the DR5::GUS reporter gene. (**C**) Absence of staining in the PMK20 plant background with the DR5::GUS reporter gene. (**D**) Staining from the DR5::GUS reporter gene in the PMK21 background. (**B** to **D**) Sepals have been removed to expose the nectaries. (LN: lateral nectary, MN: median nectary).

Discussion

The regulatory mechanisms of nectar production are largely unknown. Thus, this study was undertaken to determine JA's role in regulating nectar secretion in Arabidopsis. These results demonstrated that JA and its subsequent response are required for nectar secretion in Arabidopsis, and that JA regulates expression of nectary-specific genes previously reported to modulate nectar secretion. The synthesis mutants, *aos-2* and *dad1*, both produced no nectar while still showing proper nectary morphology but no

dehiscence of the anthers, leading to male-sterility (Figure 1a and b). When the flowers were treated with MeJA, the mutants regained nectar secretion and anther dehiscence (Figure 1e and f). *myb21-4*, the JA response transcription factor mutant, however displayed no nectar secretion in both the JA treated and untreated flowers (Figure 1c and g), which supports that proper downstream JA response is necessary for nectary function in Arabidopsis. Interestingly, *AtSWEET9*, *AtCWINV4* and *AtPIN6* all displayed decreased expression in *myb21-4*, whereas only *PIN6* was reduced in the JA synthesis mutants. AtSWEET9 and AtCWINV4 are individually required for nectar production and directly control sugar transport and metabolism, respectively. However, these results demonstrate that expression of these two genes alone is not sufficient for nectar production. Conversely, it appears that proper *PIN6* expression may be required for nectary function via a JA-dependent mechanism. With previous work by Ishiguro et al. (2001), and the data shown here it is proposed that JA may regulate nectar production via two potential mechanisms, (i) regulation of water movement in the nectaries and (ii) crosstalk between JA and auxin-dependent pathways.

Potential for JA Regulation of Nectar Secretion via Control of Water Movement

Proper timing of pollen release and nectar secretion in flowering plants can be vital for reproduction, therefore, there may be a close singular upstream mechanism between anther dehiscence and nectar secretion. Baum et al. (2001) characterized many male-sterile mutants to be deficient in nectar production. This same correlation between anther dehiscence/male-sterility and nectar production was observed in different male sterile

mutants of this study. Ishiguro et al, (2001) determined that water transport was halted in the vascular tissue of *dad1* anthers, and suggested that JA regulates water transport in the male organs. Later, Ruhlmann et al. (2010) suggested that the high amount of nectar sugars leads to the flow of water out of the nectary, creating the nectar droplet presented at the base of the sepal. Perhaps water transport in the nectaries is disrupted by the lack of JA leading to the absence of nectar in the JA synthesis and response mutants, and only when the proper JA response can be restored in the JA synthesis mutants could the water transport/nectar secretion be restored as well (Figure 1). However, the molecular mechanism by which water flow is disrupted to *dad1* stamens, and perhaps nectaries, is currently unknown.

MYB21 Regulation of SWEET9 and CWINV4

An interesting result in our study is the altered expression of *SWEET9* and *CWINV4* in *myb21-4*. *SWEET9* acts as a sucrose transporter, putatively transporting sucrose out of nectary parenchyma cells (see *Chapter 2*; Lin et al., submitted). After sucrose export, *CWINV4* converts this disaccharide into its hexose monomers, glucose and fructose (Ruhlmann et al., 2010). Null mutants for these genes phenocopy one another as neither produce nectar, while maintaining normal nectary structure, and have heavy starch accumulation in the floral receptacle (Lin et al, submitted and Ruhlmann et al., 2010). The regulation of these nectar sugar transporters and invertases is vital for proper nectar production and secretion. Thus, the *MYB21* transcription factor appears to regulate both *CWINV4* and *SWEET9,* in addition to *PIN6*. A potential explanation for the altered

expression of all three genes in only the *myb21-4* mutant and not in the synthesis mutants was shown in Reeves et al. (2012). They found that in the synthesis mutant *aos-2*, *MYB21* was still expressed in flowers by qRT-PCR, albeit at significantly lower than wild-type levels. It is currently unknown if MYB21 directly or indirectly controls *CWINV4* and *SWEET9* expression.

Crosstalk Between JA and Auxin Pathways

There are a number of well-known interactions between auxin (IAA) and JA in plants, both in terms of homeostasis and downstream response. In Arabidopsis flowers, for example, IAA acts through ARF6 and ARF8 to induce JA synthesis, leading to the expression of *MYB21* and *MYB24*, which together promote stamen and petal growth, and floral maturation (Reeves et al., 2012).

With specific regard to auxin in nectaries, the expression level of *PIN6*, a member of the auxin-efflux protein family, was previously shown to have a positive correlation with nectar production (Bender et al., 2013). Further, exogenous treatment of flowers with the synthetic auxin NAA significantly increased nectar sugar content. Conversely treatment with NPA, an auxin transport inhibitor, decreased nectar sugar (Bender et al., 2013). In all three JA mutants investigated, decreased expression of *PIN6* was observed (Figure 2). Having decreased expression of *PIN6* in all three mutants suggests that JA levels and/or response could be regulating nectar production via *PIN6* expression, which in turn alters auxin response levels within the nectary. This alteration of auxin response was also confirmed in the *aos-2* mutant carrying the DR5::GUS reporter gene (Figure 3),

again suggesting a hormonal crosstalk between JA and auxin. The molecular mechanism by which PIN6 impacts nectar production is unknown; however, the nectaries of *pin6* knockout mutants have greatly reduced auxin response (Bender et al., 2013).

Endogenous Auxin's Regulation of Nectar Secretion

Nectar production was significantly reduced in plants expressing of the *iaaL* catabolic gene. PMK20 plants also experienced a decrease in auxin activity (Figure 3), and had a significant decrease in nectar secretion as well, therefore confirming auxin's influence in proper nectary function.

Cumulatively, these results implicate JA as an indispensible regulator of nectar secretion in Arabidopsis. Two potential mechanisms related to JA's role in nectary function are presented: 1) water transport in nectary tissue, or 2) the regulation of auxin response, a known nectar-modulating hormone, via *PIN6*. These two potential mechanisms however may not be mutually exclusive, and both water transport and auxin regulation may be controlling nectar secretion due to the importance of timing between anther dehiscence and nectar secretion.

Based on the data between JA and IAA, we propose the following model (Figure 5b), relating JA and its regulation of SWEET9 and CWINV4 via MYB21, along with its crosstalk with auxin. Further studies will focus on examining this crosstalk and interaction between auxin and JA in nectar secretion, as well as determining the connection between MYB21 and CWINV4 and SWEET9 i.e. does MYB21 directly or indirectly regulate these genes.

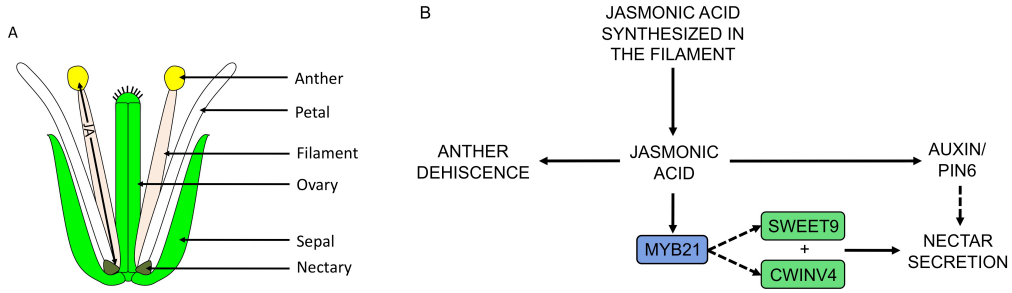

Figure 5: Proposed model of jasmonic acid's migration after synthesis and regulation of nectar secretion. (**A**) JA synthesized in the anther filament migrates down the filament to the nectary to promote nectar secretion, and travels to the anther where it promotes the dehiscence of anthers and pollen maturation. (**B**) JA's involvement in nectar secretion. JA turns on the transcription factor MYB21, which either directly or indirectly turns on SWEET9 and CWINV4, two genes previously determined to be vital for nectar secretion. JA also regulates auxin/PIN6 within the nectaries, another hormonal factor involved in nectar secretion. (Dashed lines indicate uncertainty of MYB21's direct or indirect link regulating SWEET9 and CWINV4, as well as auxin and PIN6's mode of regulating nectar secretion).

Materials and Methods

Plant Growth Conditions

All plants were grown on peat-based medium with vermiculite and perlite (Pro-Mix BX; Premier Horticulture) in individual pots. The plants were housed in a Percival AR66LX growth chamber with 16h day/8h night, photosynthetic flux of 150µmol m^{-2} sec^{-1} and at 23˚ C.

Plant Material

Arabidopsis plants, wild-type (Col-0) and *dad1* (SALK_138439) lines were acquired from the Arabidopsis Biological Resource Center. Genomic DNA was isolated from whole leaf tissue, and served as template to be amplified with the *AtDAD1* gene specific primers "*atdad1-F"* and "*atdad1-R*" which flank the T-DNA insertion site and the T-DNA specific primer "LBb1.3" to screen for the T-DNA insert as described at:

(http://signal.salk.edu/tdnaprimers.2.html). Homozygous mutants of *myb21-4* and *aos-2* (SALK_017756) and were provided by the Reed Group at the University of North Carolina at Chapel Hill.

Methyl Jasmonate Treatments

Flowers were dipped in a 500μM Methyl Jasmonate in a 0.05% Aqueous Tween 20 solution. Flowers were phenotyped for the production of nectar and opening of anthers, or RNA was isolated via the protocol described below.

RNA Isolations and Reverse Transcription PCR

To determine *AtSWEET9* expression in Arabidopsis JA mutants, RNA was isolated from whole flower tissue using Agilent Technologies' Absolutely RNA Miniprep Kit. 500μg of RNA was converted to cDNA using Applied Biosystems' High Capacity Reverse Transcription Kit. cDNA transcripts were amplified with the primer pair: "*AtSWEET9* RT-F" and "*AtSWEET9* RT-R" and ran on a 1% agarose gel to detect the presence of the *AtSWEET9*. Similar to the expression screen of *AtSWEET9, AtPIN6* and *AtCWINV4* transcripts were amplified, but the primer pairs used were "*AtPIN6* RT-F" and "*AtPIN6* RT-R" and "*AtCWINV4* RT-F" and "*AtCWINV4* RT-R" respectively. *AtGAPDH* was amplified as well and used as an internal standard using the primer pair "*AtGAPDH* RT-F" and "*AtGAPDH* RT-R."

Generation SWEET9pro::iaaL and ::iaaM Plants

The coding regions of both *iaaL* and *iaaM* were amplified using the primer pair "*iaaL* ORF-F" and "*iaaL* ORF-R" and "*iaaM* ORF-F" and "*iaaM* ORF-R" and then ligated into the plant transformation vector pPMK1 containing the *SWEET9* promoter and a Kanamycin resistance gene. These vector were given the names pPMK20 and 21 respectively. *Agrobacterium tumefaciens* (GV3101) cells were transformed to carry the pPMK20 or 21 vector and used to transform Arabidopsis using the floral-dip method described by Clough and Bent (1998). Transformed seedlings were selected on one half Muarshige and Skoog medium plates with 75µg/ml kanamycin.

Sugar Assay

Sugar assays were conducted following the protocol described by Bender et al., 2012.

GUS Histochemical Staining

Flowers were stained following the GUS staining protocol described by Jefferson et al., 1987.

Acknowledgements

I would like to thank Dr. Jason Reed at the University of North Carolina at Chapel Hill, for providing the jasmonic acid mutants, *aos-2* and *myb21-4*.

Table 1: Oligonucleotides used in this study.

Oligo Name	Sequence	Purpose
atdad1 F	AACTTTGGTGATGACGTCGTC	*atdad1* genotyping
atdad1 R	CTCTCTTTCTCCCGTACGTCC	*atdad1* genotyping
LBb 1.3	ATTTTGCCGATTTCGGAAC	T-DNA left boarder genotping
AtSWEET9 RT F	TCTCGCAGTCTTTGCTTCTCCCTT	*AtSWEET9* RT-PCR
AtSWEET9 RT R	GTTTGCCCTTCACTTCATTGGCCT	*AtSWEET9* RT-PCR
AtCWINV4 RT F	CCATATTCCAAGCATTGAG	*AtCWINV4* RT-PCR
AtCWINV4 RT R	CTGTTGAAGAGATAGAGTC	*AtCWINV4* RT-PCR
iaaL ORF-F BamHI	AAAGGATCCAGAGGACTGGCATGACT	Cloning of iaaL into pPMK1
iaaL ORF-R SalI	AAAGTCGACCACATCAGCCATTCAGT	Cloning of iaaL into pPMK1
iaaM ORF-F XmaI	AAACCCGGGGTAGAGTCGCGTTATGT	Cloning of iaaM into pPMK1
iaaM ORF-R SpeI	AAAACTAGTGCTCTCAGGACTGTTAA	Cloning of iaaM into pPMK1
AtPIN6 RT F	TCAAACCCTCATGGTCCAACTCGT	*AtPIN6* RT-PCR
ATPIN6 RT R	AACGAGTAAGCATCGGAGGAAGCA	*AtPIN6* RT-PCR
GAPDH RT F	TTCGGTGAGAAGCCAGTCACTGTT	*AtGAPDH* RT-PCR
GAPDH RT R	AAACATTGGAGCGTCTTTGCTGGG	*AtGAPDH* RT-PCR

CHAPTER 4: Concluding Remarks And Future Directions

Concluding Remarks

This study has shown the importance the sucrose transporter SWEET9 and the phytohormones jasmonic acid and auxin in the regulation of nectar production. These data provide insight into the complicated and yet still partially understood molecular mechanism of nectar secretion in the Brassicaceae. SWEET9 plays a vital role in nectar secretion by apparently transporting sucrose out of the parenchyma cells, which can be hydrolyzed into hexose sugars by CWINV4 and secreted from the nectary. JA is also important to nectar secretion, by potentially controlling water content in the nectaries and/or mediating auxin response within the nectaries. In addition, *MYB21*, a transcription factor induced by jasmonic acid, is either directly or indirectly regulating the expression of two vital nectary specific genes, *SWEET9* and *CWINV4*.

Taken together, it is proposed that JA, first synthesized in the filament (Ishiguro et al., 2001), and transported to the nectaries and anthers (Figure 5a, Chapter 3), is inducing the expression of *MYB21 and PIN6*, regulating auxin response, and the co-incidental opening of anthers (Figure 5b, Chapter 3). The expression of *MYB21* directly or indirectly induces the expression of the nectary specific sucrose transporter *SWEET9* and *CWINV4*. SWEET9 then transports newly synthesized sucrose, derived from starch, out of the cell where it can be hydrolyzed by CWINV4 and transported out of the nectary and presented as nectar. In addition to regulating genes important to nectar secretion (*CWINV4*, *SWEET9* and *PIN6*), there is an apparent crosstalk between JA and auxin, a plant hormone vital for nectar secretion. This regulation by JA allows the flowers to

present nectar and have open anthers with mature pollen at the same time, thereby potentially maximizing pollination efficiency from visitors.

Future Directions

SWEET9

GUS staining and RT-PCR supported the microarray data on *AtSWEET9* conducted by Kram et al. (2009), and mutations in *SWEET9* in both Arabidopsis and *B. rapa* lead to the absence of floral nectar. *Xenopus* oocytes were used to examine the sucrose transporter activity of SWEET9. The data indicated that SWEET9 has both sucrose influx and efflux activity, suggesting it acts as a uniport transporter. Following the starch-derived model of nectar secretion, SWEET9 is suggested to transport synthesized sucrose, derived from starch, out of the parenchyma cells were it may be hydrolyzed by CWINV4, and then moved out of the nectary as nectar. However, further work is still required to determine the mechanism of sugar modification and nectar secretion. A particularly important step in the secretion of starch-derived nectar is the break down of starch. When the starch is broken down to its monomers, they may be converted back into sucrose by a synthase, where they are exported from the cell by SWEET9. This sucrose synthase activity is an important step in nectar sugar modification and a potential key enzyme in nectar secretion. Preliminary work on discovering this synthase has been conducted, and two *SUCROSE-PHOSPHATE-SYNTHASE* (*SPS*) genes, *SPS1F* and *2F*, have been established as potential candidates. *SPS1F* and *2F* are both highly expressed in the nectary tissue. Additionally, artificial microRNA mutants of *sps1f* and *2f* in Arabidopsis phenocopy

atsweet9-3 mutants, displaying an absence of nectar and altered starch accumulation (Lin et al., submitted).

Jasmonic Acid

Mutants deficient in jasmonic acid production (*aos-2* and *dad1*) were also deficient in floral nectar production; however, treatment with exogenous JA rescued the production of floral nectar. An interesting result in the *aos-2* and *dad1* mutants was a lack of nectar secretion, while *SWEET9* and *CWINV4* were still expressed. This expression of *SWEET9*, a gene required for nectar secretion, suggests the possibility of post-translational modification to *SWEET9* regulated by JA or other unknown factors required for nectar production. Mutants of the JA induced transcription factor *MYB21*, however did not respond to JA treatments, and strongly displayed decreased expression of *PIN6*, *CWINV4* and *SWEET9*. The direct gene targets of MYB21, however are currently unknown. A promoter-binding assay for the MYB21 could shed insight into the binding site and genes regulated by MYB21.

Furthermore, to investigate the regulatory control of jasmonic acid on nectar secretion, RNA-seq may also be conducted. This would provide transcriptomic data related to genes misexpressed in the absence of JA. Another important aspect of plant hormones is their function may be modulated by their location and concentration. Exogenous JA treatments of varying concentration could further elucidate the impact of JA on nectar production in the Brassicaceae. Along with JA's regulation of *SWEET9, CWINV4,* and *PIN6*, JA appears to regulate auxin response within the nectaries. This

cross talk between hormones is a great area of interest, due to auxin's previous implication to nectar secretion (Bender et al., 2013). Examining the RNA-seq data (mentioned above) from the JA mutants could provide great insight on what auxin related genes may be misexpressed in the JA mutants. These genes may prove to be good candidates to further investigate this hormonal cross talk between JA and auxin.

Other Future Directions

Currently our group has been able to only alter and easily measure nectar sugar amounts in nectars of genetically modified plants; however, nectar is not only sugar water. There are many other solutes in the nectar, whose synthesis or transport through out the nectary has yet to be characterized. Due to the diversity of solutes in nectar and their various roles (pollinator attraction, inhibition of microbial growth, and the fending off of unwanted pollinators), determining the genetic factors that control these solutes could be vital in producing a more nutritious and/or desirable nectar. Also of interest is the extent to which the mechanisms of nectar production are conserved across species.

Also, with any potential genes determined to alter nectar production or secretion in Arabidopsis, studies should be conducted in plants that rely heavily on pollinator visitation such as particular cultivars of *Brassica sp*. Furthermore with any sort of genetic manipulation to alter nectar composition many studies must be conducted to determine the true impact of the modified nectar on pollinators. Field studies to measure pollinator preference, plant viability, and pollinator health should be conducted. As mentioned earlier while the modification of synthetic nectars such as nectars containing more proline

increased pollinator visitation (Carter et al., 2006) other studies have shown that when modified, pollinators dislike the synthetic nectars (Junior et al., 2008). This could be due to the potential co-evolution of plants and pollinators that has created a great specific preference of nectar type by the pollinator. Plants may invest a lot of energy to secrete nectar and in harsher growing environments (i.e. out of the laboratory), thus the production of modified nectar may be detrimental to their health, due to a potential improper resource allocation. Lastly, pollinator health should be considered, as manipulation of floral nectar my have unknown consequences on pollinator viability, for example, the sugar mannose was responsible for killing honeybees that ingested nectar containing that sugar (Crane, 1978).

Ultimately, these data have provided new information on the genetic and hormonal regulators of nectar production. SWEET9, the sucrose transporter is required for proper nectar secretion, and JA regulates the auxin acitivty and *PIN6* within the nectaries, as well as inducing expression of *MYB21*, a transcription factor either directly or indirectly regulating *SWEET9* and *CWINV4*. However, this model of nectar production is still only partial, and more work is required to elucidate the molecular mechanism of this biologically relevant process.

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