MNC1 Negatively Regulates Nectar Production through Auxin and Jasmonic Acid Response Pathways in *Arabidopsis thaliana*

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Dedication

This thesis is dedicated to my family and friends who supported me during the years of my thesis work.

I also want to dedicate this thesis to my favorite men's soccer team—German National Football Team—who won the 2014 Brazil World Cup during my preparation for the thesis defense. They inspired me that good teamwork and hardworking is the path that leads to success.

Abstract

Many flowering plants offer a reward for pollinators in the form of nectar. Despite the central role of pollination in reproduction of plants and the considerable amount of energy a plant devotes to produce nectar, little is known of the molecular mechanism of nectar production and its regulation.

Previous reports have suggested a significant role for the plant hormone auxin in regulating nectar production. Recent transcriptome studies have made it possible to focus research on several nectary-specific candidate genes with putative roles in the auxin response. In *Arabidopsis thaliana* this includes a gene termed *MEDIAN NECTARY CUPIN 1* (*MNC1*; At1g74820), which is highly expressed in median nectaries. *MNC1* silenced mutants (*mnc1*) showed more nectar production and increased auxin response activity while *MNC1* overexpresser mutant (MNC1 T6) showed significantly less nectar production and less auxin response activity in nectaries. A comparative sequence analysis of proteins with known function shows that MNC1 is a germin-like protein belonging to the RmlC-like cupins superfamily. MNC1 also has a conserved zinc binding domain with known Auxin Binding Protein1. Thus, we hypothesize that MNC1 negatively regulates nectar production, likely through an auxin dependent pathway. *PIN6* (At1g77110), an auxin transporter family protein, has been reported to be an auxin transporter localized to the ER modulating cytoplasmic free auxin concentration in nectaries. *Arabidopsis thaliana* mutant lines with different combinations of crossed target genes were used to understand the feedback mechanism of auxin, nectar production, PIN6, and MNC1 protein behind nectar regulation and production. Transformed *Escherichia coli* and *Pichia pastoris*, a methylotrophic yeast species, expressing MNC1 protein were employed for studying its biochemical nature, including auxin binding activity.

Jasmonic acid (JA) was also suggested to be required for nectar production, with possible crosstalk to auxin in regulation of nectar production. COI1-independent JA response pathway was found to regulate nectar production by altering the expression of nectary-specific genes such as *SWEET9*, a sucrose transporter required for nectar production, *CWINV4*, a cell wall invertase required for nectar production, and *MNC1*. A potential auxin-JA crosstalk mechanism was constructed based on results in this study and previous studies.

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Chapter 1: Introduction to nectar and nectary studies in *Arabidopsis thaliana*

Nectar and nectaries

Nectar composition

Nectar is an aqueous mixture of sugar, amino acids, vitamins, mineral and other ancillary compounds (phenolics, flavonoids, metal ions etc.), and is produced by nectaries on plants (Baker and Baker, 1983; Carter et al., 2006; Truchado et al., 2009; Varassin et al., 2001). Many angiosperms offer floral nectar as a nutritional reward for pollinators in order to achieve outcross fertilization, and some angiosperms and gymnosperms also offer extrafloral nectar to attract mutualists to prevent herbivory and increase reproductive success (Heil et al., 2001). The sugar content in floral nectar is 8~80% (w/w) depending on the species (Baker and Baker, 1983). Some species produce hexose-dominant nectar (glucose and fructose), some produce sucrose-dominant, and some produce nectars with equal amounts of sucrose and hexose, however the proportions of these sugars within species are normally constant (Baker and Baker, 1982). It is also suggested that the ratios of these different sugars can influence pollinator visitation because different pollinators might be drawn to different sugar sources. For example, long-tongued bees and hummingbirds are attracted to sucrose-rich nectar whereas short-tongued bees and flies are more attracted to hexose-rich nectar (Baker and Baker, 1983).

Nectaries

Floral nectaries are normally located at the base of flowers and covered by sepals. Most species of the Brassicaceae family (including Arabidopsis) have two lateral nectaries located under the short stamens that are responsible for producing most or all of the nectar, and two sets of median nectaries located under the long stamens that produce little or no nectar (Fig 1). However, researchers have hypothesized that median nectaries may play a role in regulation of nectar production from lateral nectaries (Kram and Carter, 2009). Floral nectaries are usually composed of three key cell types, (1) epidermal tissue, (2) parenchyma tissue, and (3) vascular tissue (Bender and Klinkenberg et al, 2012).

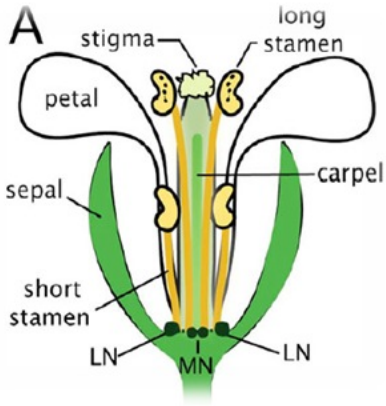

Figure 1: *A. thaliana* floral arrangement. Side view of the flower shows two sepal, two short stamen, two long stamen, two petals, two lateral (LN) and two median nectaries (MN). (Figure from Kram and Carter, 2009)

Studies with *Arabidopsis* have shown that there are four groups of floral organs (sepals, petals, stamens and carpels) regulated by a group of ABC homeotic genes including class A [*APETALA1 (AP1)* and APETALA 2 (AP2)], class B [*APETALA 3 (AP3)* and *PISTILLATA (P1)*], and class C [*AGAMOUS (AG)*]. Class A genes are responsible for the formation of sepals and the formation of petals together with B class genes; class B genes and class C genes together specify stamen formation; class C genes are responsible for the formation of carpels (Weigel and Meyerowitz, 1994).

Surprisingly, nectaries can form independently of the ABC floral genes in *Arabidopsis*, as mutants for these individual genes do not show an absence of nectaries (Baum et al., 2001). *CRABS CLAW (CRC)* is the only gene known to be essential for

nectary formation in *Arabidopsis thaliana*, and knockout mutants of this gene have no nectary formation (Bowman and Smyth, 1999). However, Baum et al. (2001) demonstrated in their study that though *CRC* is necessary, it is not sufficient for nectary formation suggesting that other genes are involved in nectary formation.

Nectar production and secretion

Although the composition of nectar is well known among different species, the molecular mechanisms of floral nectar production and secretion by nectaries remain largely unknown. The pre-nectar sugar and other minor components are generally thought to be originated from phloem sap (Pacini and Nepi, 2007), and transported into nectary tissue for storage prior to secretion. A proposed nectar synthesis and secretion pathway in *Arabidopsis thaliana* is shown in Figure 2, with epidermal cell labeled as EC, parenchymal cell as PC, and guard cell as GC. There are two possible pathways of the sugar transportation once it is loaded into the nectary: (1) the symplastic route in which the pre-nectar sugar is transported into parenchyma cells and travels through plasmodesmata, and (2) the apoplastic route in which the pre-nectar sugar travels in intercellular space. The nectar secretion method is also a largely debated topic. The two dominant ideas are 1) eccrine secretion in which the sugars are transported out of the cells by sugar transporters on the cell membrane, and 2) granulocrine secretion in which the sugars are transported out within vesicles derived from endoplasmic reticulum or Golgi apparatus (Klinkenberg, 2013).

In *Arabidopsis thaliana,* nectar secretion usually starts at stage 13 when the flower is just about to open coincidental with anthesis. Nectar production usually slows down and stops at stage 14 or 15.

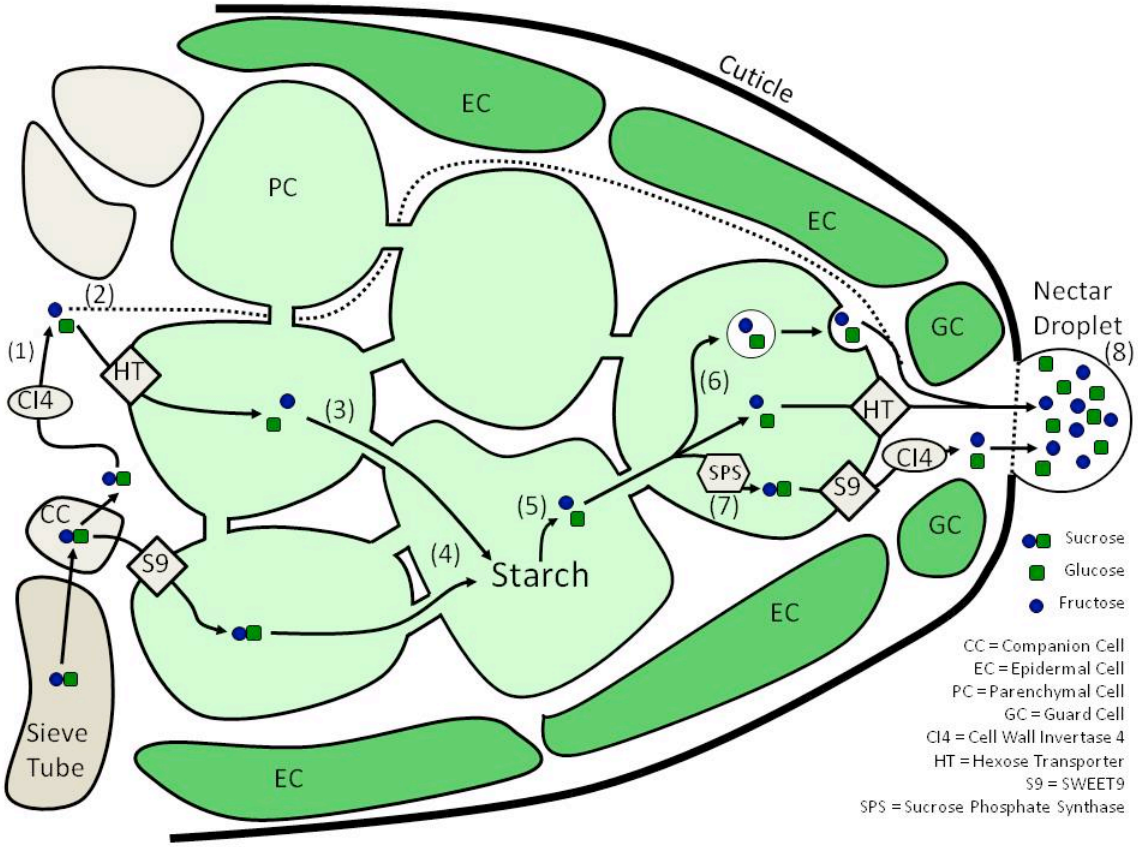

Figure 2. Nectary ultrastructure and proposed model of nectar synthesis. Nectaries are serviced with sucrose in the form of phloem sap. (1) Once in the nectary, the sucrose can be hydrolyzed into hexose sugars (glucose and fructose) by the extracellular invertase, CELL WALL INVERTASE 4 (CWINV4) (Rulhmann et al., 2010). (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 (granulocrine), or the hexose sugars may be exported via a hexose transporter (eccrine). (7) Conversely the parenchymal hexose sugars may be resynthesized by SUCROSE PHOSPHATE SYNTHASE back into sucrose, where SWEET9 transports the sucrose out of parenchyma cells (Lin et al., 2014) 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 from Kram and Carter, 2009; adapted by Jia and Klinkenberg).

Model organism and research significance

Arabidopsis thaliana

Arabidopsis thaliana (Brassicaceae) is a selfing plant with a short life cycle. The fully sequenced genome of *Arabidopsis thaliana* provides a convenient tool for plant molecular research, and recent research has demonstrated that despite the self-pollinating nature of *A. thaliana*, its nectaries are quite functional and can be functionally studied at the molecular level (Kram and Carter, 2009). A study by Davis et al. (1998) showed that *A. thaliana* produces hexose-dominant nectar, with the Columbia ecotype displaying a hexose-to-sucrose ratio close to 33:1. The hexoses in *A. thaliana* nectar are glucose and fructose in the ratio of 1:1, therefore, nectar production of *A. thaliana* in our study was represented by the results of glucose oxidation assay performed on nectar collected different mutant lines.

The floral arrangement and nectary locations are the same for *A. thaliana* and Brassica species, as shown in Figure 1. Most nectar is produced by the lateral nectaries in *A. thaliana* and secreted through modified stomata. This observation is due to the fact that lateral nectaries are heavily supplied with phloem elements, transporting more carbohydrates to these nectaries compared to median nectaries (Davis et al. 1998). A proposed nectar synthesis and secretion pathway of Brassicaceae (Figure 2) published by our lab in 2009, showed that phloem-derived sucrose is transported into nectary tissues and either stored as starch in parenchyma cells for later secretion or transported apoplastically (between cells) to stomatal apertures for secretion (Kram et al., 2009).

Biological and agricultural significance of nectar research

A. thaliana is in the same family as the *Brassica* genus which includes many agriculturally important crops such as rapeseed, broccoli, and cabbage (USDA Classification). Pollination success in these Brassica species rely heavily on effective pollinator attraction by nectar volume and sugar concentration, therefore, a complete understanding of the molecular processes underlying nectar production in representative Brassicaceae is very important. The *Brassica* crops largely depend on insects for their pollination, and unsuccessful pollination can reduce yield by more than 50% (nectarygenomics.org, 2013). For example, the seed yield of *Brassica napus*, or canola, was increased by 46% with the presence of multiple hives of honeybees (Sabbahi et al. 2005). Pollination success in these species rely on effective pollinator attraction positively correlated with nectar volume and sugar concentration; therefore, identifying genes, cellular structures, and molecular processes underlying nectar production in representative Brassicaceae is very important.

Nectar research is often overlooked of its significance in pollinators' ecology and the honey industry. A tremendous amount of material and energy are transferred from plants to pollinator colonies through the process of nectar. One study showed that 10% of total forage crop energy of alfalfa can be transferred into a bee colony and converted into honey (Southwick, 1984). This number may vary with different types of nectar containing different sugar contents. Therefore, understanding the production and regulation of nectar is crucial to bee ecology research and the honey industry.

Genes important for nectar production and hormone regulation

Many questions and hypotheses have been proposed in the area of hormonal control and biochemical pathways of floral nectar processing and secretion. A recent transcriptome study identified 270 nectary-enriched genes involved in functions including gene regulation, carbohydrate and lipid metabolism pathways, and hormone homeostasis and response (Kram et al., 2009). Several important and targeted candidate genes and plant hormones our lab has focused on in the nectary research project are discussed in detail as follows.

Targeted genes

CWINV4

CELL WALL INVERTASE 4 (*CWINV4*) is a nectary specific enzyme in *Arabidopsis thaliana* that catalyzes the hydrolysis of sucrose into glucose and fructose. An orthologue from *Brassica rapa* also has nectary-enriched expression of the gene. Knockout *cwin4* flowers in Arabidopsis did not produce nectar, but starch accumulated at the receptacle (Ruhlmann, et al, 2010) indicating that *CWINV4* is responsible for hydrolyzing the sucrose from phloem sap. It is also possible that *CWINV4* is responsible for post secretory hydrolysis during nectar secretion (Fig 2).

SWEET9

RT-PCR results from our lab have confirmed the strong expression of SWEET9, a member of the SWEET family sucrose transporters, in mature median nectaries,

immature lateral nectaries, and mature lateral nectaries (Kram et al, 2009). A study done in collaboration with Lin et al. showed that SWEET9 knock out mutant plants did not produce nectar, and starch staining on the mutants showed starch accumulation at all parenchyma cells in nectary tissues. It was concluded that SWEET9 is the sucrose efflux transporter in nectary tissue and it is responsible for exporting sucrose during nectar secretion (Lin et al., 2014). We have further found that SWEET9 was not expressed in the background of *MYB21* mutant plants. *MYB21* is a transcription factor that can be induced by the hormone jasmonic acid (JA) (Reeves et al., 2012). Thus, the JA regulated pathway of *MYB21* expression and the resulting expression of SWEET9 is a positively regulated pathway leading to nectar production (Klinkenberg and Carter, unpublished data).

PIN6

A very recent study in our lab focused on *PIN6*, a nectary-enriched gene whose expression level is positively correlated to total nectar production in Arabidopsis, and whose function is required for the proper development of short stamens (Bender et al., 2013). The study demonstrated that *PIN6* is involved in auxin-dependent responses in nectaries (via the use of auxin responsive reporters DR5::GFP or DR5::GUS). The auxin responsive reporters showed intense signals in lateral nectaries of wild-type plants, but little or no signal in the lateral nectaries of *pin6* knockout mutant plants. *PIN5*, another PIN family protein, has been shown to be located in the endoplasmic reticulum of Arabidopsis cells and it could be transporting auxin into ER to regulate intracellular

auxin concentration (Mravec et al. 2009). PIN6 may have a similar role as to PIN5 (Fekete, 2011). In the auxin regulated pathway, *PIN6* has an indirect positive effect on nectar production through auxin.

MNC1

MEDIAN NECTARY CUPIN 1 is another gene that stood out in the previous microarray data because it is only expressed in median nectaries. *PIN6* has been reported to be an auxin transporter localized to the ER previously by Bender et al. (2013), interestingly, *pin6* knockouts resulted in over-expression of *MNC1* in lateral nectaries, along with decreased auxin response and decreased nectar production. A comparative sequence analysis of proteins with known function shows that MNC1 is a germin-like protein belonging to the RmlC-like cupins superfamily. MNC1 also has a conserved domain found in auxin binding proteins. Thus, we hypothesize that *MNC1* negatively regulates nectar production, likely through an auxin dependent pathway. A detailed introduction and study of *MNC1* is presented in chapter 2 of this study.

Plant hormones in nectar production

Auxin

The phytohormone IAA (indole-3-acetic acid), also known as auxin (Table 1), regulates plant growth and behavior including root growth, apical dominance, flower development, fruit development and many other aspects (Aloni et al., 2006; Dun et al., 2006; Overvoorde et al., 2010; Pattison et al., 2013). Previous studies have suggested that auxin

plays a dual role in nectar production and regulation (Bender et al., 2013; Shuel, 1964). Bender et al. (2013) showed that exogenous application of naphthaleneacetic acid (NAA, a synthetic auxin) up to 100 μM had positive effect on nectar production but a sharp decline of nectar production was observed at higher concentrations. Another synthetic auxin commonly used is 2,4-Dichlorophenoxyacetic acid (2,4-D, a synthetic auxin). A detailed introduction and report of study on auxin regulation of nectar production is discussed in chapter 2 and 3 of this study.

Jasmonic Acid

Jasmonic acid (JA) is another plant hormone (Table 1) that has been heavily researched for its important role in plant defense systems and flower development (Reeves et al., 2012; Nagpal et al., 2005), and has also been recently suggested to affect nectar production in Arabidopsis and related species (Radhika et al., 2010). JA biosynthesis mutants *aos2* and *dad1* are both male sterile and neither produces any nectar unless treated with exogenous MeJA (Klinkenberg and Carter, unpublished data), indicating that JA is required for nectary production or secretion. A detailed introduction and study of JA regulation in nectar production is discussed in Chapter 3 of this study.

This study focuses specifically on determining the role of a nectary-enriched gene *MEDIAN NECTARY CUPIN 1 (MNC1)* and its role in regulation of nectar production through auxin pathway. Furthermore, this study endeavors to decipher the potential crosstalk of auxin and jasmonic acid in regulation of nectar production and secretion (Klinkenberg, 2013; Reeves et al., 2012).

Table1. Abbreviation table for plant hormones and hormone related molecules.

Abbreviation	Name	Nature
IAA	Indole-3-acetic acid	Natural auxin
NAA	1-Naphthaleneacetic acid	Synthetic auxin
2,4-D	2,4-Dichlorophenoxyacetic acid	Synthetic auxin
AUX/IAA	AUX/IAA genes	Auxin response repressor genes
JA	Jasmonic Acid	Plant hormone
MeJA	Methyl Jasmonic Acid	JA derivative
$SCF^{TIR1/AFB}$ (TIR1)	SCF TIR1/AFB complex	Auxin receptor containing Skp, Cullin, F-box containing complex and TIR1 gene.
SCF^{COI1} (COI1)	SCF COI1 complex	Jasmonic receptor containing Skp, Cullin, F-box containing complex and COI1 gene.

Chapter 2: MNC1 negatively regulates nectar production through the auxin response pathway in *Arabidopsis thaliana*.

Introduction

The Cupin Protein family

MEDIAN NECTARY CUPIN 1(MNC1) is a gene that stood out among the RT-PCR validation profile of nectary-enriched genes (Kram et al., 2009) because it is only highly expressed in median nectaries, with little if any expression in lateral nectaries (Figure 1). *MNC1* is a germin-like protein belonging to the RmlC-like cupins superfamily. The cupin superfamily was originally discovered as a conserved motif that forms a β-barrel found in germin and germin-like proteins, which accumulated highly in germinating wheat (Dunwell et al., 2003). Many proteins with diverse functions have since been identified in the cupin superfamily including various germin proteins, germin-like proteins (GLP) such as Nectarin 1 in tobacco nectar, Auxin Binding Protein 1 (ABP1) in both maize and Arabidopsis, and AtPIRIN that functions downstream of G-protein alpha subunit to regulate seed germination in Arabidopsis (Carter et al., 1999; Woo et al., 2002; Lapik and Kaufman, 2003).

Median Nectary Cupin 1 (MNC1)

The MNC1 protein is a 22kDa Rmlc-like cupin family protein that has a consensus sequence of zinc ion binding site which is also found in ABP1 in both *Arabidopsis thaliana* and *Zea mays* (Woo et al., 2002). It is crucial to understand the role of *MNC1* since it is one of the few negative regulators found in the nectar production pathway and it is required for the normal development of median nectaries. *MNC1* transcript level was shown to be more than 12,000-fold higher in mature median nectaries

than in lateral nectaries and other plant tissues (Figure 1) from previous microarray data (Kram et al., 2009).

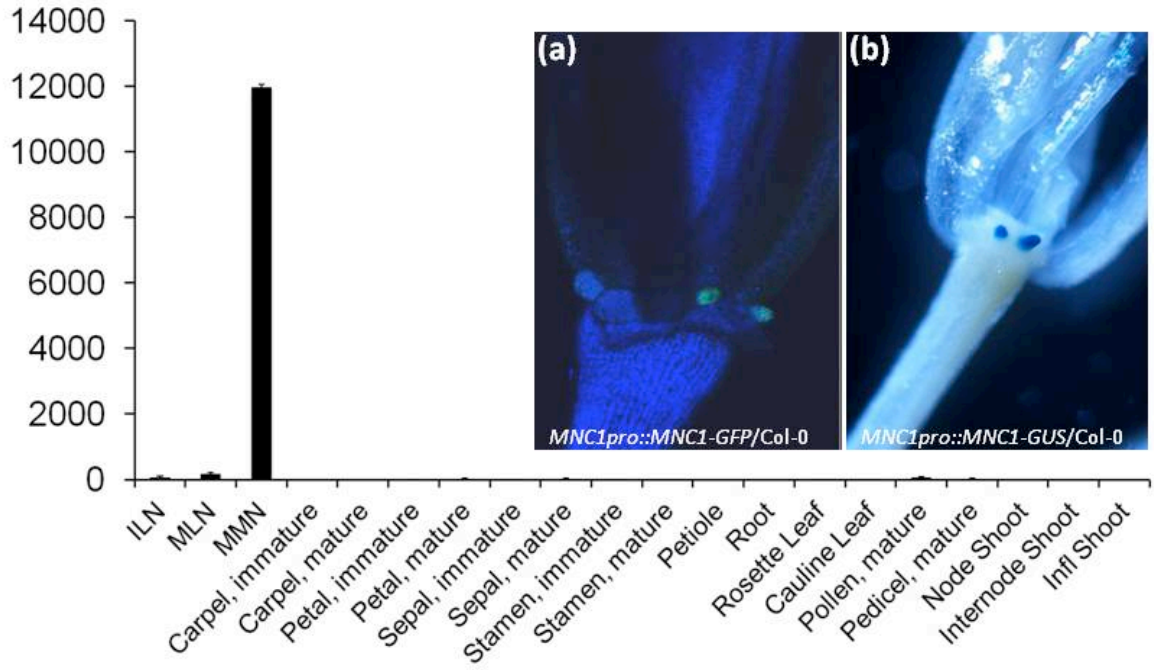

Figure 1: Microarray data suggested *MNC1* expression is highly median nectary specific. (Figure adapted from Bender et al, 2012a) **(a)** Green Fluorescence Protein (GFP) expression under *MNC1* promoter in wild-type *Arabidopsis thaliana*. MNC1-GFP fusion protein expression is highly median nectary-specific showed in bright green signal. **(b)** β-glucuronidase (GUS) reporter gene expression under *MNC1* promoter in wild-type *Arabidopsis thaliana*. MNC1-GUS expression is highly median nectary-specific showed in dark blue signal.

To probe the function of MNC1, the gene was silenced by artificial microRNA (amiRNA). The *mnc1* silenced plants (knock down of *MNC1*) showed extremely reduced median nectary development or no median nectary development (Figure 2A), and increased nectar production (Figure 2B). This observation strongly suggests that *MNC1* has a negative effect on nectar production and/or secretion.

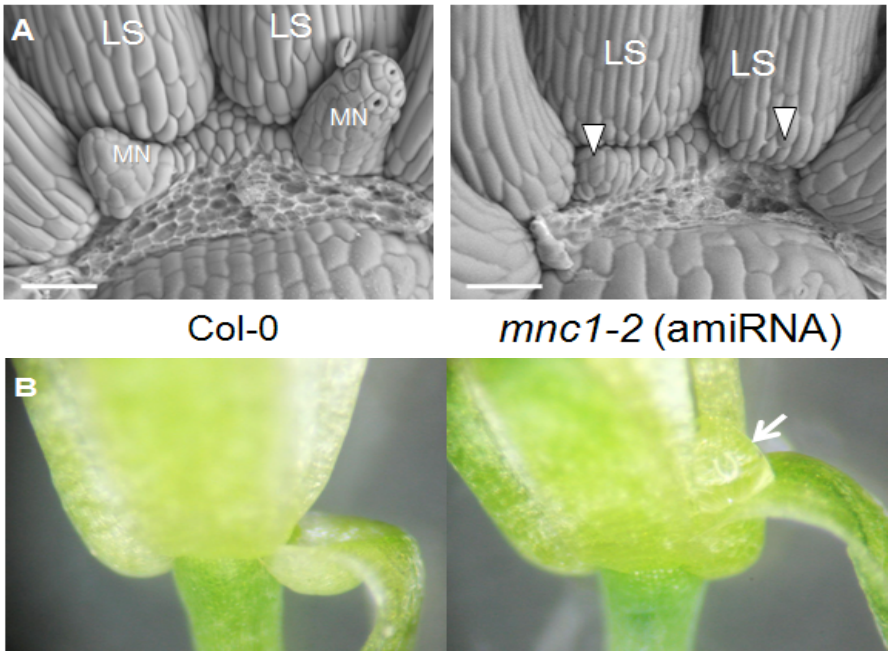

Figure 2. Nectar volume and median nectary development in *mnc1*. (A) Differential nectary development in *mnc1*. Col-0 (wild type) median nectaries (MN) are present, and *mnc1-2* (MNC1 knockout mutant) median nectaries are missing (LS = long stamen). **(B) *mnc1* knock down mutant has increased nectar volume.** Note the large bubble of pooled nectar observed in the *mnc1-2* mutant (Figure adapted from Bender et al., 2012a).

Relationship between MNC1, PIN6 and Auxin

Auxin is one of the targeted hormones our lab has been studying in the project to discover the role of phytohormones in nectary development and function. As mentioned before, auxin plays a diverse role in plant growth and development. Bender et al. (2013) suggested that auxin is also important in the regulation of nectar production. They showed that the exogenous auxin treatment on Arabidopsis flowers (by placing peduncles in up to 100 µM synthetic auxin NAA in 10% sucrose) significantly increased nectar production.

PIN6 was characterized as a nectary-specific auxin transport protein localized at the endoplasmic reticulum (ER) membrane. Its expression is required for short stamen

development, auxin response, and its expression level is positively correlated with total nectar production in Arabidopsis (Bender et al., 2013). Interestingly, Bender et al. found that in both *PIN6* overexpresser and knock out mutant, auxin-responsive DR5::GFP or DR5::GUS reporters had decreased signal in lateral nectaries when compared to wild-type plants. They suggested that this is due to sequestering of cytosolic auxin into the lumen of ER by *PIN6* in order to maintain the homeostasis of intracellular auxin. *MNC1* was also observed to be up-regulated by 24-fold in *pin6-2* (knockout mutant of *PIN6*) lateral nectaries (Figure 3). *MNC1*, as discussed earlier, is in the same family with ABP1 and has many similarities in amino acid sequence with ABP1. Together with the decreased auxin response in both *pin6-1* (overexpresser) and *pin6-2* lateral nectaries, we hypothesized that the up-regulation of *MNC1* in pin6-2 might be a result or partial cause of the decreased auxin response in *PIN6* mutant plants.

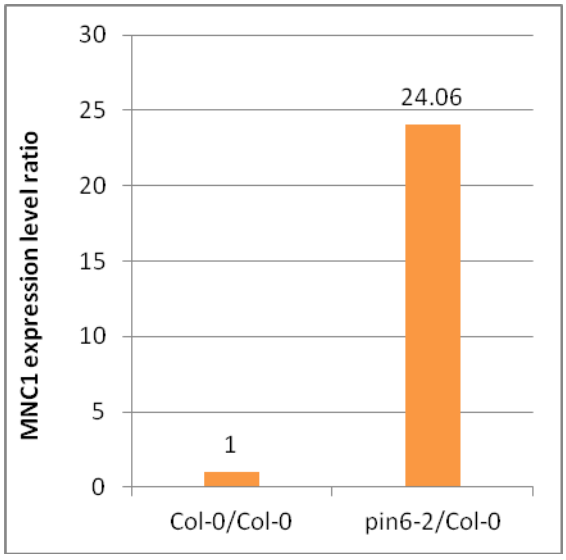

Figure 3. RNA sequencing *MNC1* expression in *pin6-2*. RNA sequencing data showed that *MNC1* had a 24-fold increased expression in *PIN6* knock out plant *pin6-2*. (RNA sequencing data obtained from Bender et al., 2013).

This chapter will focus on the characterization of *MNC1* in *Arabidopsis thaliana* regarding to its role as a negative regulator of nectar production possibly through auxin pathway.

Results

Localization

MNC1 was predicted to be secreted out of cells by PSORT (Prediction of Protein Localization Sites version 6.4, http://psort.hgc.jp/form.html) with a certainty of 0.820. The report also gives a possible N-term signal sequence for secretion consisting of 27 amino acids. The microarray data shown in Figure 1 suggested that MNC1 is highly expressed in the median nectary. To confirm the localization of MNC1 on organelle and cellular level, *Agrobacterium tumefaciens* transformed mutant plant MNC1pro:MNC1-GFP (MNC1::GFP) was made to create a fusion protein of MNC1 and Green Flourescence Protein under the *MNC1* promoter. MNC1-GFP signal was repeatedly observed to be outlining the cell of median nectaries in *A. thaliana* (Figure 4a, 7c). Mannitol treatment on MNC1::GFP flower was done to verify that MNC1 is mostly localized between plasma membrane and cell wall. Concentrations of both 0.5M and 1.0M mannitol were used in the mannitol treatment and GFP signal was more diffused in the median nectary cells in the mannitol treated flowers (Figure 4b) when clear cell-boarder outlining pattern was observed in PBS treated cells (Figure 4a). This was repeated three times to prevent any random causation to the phenomenon observed.

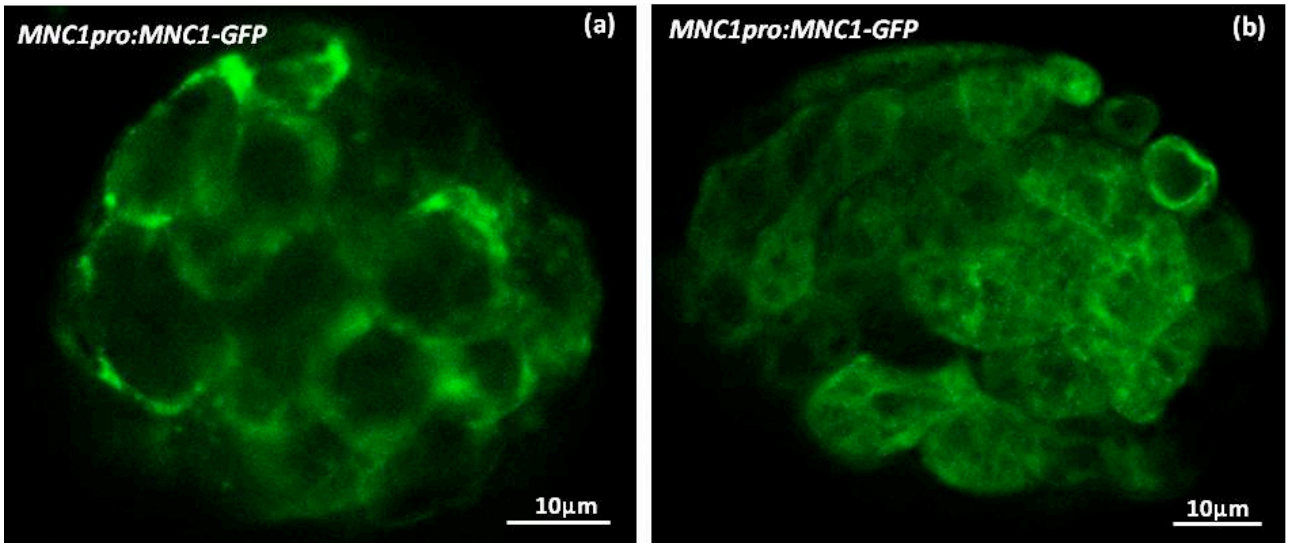

Figure 4: *MNC1* Locolization in cell. MNC1::GFP *A. thaliana* flowers were treated with either Phosphate Buffer Saline (PBS) or 1.0M mannitol, green fluorescence GFP signal in median nectary was then imaged with confocal fluorescence microscope with 20X objective, (a) Median nectary of PBS treated MNC1::GFP flower (b) Median nectary of 1.0M mannitol treatment.

Circadian expression

Nectar secretion in *Brassica* sp. was observed to undergo circadian oscillation with maximum secretion between 4 -8 hours after dawn (h.a.d.) (Búrquez and Corbet, 1991), and the same pattern was observed in *Arabidopsis thaliana* nectar production (Bender et al., 2012b). It is logical to speculate that the genes involved in nectar production regulation might undergo circadian oscillation in their expression level that rhymes with the nectar production circadian oscillation. Wild-type flowers were collected every four hours for total of 36-hour duration to examine the expression of *MNC1* and *PIN6*. Results shown in Figure 5 demonstrated that expression of both *MNC1* and *PIN6* did undergo circadian oscillation that is similar to that of nectar production. *MNC1* expression level peaked at 4 h.a.d. on both days and expressed at a lower level at other time points (Figure 5a). *PIN6* was expressed at 4 h.a.d. on both days and was expressed

the highest at 12 h.a.d., however, it was almost not expressed in other time points (Figure 5b). This expression examination was repeated twice with different collections of flowers.

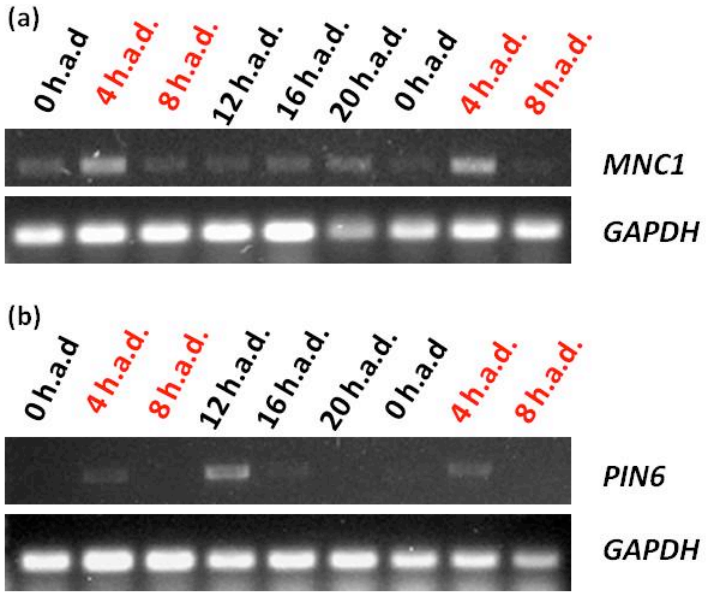

Figure 5: Circadian oscillation of *MNC1* and *PIN6* expression level in wild-type Arabidopsis whole flowers. (a) *MNC1* expression level peaks at 4 h.a.d. and expressed in lower level at other time points. (b) *PIN6* expressed at 4 h.a.d. and 12 h.a.d. and was almost not expressed in other time points. *GAPDH* was used as internal standard for these expression examination. Maximum nectar secretion time points are labeled in red. (h.a.d. = hour after dawn)

Nectar production in *MNC1* mutant lines

To examine the role of *MNC1* on nectar production, *Arabidopsis thaliana mnc1*-silenced plants were made by artificial miRNA gene silencing, the *MNC1* overexpresser line MNC1 T6 was made through recombinant DNA by linking *MNC1* to SWEET9 promoter which is a strong nectary enriched promoter. Assays of glucose content in nectar were performed on these mutant lines to evaluate the abundance of glucose in the secreted nectar. MNC1 T6 was found to have significantly less nectar glucose (about

53.68% of wild-type, p<0.001) when comparing to that of wild-type nectar. However, an 11.5% increase of nectar glucose was found in *mnc1* (Figure 6).

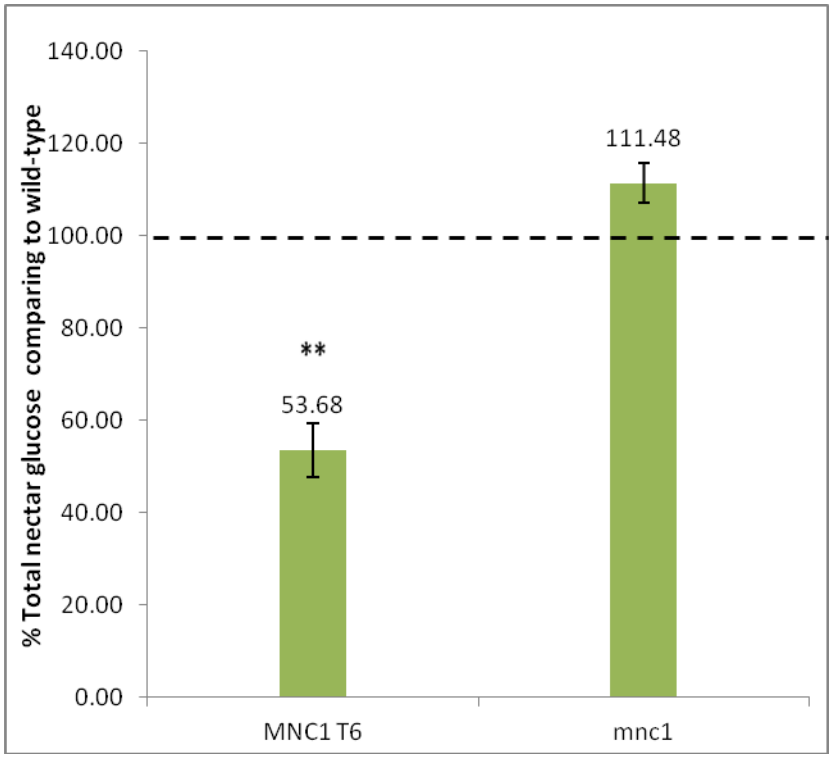

Figure 6: Nectar production in *MNC1* mutant lines. Nectar glucose assay was performed on samples collected between 4 and 8 h.a.d., which is the maximum nectar secretion time. 10 flowers were collected for each line with a repetition of 3 on different days. The mean percentage of glucose content in each mutant line comparing to wildtype is shown. Wild-type glucose content is set as 100% showing in dashed line. (Error bar= standard deviation, p<0.001 for MNC1 T6, p=0.058 for *mnc1*)

Auxin response in *mnc1* mutant lines

We hypothesized that MNC1 might be regulating nectar production through an auxin response pathway. To examine the auxin response in *MNC1* mutant plants, DR5::GUS and DR5::GFP reporter genes were used. The DR5 promoter is an auxin-inducible promoter (Ulmasov et al., 1997). Researchers have used DR5 promoter driven GUS/GFP (DR5::GUS/GFP) plants to study auxin response activity in many plants (Bierfreund et al., 2002; Chen et al., 2013; Bender et al., 2013). DR5::GUS/GFP plants were crossed to both *MNC1* T6 (overexpresser) and

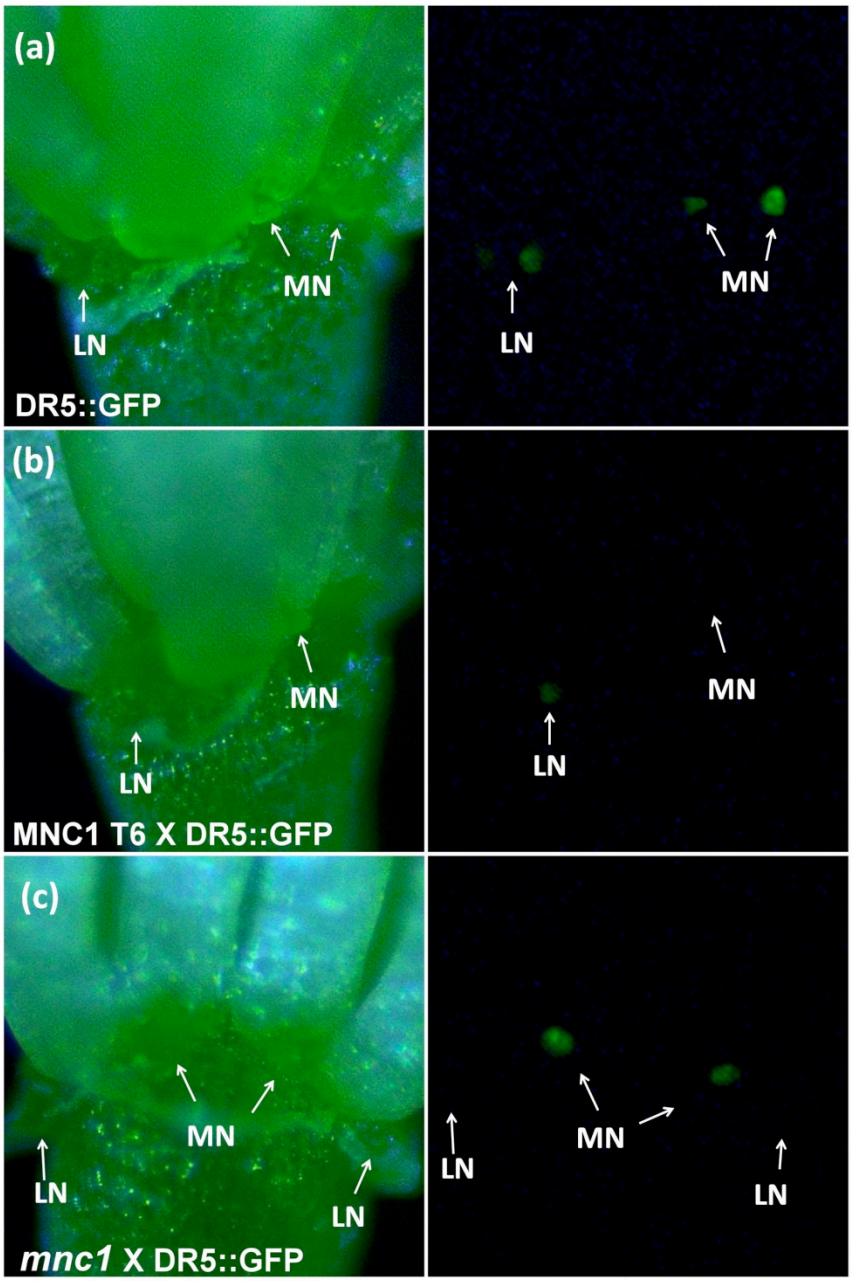

Figure 7: Auxin response activity of MNC1 mutants through EGFP imaging. (a) Auxin response activity in wild-type DR5::GFP plants displayed strong GFP signals in median nectaries (MN) and weaker signals in lateral nectaries (LN). **(b)** Auxin response activity in MNC1 T6 mutant background displayed considerably weaker signal in median nectary comparing to wild-type plants. **(c)** Auxin response activity in *mnc1* background showed slightly stronger signal in median nectary compared to that of wild-type plants.

mnc1 (silenced) mutant lines. Fluorescence imaging for GFP signals on MNC1 T6 x DR5::GFP plants showed weaker GFP signals consistently representing lower auxin response activity in lateral nectaries and median nectaries (Figure 7b) when compared to wild-type DR5::GFP plants (Figure 7a). However, *mnc1* x DR5::GFP showed what appeared to be more GFP signal compared to that of wild-type in median nectaries (Figure 7c) and sometimes in lateral nectaries as well (data not shown).

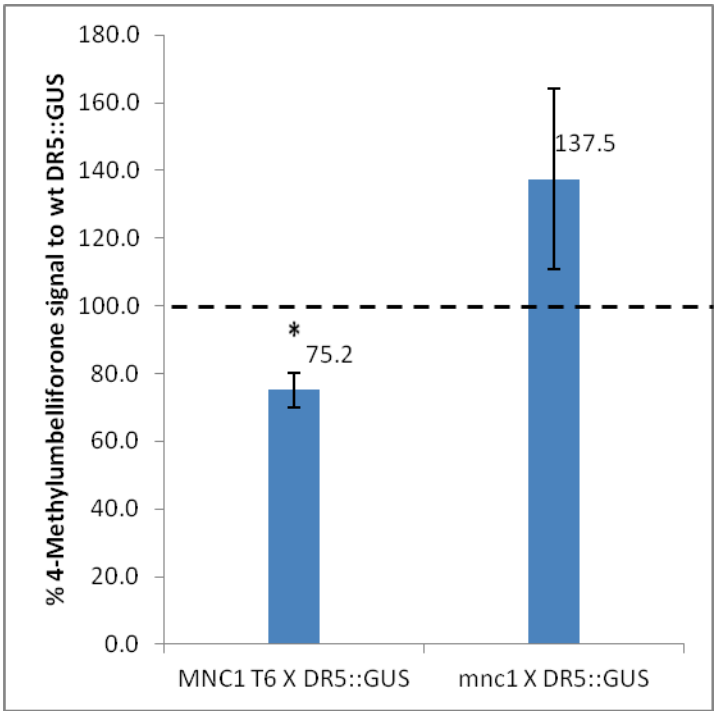

Figure 8: MUG assay on *MNC1* mutant plants. 4-methylumbelliforone signal was measured with a Nanodrop fluorescence spectrophotometer to quantify auxin response activity in MNC1 mutant lines. MNC1 T6 showed significantly less auxin response activity while *mnc1* showed more auxin response activity than that of wildtype. Bradford protein assay was performed to standardize protein quantity before MUG assay was conducted. Boiled protein control showed no fluorescent signal. (Error bar= standard deviation, $p<0.05$)

Similar results were obtained through GUS staining in the flowers of DR5::GUS in wild-type background and both *MNC1* mutant background (data not shown). Because the GUS/GFP imaging results on *mnc1* x DR5::GFP showed various signal levels when compared to wild-type plants, fluorescent assays quantifying GUS activity by measuring the product signal of MUG and GUS (4-methylumbelliforone) (Jefferson et al., 1987) were performed to quantify the auxin response activities among wildtype DR5::GUS, MNC1T6 x DR5::GUS and *mnc1* x DR5:: GUS. Auxin response activity in the *MNC1* T6 background was significantly lower (24.8%) than that of wild-type DR5::GUS plants while the mnc1 showed about 37.5% more auxin response activity (Figure 8). Taken together, it was concluded that *MNC1* alters auxin response activity negatively.

MNC1 expression in auxin and *PIN6* mutant lines

Since *MNC1* expression and auxin response was altered in *pin6-2* mutant plants, we examined *MNC1* expression in both *pin6-1* (overexpresser) and *pin6*-2 (knock down). *MNC1* was highly expressed in both mutant lines (Figure 9a), while the overexpression of *MNC1* in *pin6-2* was consistent with what Bender et al. (2013) found in the RNA sequencing data where *MNC1* was up-regulated by 24-fold in lateral nectary of pin6-2 knock down mutant (Figure 3). To confirm the overexpression of *MNC1* in *pin6-1*(Figure 9a), we crossed *MNC1*::GFP plants with *pin6-1* plants to express *MNC1*::GFP in *pin6-1* background. Both *MNC1*::GFP in wild-type *Arabidopsis thaliana* and in *pin6-1* background were examined with confocal fluorescence microscopy (Figure 9c, 9d).

MNC1-GFP signal was much stronger in *pin6-1* median and later nectaries (Figure 9c) than that of wild-type (Figure 9d).

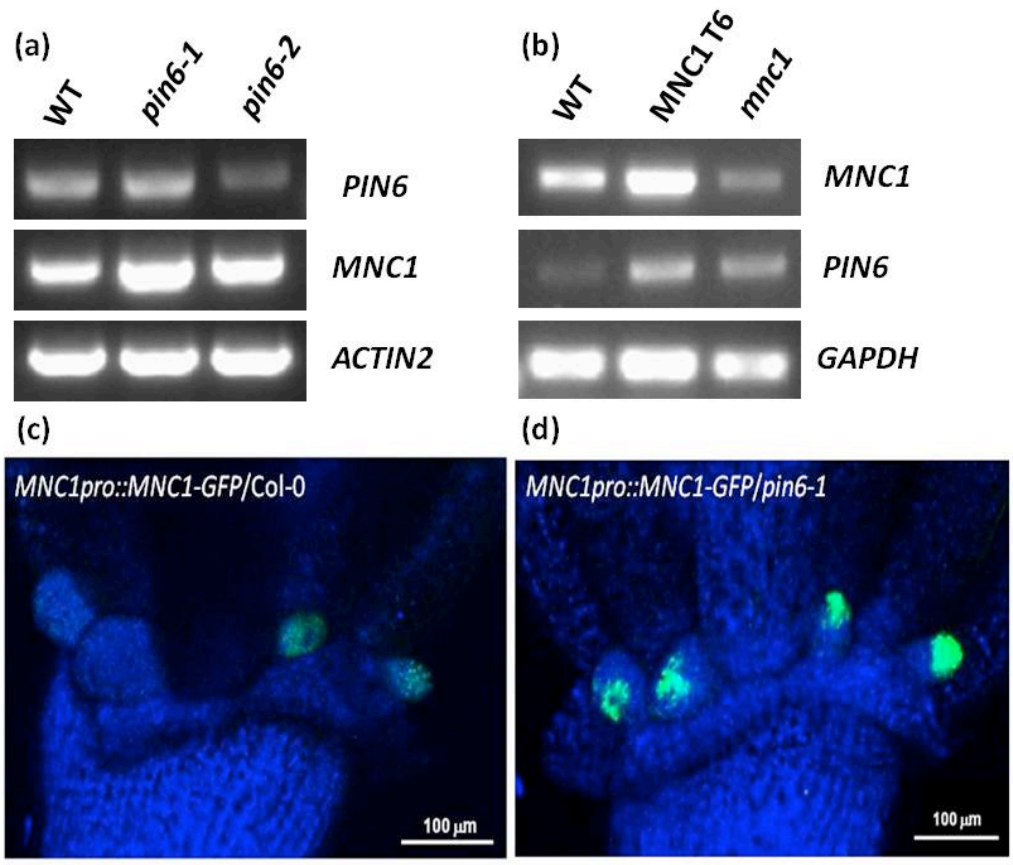

Figure 9: Expression examination of *pin6* and *mnc1* mutants. (a) *PIN6* and *MNC1* expression level in *PIN6* overexpresser (*pin6-1*) and *PIN6* knock down (*pin6-2*). MNC1 is slightly overexpressing in both *PIN6* mutant lines. **(b)** *PIN6* and *MNC1* expression level in *MNC1* overexpresser (MNC1 T6) and knock down (mnc1). PIN6 is overexpressing in both MNC1 mutant lines. ACTIN2 and GAPDH are used as internal standard respectively. **(c)** MNC1::GFP confocal imaging in wild-type *Arabidopsis thaliana* showed GFP signal in median nectary only (MN). **(d)** MNC1::GFP confocal imaging in *pin6-1* background showed strong GFP signal in both median and later nectary (LN).

The complex relationship of *MNC1* and *PIN6* showed in the result of expression examinations led us to examine the *MNC1* expression level in auxin mutants. The auxin co-receptor *TIR1* knockout mutant *tir1-1* was previously found to have significantly

increased nectar glucose that phenocopied the phenotype of increased nectar glucose in *pin6-1* knock-up mutant (Bender et al., 2013). *MNC1* expression in the *tir1-1* knockout mutant was down-regulated (Figure 10a, Bender and Carter, unpublished data). *iaaL* and *iaaM* are auxin modulating genes that have been characterized to be involved in pathogen invasion of plants. *iaaL* can inactivate auxin by conjugating a lysine to IAA (natural auxin) leading to significant reduction of active auxin (Romano et al., 1991), *iaaM* can promote the production of excess IAA in petunia plants by 10-folds (Klee et al., 1987). Plant transformation vector with a strong nectary specific promoter (*SWEET9pro*) were used to highly express these genes in *Arabidopsis thaliana* nectaries (Klinkenberg and Carter, unpublished result). Expression of *MNC1* in these *iaaL* and *iaaM* mutant plants showed relatively similar level of MNC1 expression between the two mutant lines that is slightly higher than wild-type (Figure 10b). The *iaaL* plant with less endogenous active auxin did not have the same MNC1 expression pattern with the auxin co-receptor knockout mutant *tir1-1* (Figure 10).

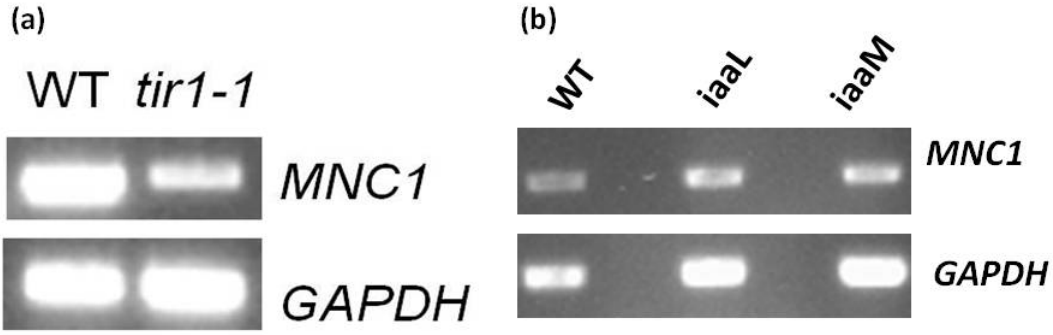

Figure 10: MNC1 expression in auxin mutants. (a) *MNC1* expression level is less in *TIR1* knock out mutant than wild-type (Bender and Carter, unpublished data). **(b)** *MNC1* expression level is slightly up-regulated in both *iaaL* and *iaaM* plants. GAPDH is used as internal standard.

MNCI Biochemistry

Protein alignment

As stated previously, MNC1 is in the same cupin family with Auxin Binding Protein 1 (ABP1). ABP1 is a known auxin receptor that is ubiquitous among green plants found both in ER and extracellular space (Woo et al., 2002). ABP1 was shown to be dimeric and can bind free auxin at physiological concentrations (Löbler and Klämbt, 1985; Shimomura et al., 1986). Woo et al. (2002) solved the ABP1 crystal structure in *Zea mays*, which revealed the auxin binding pocket on ABP1 is predominantly hydrophobic with a zinc ion inside interacting with three histidines and a glutamate (His 57, His 59, Glu 63, His 106). An alignment of the AtMNC1 and ZmABP1 protein sequence with MUSCLE protein alignment using Geneious software showed an identity score of 16% with 29% positive sites (Figure 11). This concensus sequence for zinc ion coordination site was also found in AtMNC1 shown as grey-shaded amino acid on Figure 11.

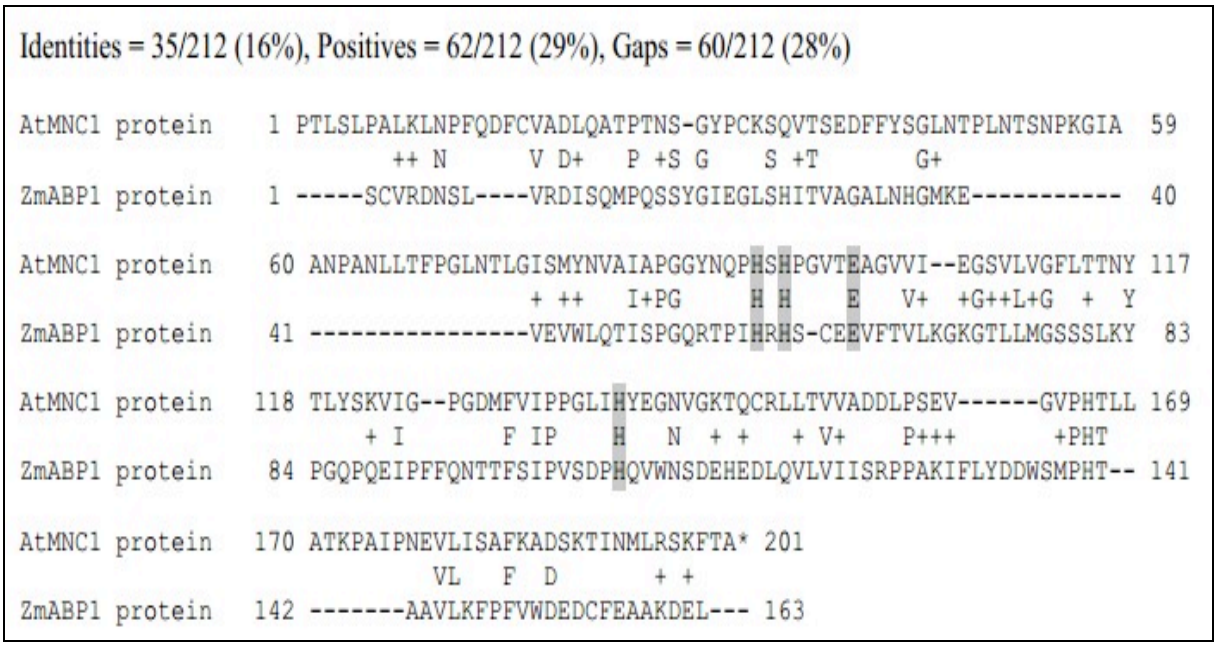

Figure 11: AtMNC1 alignment with ZmABP1 with MUSCLE protein alignment. Highlighted amino acid sequences (His 57, His 59, Glu 63, His 106) are the conserved sites for zinc interaction on ZmABP1 and AtMNC1. Signal peptide sequences for both AtMNC1 (27aa) and ZmABP1 (39aa) were deleted for alignment purpose.

Auxin Binding Protein 19 (ABP19) in *Prunus persica* (peach) is another germin-like protein with auxin binding properties (Ohmiya et al., 1998) that has highly conserved sequence with AtMNC1. Alignment of PpABP19 and ZmABP1 has only 15% identity and 28% positive sites while Alignment of PpABP19 and AtMNC1 had 32% identity and 43% positive sites (Alignments not shown). This shows that even proteins with limited identity to ABP1 can bind auxin. Therefore, it is reasonable to hypothesize auxin binding properties in AtMNC1.

Expression in E.coli and P.pastoris

In an endeavor to decipher the possible interaction of MNC1 with zinc and with auxin, constructs were made to express MNC1 in both *Escherichia coli* (pRLB11) and *Pichia pastoris* (pMYJ2.9) for auxin binding activity examination. Standard SDS PAGE and western blot (His-tag rabbit antibody as 1˚Ab and Goat anti-rabbit antibody as 2˚Ab) was used to detect MNC1.

Cupin-family proteins are known to form homo-multimeric proteins (Dunwell et al., 2000). As shown in Figure 12a, when the protein lysate was not boiled prior to SDS PAGE, a thinner band was observed at 22 kDa, and when the protein lysate was boiled prior to SDS PAGE, the band at 22kDa was thicker, indicating possible oligomer formation. After protein purification with Cobalt His-column, the same size (~22kDa) protein bands showed up in all elution fractions (Figure 12a). Protein lysate of *E. coli* carrying empty vector PET21a that was used to construct pRLB11 to express MNC1 in *E. coli* was used as a negative control and did

not have any band show up in the western blot at 22kDa. The possible oligomer formation was observed in both the non-boiled protein lysate (Zn^{2+} added to culture) and the non-boiled Cobalt His-column purified then dialysed protein (Figure 12b). The expression of MNC1 in *Pichia pastoris* resulted in multiple bands even when protein lysates were boiled, however these bands were not observed on the western blot of non-transformed *P. pastoris* cell lysate (Figure 12c).

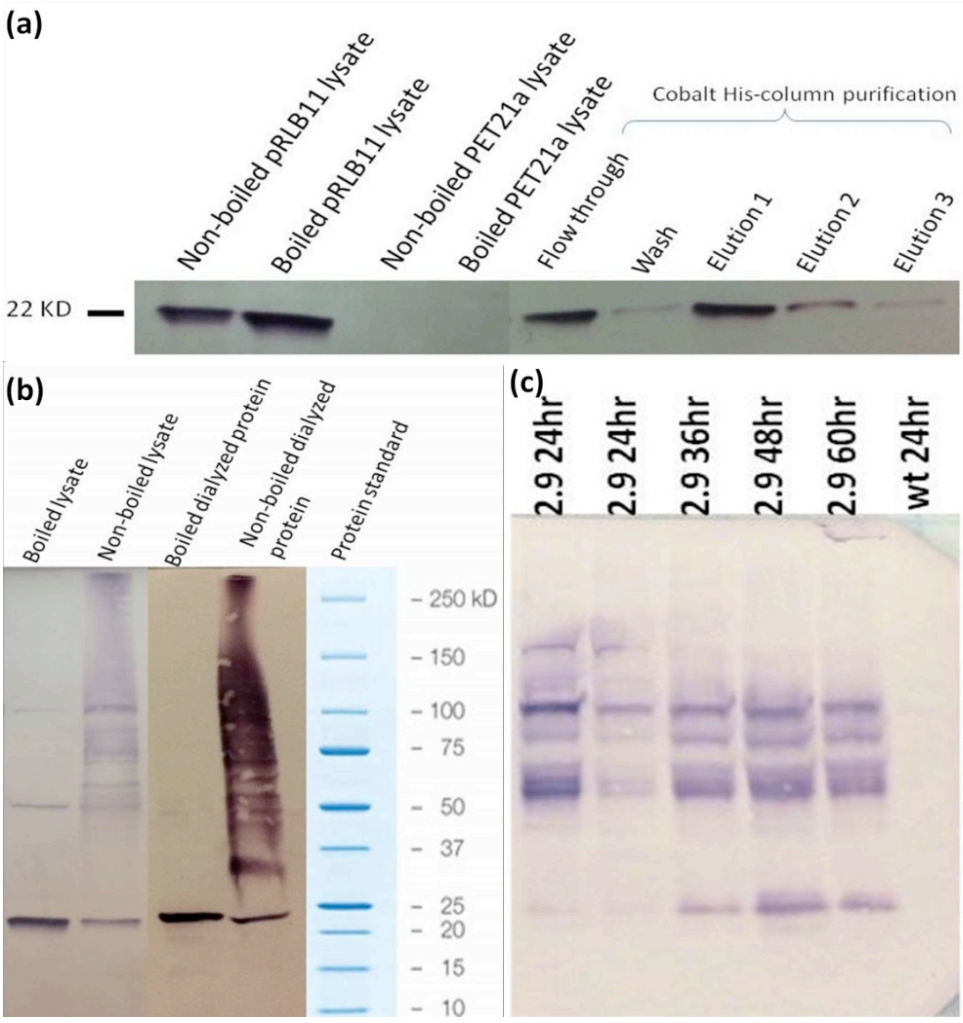

Figure 12: *MNCI* Expression in prokaryote and eukaryote expression system. (a) Western blot of MNC1 expression in *E. coli* and His-Column protein purification of MNC1 expressed in E. coli (Zn^{2+} added to culture). PET21a is the empty vector used to construct pRBL11 carrying MNC1. No 22kDa band was observed in PET21a protein lysate. **(b)** Oligomerization pattern seen in non-boiled protein lysate and non-boiled purified-dialyzed protein. **(C)** Western blot of MNC1

expressing in *P. pastoris* with pMYJ2.9 construct. No band was observed in the wild-type strain *x33* used for transformation.

Auxin Column

Auxin affinity columns were constructed by linking synthetic auxin NAA and 2,4-D to CarboxyLink™ immobilization column (Thermo Scientific) in attempt for purification of MNC1 and to show its auxin binding affinity. SDS PAGE and western blot were performed to confirm the presence of protein in column flow through, wash and different elution fractions (Figure 13). A 22kDa band was oberved in the NAA column with glycine elution when the column was used for the first time. The band became very faint starting from the second time the column was used. There was no band observed in the flow through or wash in the first two times of usage. However, since the third time, most protein came out in the flow through and wash with no visible 22kD protein band in elution fractions (Figure 13a).

Similar results were observed in 2,4-D column. It should be noted that the 22kDa band in the first time usage of 2,4-D column was fainter than that of the NAA column(Figure 13b). To test whether the column itself have affinity for MNC1, a mock column was constructed by linking acetate rather than synthetic auxin to the CarboxyLink™ column. A 22kDa band was observed in the flow through and no visible bands in wash or elution fractions was observed (Figure 13c). Proteins from each of the column slurries were then run on a SDS PAGE and western blot in order to locate the majority of MNC1 protein that went into the auxin affinity columns and never came out of flow through, wash or elution fractions in the later usage of the columns. MNC1 was found in the NAA columns and acetate column slurry (Figure 13d). 2,4-D column slurry

on another SDS PAGE and western blot experiment showed the same size band (data not shown). Thus, we were unable to conclude if MNC1 has auxin binding properties.

Germin-like protein properties

MNC1 is in the Rmlc-like cupin superfamily along with many germin and germin-like proteins. It is resonable to examine some biochemical activities that are present in other cupin superfamily proteins. IAA oxidase activity was examined because both germin and some GLPs has oxalate oxidase activity (Carter and Thormburg, 1999, Dumas, et al., 1993, Lane, et al., 1993). An IAA oxidase activity assay was performed on partially purified MNC1 protein since IAA has similar structure with oxalate, however, the IAA oxidase activity assay did not show an obvious difference when IAA was incubated with transformed culture and non-transformed control of *E. coli* culture. Nor did it show significant difference in spectrophotometer reading when IAA was incubated with purified-dialyzed protein product from transformed *E. coli* (data not shown).

A separate superoxide dismutase activity assay was employed because researchers have also shown that some native germin protein and some GLPs from mosses and higher plant exhibit superoxide dismutase (SOD) activity (Dunwell et al., 2004; Ohmiya et al., 2002). However, the SOD activity assay did not show a difference between transformed culture and non-transformed culture or between the His-tag purified MNC1 protein product of transformed *E. coli* culture and purified protein product from non-transformed (data not shown).

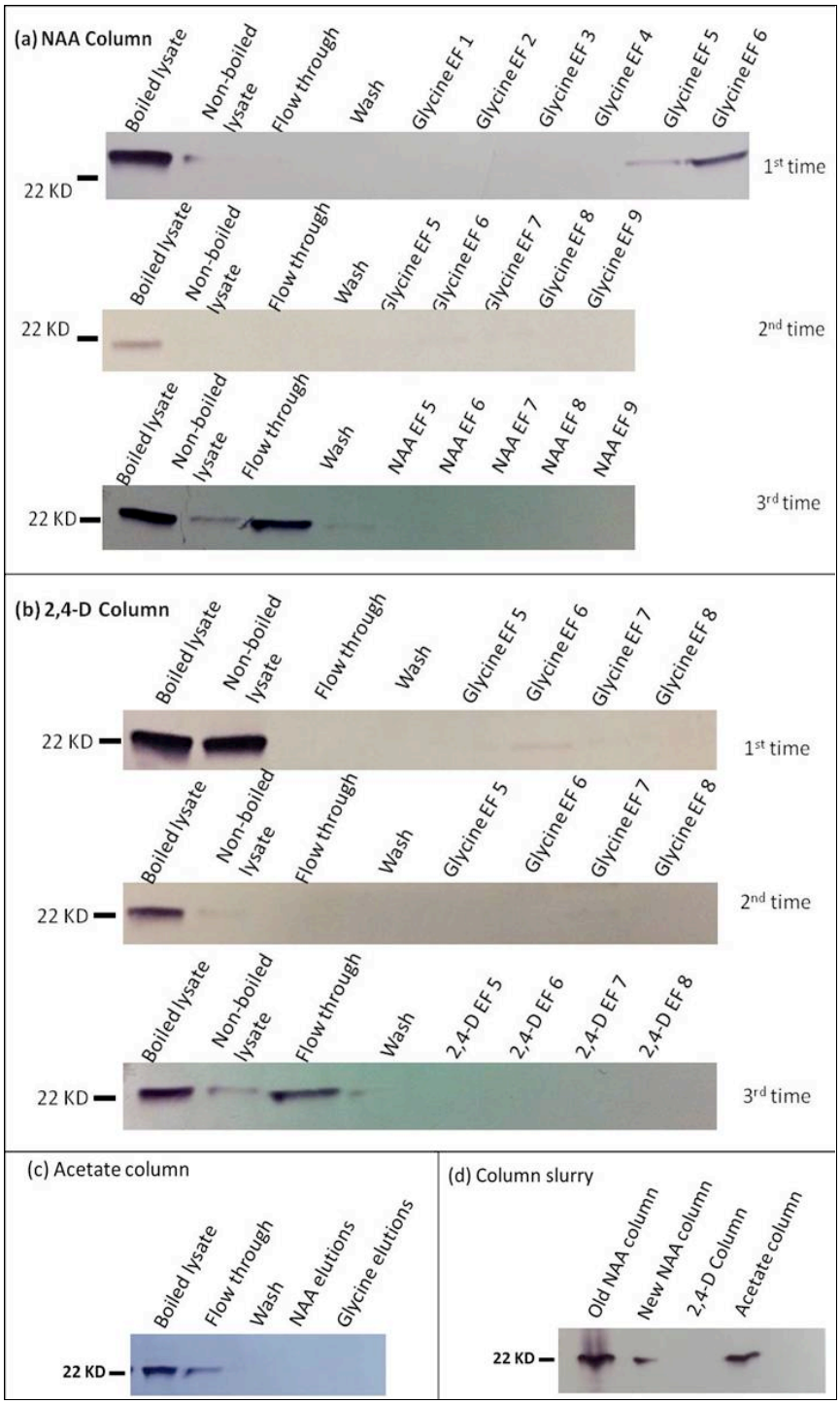

Figure 13: Auxin affinity column and control experiments. (a) NAA affinity column. 22kDa protein band observed in glycine elutions for the first two time use of the column. 22kDa protein band observed in flow through and wash since the third time of usage.**(b)** Faint 22kDa protein band observed in glycine elutions for the first two times of use of the column. Dark 22kDa protein band observed in flow through and wash in the third time of usage.2,4-D affinity column. **(c)** Control acetate column. 22KDa protein band observed only in flow through of the column. **(d)** Column slurry was run directly on SDS PAGE and western blot. 22kDa protein band in NAA column and acetate column slurry.

Discussion

Localization

MNC1 was predicted to be highly expressed in median nectary cells of *Arabidopsis thaliana* according to microarray data found in Kram et al., (2009). Direct imaging of MNC1::GFP flowers via confocal fluorescent microscope confirmed this prediction (Figure 9c). GUS histochemical staining of MNC1::GUS flowers showed the same result (data not shown). A small amount of ABP1 was also shown to be secreted from cell to perform important role in regulation of cell expansion and elongation through auxin-dependent responses (Woo et al., 2002).

MNC1 was also predicted to be secreted out of the cell by computational analysis. High concentration of mannitol treatment on plant cells can induce plasmolysis and create gap space between plasma membrane (PM) and cell wall. We expected that if MNC1 is localized in the area between PM and cell wall, GFP labeled MNC1 should exhibit diffused localization pattern in the gap space after the mannitol treatment rather than a sharp outline of the cell border observed in untreated cells. This predicted phenomenon was observed three times with confocal microscopy for the mannitol treated samples (Figure 4). It should be noted that 0.5M mannitol treatment was also used and resulted in an intermediate state of GFP signal diffusion between the untreated control and 1M mannitol treatment (data not shown) indicating the diffusion of GFP signal is dependent on the mannitol concentration dependent plasmolysis. Mannitol was used as an osmotic stress inducer in this treatment because it cannot be used as sugar source by plant

cells. Together with the PSORT prediction, it strongly indicates that MNC1 is indeed localized outside of the plasma membrane of median nectary cells.

Circadian Expression of *MNC1* and *PIN6*

PIN6 and *MNC1* were both hypothesized to have circadian oscillating expression pattern due to their important role and close correlation in regulating nectar production. An examination of expression of these genes from flowers collected every four hours for total of 36-hour duration showed that both *PIN6* and *MNC1* did have circadian expression patterns (Figure 5) coinciding with the nectar production circadian oscillation in *A. thaliana* (Bender et al., 2012b). *MNC1* expression coincided perfectly with nectar production circadian oscillation by having maximum expression at 4 h.a.d. (Figure 5a) which is also the maximum nectar production time point. Nectar production result (Figure 6) in this study has led us to hypothesize that *MNC1* is a negative regulator of nectar production possibly by controlling the maximum nectar production, which might explain why *MNC1* is only expressed at higher levels during the maximum nectar production period. *PIN6* was also expressed at 4 h.a.d., however the highest expression level for *PIN6* was at 12 h.a.d (Figure 5b). The exact reason for this expression level alternation is not clear, in order to decipher this particular result, future experiments can be done to collect *PIN6* mutant plants at 12 h.a.d. to examine expression levels of different genes and nectar production level.

Nectar production in *MNC1* mutant lines

MNC1 knock down mutant *mnc1* in Arabidopsis showed a 11.5% increase of glucose content in nectar (Figure 6). Although this result was not significant, the p-score for a paired student T-test was 0.058, which is almost at the 99.5% confidence interval. It was also observed that the nectar droplets under *mnc1* flower sepals were significantly bigger when compared to wild-type (Figure 2B), indicating MNC1 might be regulating nectar secretion at both sugar content and volume level. The MNC1 overexpresser (MNC1 T6) in Arabidopsis had a significant decrease of nectar glucose content compared to the wild-type plant (Figure 6), indicating the negative regulation by MNC1 on nectar production. The results showed in this section confirmed the specific role of MNC1 as a negative regulator of nectar production. Preliminary nectar production on *Brassica rapa mnc1* knockdown mutant plant also showed 7% increased nectar production (Larson and Carter, unpublished data) confirming this conclusion.

Auxin response

MNC1 was hypothesized to bind to auxin in our study because its sequence is similar to ZmABP1 and PpABP19, encoding two auxin binding proteins in *Zea mays* and *Prunus persica* respectively. The DR5::GUS/GFP auxin response activity reporter systems were employed in this study. MUG assay results confirmed that the MNC1 T6 overexpresser mutant showed significantly less auxin response activity (24.8% less than wild-type), while auxin response activity in *mnc1* was 37.5% more than wild-type on average, although, due to large error bar, the result was not statistically significant (Figure 8). The MUG assay result was consistent with direct GFP imaging result in which GFP signal in *mnc1* knockout mutant background showed various intensity levels in

lateral nectary. It was therefore concluded that *MNC1* can affect auxin response activity negatively when it is overexpressed, leading to the speculation that overexpressed *MNC1* in *pin6-2* knockout mutant lateral nectary and reduced auxin response activity in *pin6-2* (Bender et al., 2013) might be related.

MNC1 expression in *PIN6* mutants

An interesting result in Bender et al. (2013) was that the mutant *pin6-1,* which had an increase in PIN6 expression level, also had decreased auxin response activity in lateral nectaries. To investigate a possible role that *MNC1* plays in this phenomenon, *MNC1* expression level in *pin6-1* flowers was also examined. *MNC1* expression level was up-regulated in *pin6-1* at same level or even slightly more than its expression in *pin6-2* flowers (Figure 9a). Since the expression was examined from whole flower RNA extraction rather than nectary-specific RNA, MNC1::GFP was crossed to *pin6-1* mutant plant to image for the examination of localization and expression of *MNC1*. *MNC1* was found to be up-regulated in both lateral and median nectary of *pin6-1* flowers (Figure 9d) when compared to MNC1::GFP in the wild-type background (Figure 9c). This result can partially explain the reduced auxin response activity showed in *pin6-1* lateral nectary since *MNC1* is a negative regulator of auxin response and is up-regulated in lateral nectary of *pin6-1*(Figure 9a, 9d)

PIN6 expression levels were also up-regulated in both *MNC1* overexpresser and knock down mutants (Figure 9b). However, in the circadian expression result, *PIN6* was not up-regulated at all time points when *MNC1* was expressed in lower level as what was

observed in *PIN6* expression in *MNC1* mutants (Figure 9b). And when *PIN6* was not expressed in circadian time points or when it was highly expressed at 12 h.a.d., *MNC1* was not up-regulated either as what was observed in *pin6-2* and *pin6-1* mutants (Figure 9a). It was therefore concluded that the altered expression of *PIN6* and *MNC1* in the mutant lines of each other is not due to a direct cause of the expression level of the other gene. These results together suggested that *PIN6* and *MNC1* are likely working together instead of being antagonistic of each other in regulation of auxin level in nectaries.

MNC1 expression in auxin mutants

MNC1 expression in different auxin mutants was examined and showed down-regulation in the *tir1-1* mutant and slight up-regulation in both *iaaL* and *iaaM* mutant lines (Figure 10). It should be noted that in *iaaL*, there is less bioactive auxin, whereas in *tir1-1*, active auxin is still present in the cell but not perceived through the $SCF^{TIR1/AFB}$ pathway (Dharmasiri et al., 2005). The auxin response through $SCF^{TIR1/AFB}$ pathway has been well studied (Zheng et al., 2002), however, much evidence was presented for $SCF^{TIR1/AFB}$ -independent auxin signaling pathways such as ABP1 regulation, MAP kinase activation, INDOLE-3-BUTYRIC ACID-RESPONSE5 (IBR5) dual-specificity phosphatase regulation (Monroe-Augustus et al., 2003; Steffens et al., 2001; Mockaitis and Howell, 2000; Quint and Gray, 2006). From the expression results (Figure 10), we concluded that *MNC1* expression is more dependent on how much auxin is perceived through $SCF^{TIR1/AFB}$ pathway rather than available active auxin in plant cell. However, exogenous auxin treatment on MNC1::GFP/GUS plants did not show a significant difference in imaging result of either GFP direct imaging or GUS histochemical staining

(data not shown). MUG assays on exogenous auxin treated MNC1::GUS plants can be done in the future to further investigate this issue.

It should be noted that the result of *MNC1* expression being dependent on auxin signaling through $SCF^{TIR1/AFB}$ pathway (Figure 10a) was completely opposite from the nectar production pattern shown in *tir1-1* mutant and *iaaL* mutant. *iaaL* mutants produced significantly less nectar than wild-type plants (Klinkenberg, 2013) while *tir1-1* mutant produced significantly more nectar than wild-type plants (Bender et al., 2013), indicating that bioactive auxin available in cell is more important to nectar production, possibly through a $SCF^{TIR1/AFB}$-independent auxin pathway. Future experiment can be done to further confirm this finding by treating *iaaL* mutant plants with exogenous auxin (NAA) and conducting nectar production assay on treated *iaaL* flowers to check if the glucose content will be restored to the level of wild-type flower.

MNC1 Biochemistry

Although the alignment of AtMNC1 with ZmABP1 only showed conserved zinc binding residues and failed to show any of the predicted conserved auxin binding residues (Figure 11) from ZmABP1 by Woo et al. (2002), it is still reasonable to hypothesize that auxin binding property can be present in AtMNC1 for reasons followed: PpABP19 was previously characterized as an auxin binding protein in *Prunus persica*. Alignment of PpABP19 and ZmABP1 has only 15% identity and 28% positive sites with all the zinc binding sites conserved and only 2/7 proposed auxin binding related amino acids conserved in both proteins (Ohmiya et al., 2002, Woo et al., 2002). This indicates

that the proposed auxin binding related amino acids on ZmABP1 by Woo et al. (2002) are not strictly required in other auxin binding proteins. It is important to note that amino acid sequence alignment of AtMNC1 with PpABP19 had more identitive (32%) and positive (43%) sites than the alignment of PpABP19 and ZmABP1(identity=15%, positive=28%), indicating AtMNC1 is more closely related to PpABP19 than PpABP1 is to ZmABP1.

The expression of MNC1 in *E. coli* confirmed the size of AtMNC1 protein at 22kDa and indicated possible oligomerization with zinc interaction (Figure 12a, 12b). However, a confounding factor for expressing this eukaryotic protein in prokaryotic *E. coli* cell is that whether the protein was correctly folded to provide enzymatic activity such as superoxide dismutase activity or IAA oxidase activity was unknown. We propose that this might be the reason for the negative result of those enzymatic activity assays.

MNC1 is speculated to be secreted from nectary cells, and the MNC1::GFP imaging showed what appeared to be localization of MNC1 outside of plasma membrane (Fig 4). This is important since that the ABP1 was reported to bind auxin extracellularly as well (Woo et al, 2002). However, when MNC1 was expressed in *P. pastoris* with pPICZαA, which is a vector that targets its insert for secretion, MNC1 was not detected in media by western blot (data not shown), indicating it was not properly secreted. Multiple protein bands larger and smaller than 22kDa were detected in western blot of the transformed *P. pastoris* cell lysate (Figure 12c). The exact reason for the different sized protein bands is unclear. We speculate that the smaller sized bands could be due to partial

or unsuccessful secretion while the larger size bands could be due to oligomer formation observed in MNC1 expression in *E. coli* (Figure 12b).

Although auxin affinity column studies initially showed protein binding to both NAA and 2,4-D affinity columns, most protein came out of flow through and wash fractions in the later usage of the auxin columns (Figure 13a, b), indicating the columns were possibly saturated with protein bindings. Washing of the columns with both acidic solution and free auxin was not able to elute out proteins that were stuck in the column in later usages. Protein bands of 22kDa were detected in the column slurry itself of all auxin affinity columns (Figure 13d) showing proteins were permanently bound to the columns. A control column linked with acetate was constructed to be a negative control since MNC1 was not expected to show affinity toward acetate binding. A 22kDa protein band was observed in the flow through in the first time the column was used (Figure 13c), which was not observed in the first or second usage of auxin affinity columns (Figure 13a, b), indicating the interaction between MNC1 and auxin in auxin column is stronger than interaction between MNC1 and acetate in the acetate column. Unfortunately, a protein band sized 22kDa also showed up in the acetate column slurry (Figure 13d) showing that MNC1 could also be stuck in the column itself when there was no auxin present.

Future experiments can be done with radioactive isotope labeled protein to better locate the specific position of protein in affinity columns. It is also possible to run specific concentration of auxin through site-specific immobilized protein (Camarero, 2008) and detect flow through auxin concentration with a mass spectrometer. It is also

important to ensure proper folding of the protein expressed heterologously so that the protein is folded correctly or enzymatically active in future experiments.

Conclusion: Potential feedback loop of *MNC1*, *PIN6* and Auxin

Taking the results in this chapter together (Figure 14), *MNC1* is a negative regulator of nectar production in controlling maximum production of nectar. *MNC1* expression is partially auxin-induced through the TIR1-dependent pathway. *MNC1* works together with *PIN6* to control the homeostasis of cellular auxin level in lateral and median nectary cells by negatively regulate auxin. A construct overexpressing *MNC1* in whole plants (pMYJ1.3) has been made and is currently being transformed into DR5::GUS/GFP plants directly for further study of MNC1 and auxin relationship. As stated previously, auxin has a dual role on nectar production in relation to its concentration. Exogenous auxin treatment up to 100 μM NAA showed a positive correlation with nectar production and a sharp decrease was observed in high concentrations (Bender et al., 2013). Nectar production in *iaaL* and *tir1-1* showed that endogenous free auxin can effect nectar production positively through a TIR1-independent pathway. Therefore this potential feedback loop is crucial for nectary cells to maintain the homeostasis of free auxin through *PIN6* and *MNC1*in regulation of nectar production.

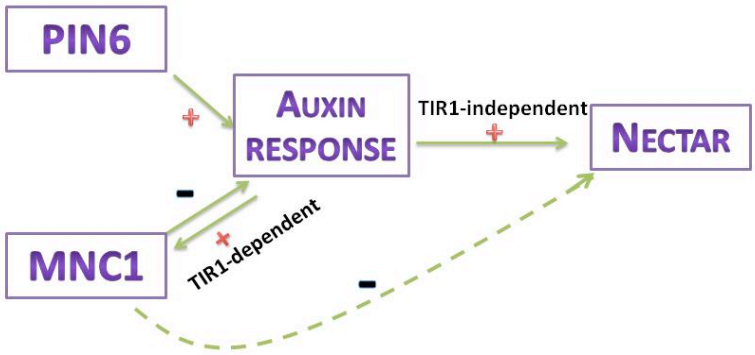

Figure 14: Feedback loop of *MNC1*, *PIN6*, auxin and nectar production. Positive sign over an arrow refers to a positive effect the factor at end of the arrow has on the factor at the head of the arrow; negative sign refers to negative effect; dash line arrows indicate indirect effect.

Material and Methods

Plant growth condition

All plants were grown on peat-based medium with vermiculite and perlite (Pro-Mix BX; Premier Horticulture) in individual pots. The plants were either housed in a Percival AR66LX growth chamber with 16h day/8h night, photosynthetic flux of 150µmol m^{-2} sec^{-1} and at 23˚ C or in a growth room with same 16h day/8h night with slightly lower photosynthetic flux at 22˚ C. It should be noted that plants for any direct comparisons were under same growth condition.

Mutant Lines

Arabidopsis thaliana MNC1 knock down mutant *mnc1* was made by artificial miRNA technique, the *MNC1* overexpresser mutant MNC1 T6 were made through DNA recombination by linking *MNC1* to SWEE9 promoter which is a strong nectary enriched

promoter (by Ricci Bender). Mutant plant that overexpresses *MNC1* in the whole plant was constructed by inserting *MNC1* gene into the PORE-E3 plasmid (with primers "*MNC1*-F Xho1" and "*MNC1*-R Xmal") and the construct was then transformed with Agrobacterium into wildtype DR5::GUS/GFP plants to observe the effect of overexpressing *MNC1* on auxin response activity in whole plant.

MNC1 localization

The localization of the *MNC1* gene was confirmed by the transcriptome data (Kram et al., 2009) and *MNC1*:: GFP confocal imaging and *MNC1*:: GUS histochemical staining (Jefferson et al., 1987)

MNC1 Biochemistry

Escherichia coli (Shuffle) and *Pichia pastoris* (*X33*) were used to heterologously express the MNC1 protein in order to study the biochemistry of MNC1 and auxin interaction. PET21 vector was used to construct the vector pRLB11 to express MNC1 in E.coli and pPICZαA was used to construct pMYJ2.9 vector to express MNC1 in *P. pastoris*. Zinc ion was added in the culture to facilitate protein function. Nickel and Cobalt His-tag column was used to partially purify MNC1 protein from *E.coli* and *P. pastoris*. SDS PAGE gels were done on protein lysate, flow through sample, wash sample and elution samples of different columns with boiled and un-boiled conditions. Western blot immunoassays were used as protein detection methods (anti-His-tag rabbit antibody as 1˚ab and Goat anti-rabbit antibody as 2˚ab). Auxin affinity columns were made by linking 2,4-D and NAA to CarboxyLink™ Immobilization Kit (Thermo Scientific). IAA oxidase activity at 245nm and 280nm was checked and super oxide dismutase activity

assays (Beauchamp and Fridovich, 1971) were done to further analyze the interaction between MNC1 and auxin.

RNA Isolation and Reverse Transcription PCR

To determine *MNC1* expression in Arabidopsis *PIN6* mutants, auxin mutants, and JA mutants, RNA was isolated from whole flower tissue using Agilent Technologies' Absolutely RNA Miniprep Kit. 500ng of RNA was converted to cDNA using Promega Reverse Transcription Kit. cDNA transcripts were amplified with the primer pair: "*AtMNC1* RT2-F" and "*AtMNC1* RT2-R" and ran on a 1% agarose gel to detect the presence of the *AtMNC1*. Similar to the expression screen of *AtMNC1, AtPIN6* transcripts were amplified in *MNC1* and *PIN6* mutants, but the primer pairs used were "*AtPIN6* RT-F" and "*AtPIN6* RT-R". *AtGAPDH* or *AtACTIN* was amplified as an internal standard using the primer pair "*AtGAPDH* RT-F" and "*AtGAPDH* RT-R" and "*AtACTIN* RT-F" and "*AtACTIN* RT-R" respectively.

GUS and GFP reporter system

The GUS (β-glucuronidase) enzyme can react with X-Gluc to change blue for histochemical staining. GFP (Green fluorescent protein) turns green when imaged under fluorescent microscope between 490-520nm. Mutant plants carrying MNC1 promoter driven MNC1-GUS/GFP fusion gene constructed (by Ricci Bender) to detect the localization of MNC1 expression. Confocal florescence and GUS stain imaging were performed on these mutant lines.

MNC1 overexpresser (MNC1 T6) and knock down (*mnc1*) mutants were crossed into to DR5:: GUS/GFP plants to compare auxin response activity between wildtype, MNC1 mutants plants by confocal direct imaging, GUS histochemical staining and MUG assay (Jefferson et al., 1987).

Fluorimetrical 4-methylumbelliferyl-beta-D-glucuronide assay (MUG)

10 flowers from each line were collected during the maximum nectar production time period for protein extraction and Bradford protein assay was employed to ensure 20 μg of protein were used in the MUG assay for each line. fluorescent nanodrop was used to measure the product of MUG assay reaction 4-methylumbelliforone signal (Jefferson et al., 1987) to compare the β-glucuronidase (GUS) activity among wildtype DR5::GUS, MNC1T6 x DR5::GUS and *mnc1* x DR5:: GUS in order to quantify the auxin response activity.

Nectar production assay

Amp-red glucose assay (Bender et al., 2012) was used to determine the sugar contents in the nectar of *Arabidopsis thaliana*. Comparisons were made between wildtype *A. thaliana* and MNC1 mutant lines.

Exogenous Auxin Assay

Exogenous NAA was added into ½ MS 10% Sucrose media to incubate crowns of wildtype and MNC1 mutant lines flowers (Bender et al., 2012). Exogenous NAA assays

were performed on MNC1::GUS and MNC1::GFP flowers to see the effect of auxin level on MNC1 expression and localization.

Mannitol Treatment

Mannitol treatment was used to induce plasmolysis with concentration of 0.5M and 1.0M mannitol dissolved in MilliQ water. Flowers were fully emerged in solution for more than one hour incubated in dark. PBS solution at pH 7.0 was used as control. Flowers was examined with confocal microscopy immediately after incubation.

Acknowledgment

I would like to thank the Hicks Lab at University of Minnesota Duluth for their fluorescence nanodrop, Dr. Charles Anderson at Pennsylvania State University for mannitol treatment suggestion, Dr. Carter for his patient confocal training, Ms. Lisa Wiesen for her help in collection of circadian flowers, Ricci Bender for many preliminary result and construct of *MNC1* mutant plants.

Table 1: Oligonucleotides used in this chapter**.**

Oligo Name	**Sequence**	**Purpose**
AtPIN6 RT F	TCAAACCCTCATGGTCCAACTCGT	*AtPIN6* RT-PCR
AtPIN6 RT R	AACGAGTAAGCATCGGAGGAAGCA	*AtPIN6* RT-PCR
AtMNC1 RT F	TCGAACCCAAAAGGTATTGCCGC	*AtMNC1* RT-PCR
AtMNC1 RT R	AGCAGTAAACTTTGACCTGAGCATGTT	*AtMNC1* RT-PCR
GAPDH RT F	TTCGGTGAGAAGCCAGTCACTGTT	*AtGAPDH* RT-PCR
GAPDH RT R	AAACATTGGAGCGTCTTTGCTGGG	*AtGAPDH* RT-PCR
ACTIN2 RT F	GTTGGGATGAACCAGAAGGA	At*ACTIN2* RT-PCR
ACTIN2 RT R	GAACCACCGATCCAGACACT	At*ACTIN2* RT-PCR
MNC1-F Xho1	AAACTCGAGAATCATCTCACTCACTAGCA	Cloning of MNC1 into PORE-E3
MNC1-R Xmal	AAACCCGGGTGGGAACTTGGGAGGCTAA	Cloning of MNC1 into PORE-E3
MNC1 PICHIA EcoR1 F	AAAGAATTCAACCCTACTCTCTCACTCCC	Cloning of MNC1into pPICZαA
MNC1 PICHIA Xba1 R	AAATCTAGATCAATGATGATGATGATGAT	Cloning of MNC1into pPICZαA

Chapter 3: Hormonal Regulation of Nectar production and secretion in *Arabidopsis thaliana*.

Introduction

Jasmonic Acid

Jasmonic acid (JA) is another plant hormone that has been heavily studied due to its important role in plant responses to herbivory and pathogens, as well as its involvement in root growth and flower development (Stotz et al., 2011; Staswick et al., 1992; Ishiguro et al., 2001; Reeves et al., 2012; Nagpal et al., 2005) It has been recently suggested that JA also affect nectar production in Arabidopsis and Brassica as JA was detected to reach peak levels prior to nectar secretion (Radhika et al., 2010). Additionally, research has also suggested that anther dehiscence and the initiation of nectar secretion are coincident (Schmid and Alpert 1977), both regulated by JA.

In Arabidopsis flowers, JA is synthesized in stamen filaments by converting phospholipids into jasmonic acid and requires a series of genes including *DEFECTIVE IN ANTHER DEHISCENCE1 (DAD1), LIPOXYGENASE (LOX), ALLENE OXIDE SYNTHASE (AOS), ALLENE OXIDE CYCLASE (AOC),* and *12-OXO-PHYTODIENOIC ACID REDUCTASE (OPR)* as shown in Figure 1 (Ishiguro et al., 2001, Turner et al., 2002). The most established JA signaling pathway is through the SCF^{COI1} JA receptor pathway (Figure 1). In contrast, research has also been done to show COI1-independent JA signaling pathway involved in plant defense responses and embryo development through OPDA which is the precursor of JA made by AOC (Taki et al., 2005; Stotz et al., 2011; Wasternack et al., 2012).

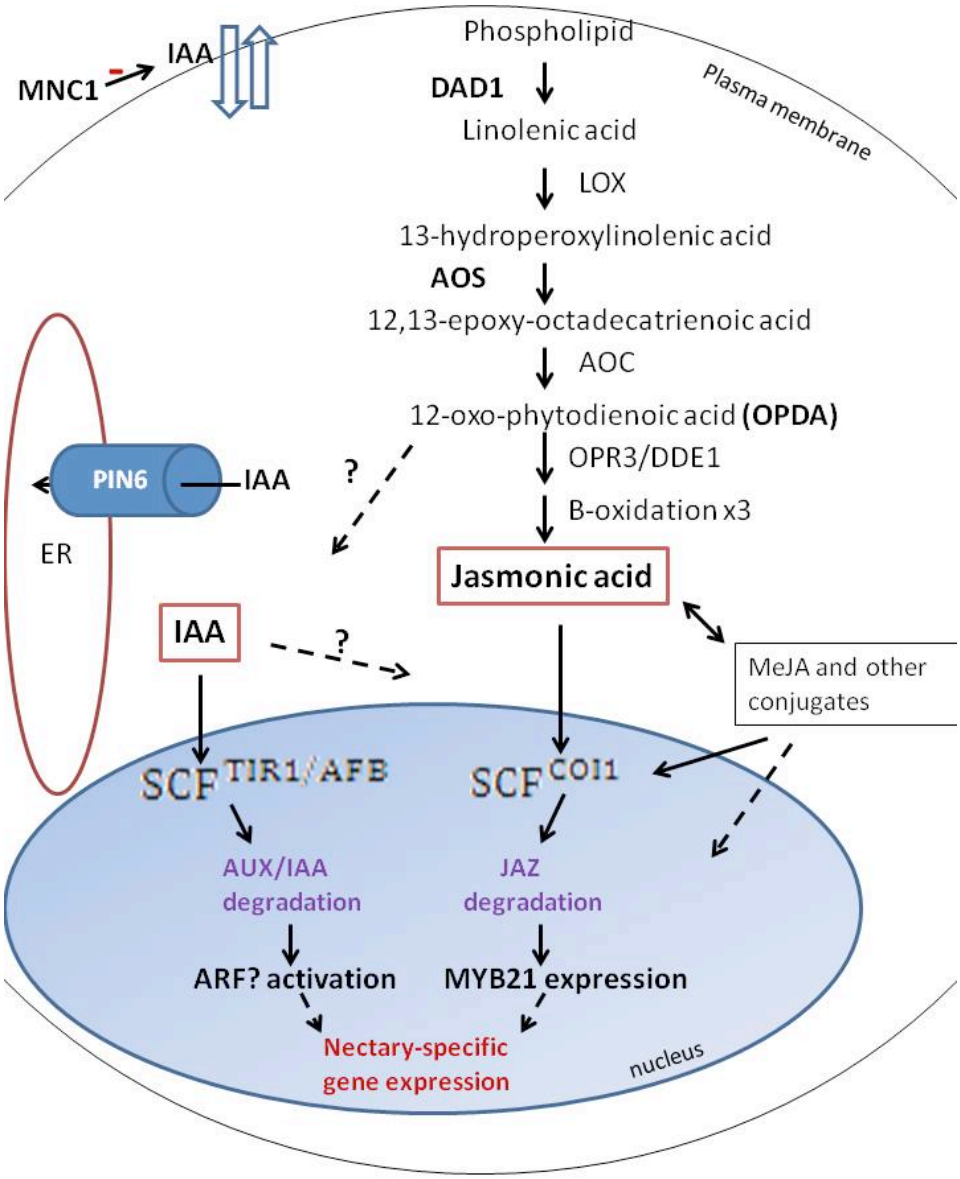

Figure 1: Possible auxin and JA crosstalk in regulation of nectar production and secretion. JA biosynthesis pathway from phospholipid to JA and JA-elle signaling through SCF^{COI1} pathway is presented on the right side of the cell with important genes bolded (*DAD1, AOS2, COI1* and *MYB21*). Auxin signaling pathway through $SCF^{TIR1/AFB}$ pathway is shown on the left side of the cell along with *PIN6* and possible *MNC1* regulation of auxin. Nectary specific genes are referring to *SWEET9, CWINV4, PIN6, MNC1,* and other possible nectary-specific genes that are induced by auxin and/or JA pathways.

This COI1-independent but OPDA-dependent JA signaling pathway (shown in Figure 1 with dashed arrows from OPDA) might be important to our study because previous studies found that JA biosynthesis mutants *aos2* and *dad1* are both male-sterile

and neither produces floral nectar unless treated with exogenous methyl jasmonate (MeJA) which is a natural derivative of JA (Klinkenberg and Carter, unpublished data), indicating that JA is required for nectary production or secretion. However, in this study, we found that the *COI1* knockout mutant *coi1-1* does produce nectar in old flowers which are the 2nd or 3rd opened flowers on a stem (Figure 2), indicating a COI1-independent pathway is involved in the JA signaling of nectar regulation, possibly through an OPDA-dependent pathway or other JA derivative signaling pathway.

Auxin

The phytohormone IAA (indole-3-acetic acid), also known as auxin, regulates plant growth and behavior including root growth, apical dominance, flower development, fruit development and many other aspects (Aloni et al., 2006; Dun et al., 2006; Overvoorde et al., 2010; Pattison et al.,2013). Previous studies have suggested that auxin plays a dual role in nectar production and regulation (Bender et al., 2013; Shuel, 1964). Bender et al. (2013) showed that exogenous application of NAA (synthetic auxin) up to 100 μM had positive effect on nectar production but a sharp decline of nectar production was observed with higher concentrations. Bender et al. also identified an auxin transporter *PIN6* that is required for auxin response and short stamen development (2013).

There is evidence for more than one auxin response pathway as discussed in Chapter 2 including the $SCF^{TIR1/AFB}$ pathway, ABP1 regulation, MAP kinase activation, and IBR5 dual-specificity phosphatase regulation (Monroe-Augustus et al., 2003;

Steffens et al., 2001; Mockaitis and Howell, 2000; Quint and Gray, 2006). We hypothesized that auxin can regulate nectar production in Arabidopsis through a TIR1-independent pathway because the *tir1-1* knock out mutant showed significantly more nectar glucose content than that of wild-type (Bender et al., 2013). However, *MNC1* expression in *tir1-1* was down-regulated (Figure 8 in Chapter 2) indicating *MNC1* is partially involved in TIR1-dependent auxin signaling pathway. It was also shown in Chapter 2 that *MNC1* expression levels in endogenous auxin mutants driven by nectary-specific *SWEET9* promoter *SWEET9pro::iaaL* (less auxin in nectary) and *SWEET9pro::iaaM* (more auxin in nectary) were not altered, indicating the amount of free auxin present in nectary do not directly regulate *MNC1* expression.

Although auxin regulation of nectar production and auxin regulation of *MNC1* expression might utilize different auxin signaling pathways, the down-regulated *MNC1* expression in both auxin receptor *TIR1* loss-of-function mutant and JA receptor *COI1* loss-of-function mutant show strong evidence that *MNC1* is involved in both phytohormone pathways of nectar regulation. Much evidence exists for auxin and JA crosstalk in flower maturation (Reeves et al., 2012; Cecchetti et al., 2008; Nagpal et al., 2005), thus, it was reasonable to hypothesize that nectar regulation is also under the control of potential auxin and JA crosstalk.

Potential genes involved in auxin and JA crosstalk in nectar regulation

TRANSPORT INHIBITOR RESPONSE 1 (TIR1) is a gene that encodes a protein containing an F-box motif and serves as an auxin receptor. Upon reception of auxin in

nucleus, TIR1 is responsible for mediating AUX/IAA (auxin response repressor genes) degradation, which leads to activation of auxin-regulated gene transcription (Ruegger et al., 1997; Dharmasiri et al., 2005). As stated previously *TIR1* loss-of-function mutant resulted in significantly increased nectar production (Bender et al., 2013) and down-regulated expression of *MNC1* (Bender and Carter, unpublished data).

AUXIN RESPONSE FACTORS (*ARF*) are transcription factors under control of the TIR1-dependent pathway of auxin regulation. *ARF6* and *ARF8* were shown to induce JA production for proper petal, stamen development and nectary formation (Reeves et al., 2012). According to the microarray data that identified nectary-enriched genes obtained by Kram et al. (2009), ARF6 and ARF8 were not particularly up-regulated in either median or lateral nectaries of *Arabidopsis thaliana*. The exact ARF(s) possibly involved in the auxin-JA crosstalk in nectar regulation is yet to be identified. Through data analysis, it was found that *ARF3* expression was up-regulated by 2-3-fold in both median and lateral nectaries when compared to other floral organs including carpels, sepal, petals and stamens (Kram et al., 2009). In the RNA-seq profile of *pin6-2* (knock down mutant of *PIN6*) by Bender et al. (2013), *ARF4* was also shown to have a 3.8-fold increase of expression in *pin6-2* mutant nectaries comparing to wild-type flower nectaries. Possible roles that ARF3 and ARF4 might play in auxin-JA crosstalk in nectar regulation were hypothesized.

CORONATINE INSENSITIVE 1 (*COI1*) is also a gene containing F-box that is functioning as the receptor of JA (Xie et al., 1998, Turner et al., 2002) which targets JAZ1 (repressor of JA-regulated genes) for degradation and results in JA-regulated gene

transcription upon JA binding (Thines et al., 2007). R2R3 MYB transcription factors MYB21 and MYB24 are encoded by two downstream genes (*MYB21and 24*) that are induced by this JA signaling pathway. Both of MYB21 and 24 promote petal and stamen growth (Reeves et al., 2012). *MYB21* was purposed to induce the expression of *SWEET9*, *CWINV4* and *PIN6*, indicating that JA signaling pathway is upstream of auxin signaling pathway (Klinkenberg, 2013). It was therefore hypothesized that *MYB21* can also induce the expression of *MNC1*.

The *MNC1-PIN6* feedback discussed in Chapter 2 is also important in the auxin-JA crosstalk since *PIN6* and *MNC1* expression are both altered in the JA synthesis mutants and the mutants of *MNC1* and *PIN6* both exhibit altered auxin response activity (Chapter 2; Bender et al., 2013; Klinkenberg, Jia, and Carter, unpublished data).

This chapter discusses the current evident for an auxin-JA crosstalk in regulation of nectar production and secretion and proposes future experimental direction for deciphering the mechanism of this crosstalk.

Results

JA is required for nectar secretion in Arabidopsis

Previous results showed that JA biosynthesis mutant *aos-2*, *dad1* and JA induced transcription factor MYB21 mutant (*myb21-4)* did not produce nectar in *Arabidopsis thaliana*. Exogenous MeJA treatment restored nectar production in *aos-2* and *dad1* flowers but not in *myb21-4* flowers (Klinkenberg and Carter, unpublished data). In light of these results, nectar production was examined in the loss-of-function mutant of JA

receptor COI1 (*coi1-1*). Surprisingly, *coi1-1* old flowers (usually 2nd or 3rd and older opened flowers that are older than stage 13 on a peduncle) produced significantly more nectar both in volume and in glucose content when comparing to that of wild-type, but young flowers (usually 1st or 2nd opened flowers that are older than stage 13 on a peduncle) did not produce any nectar (Figure 2). Anther dehiscence was not observed in either young or old *coi1-1* flowers.

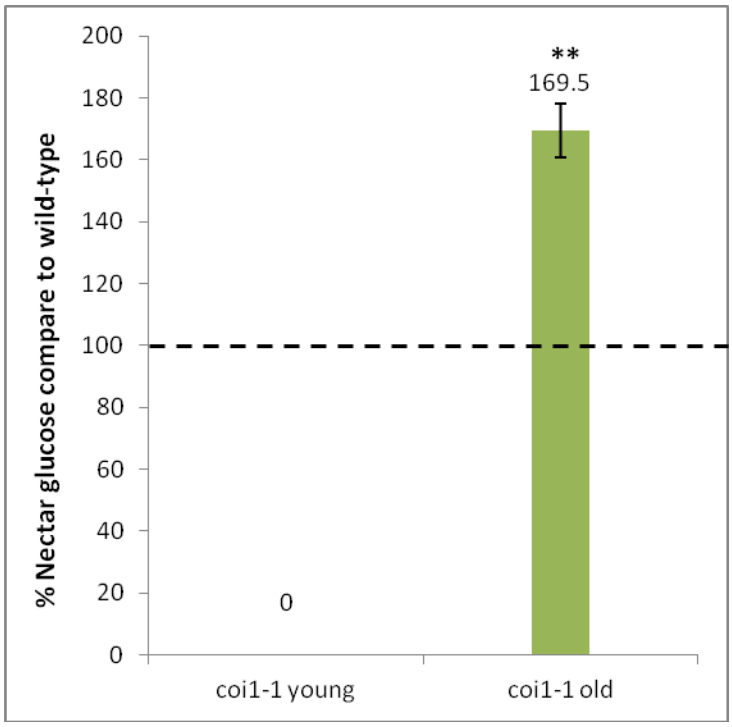

Figure 2: Nectar production in *coi1-1* young and old flowers. Nectar glucose assay was done between 4 and 8 h.a.d. which is the maximum nectar secretion time. Ten flowers were collected for each line with a repetition of 3 on different days. The mean percentage of glucose content in each type of *coi1-1* flowers comparing to wild-type was shown. Wild-type glucose content was set as 100% showing in dashed line. (Error bar= standard deviation, $p<0.001$)

Auxin response in JA mutants

The JA biosynthesis mutant *aos-2* showed a complete loss of DR5::GUS signal in nectaries and *dad1* showed a loss of DR5::GUS signal in young flowers with no nectar

but restoration of DR5::GUS signal in old flowers that produce nectar (Klinkenberg and Carter, unpublished data). To further elucidate the cross talk of auxin and JA in regulation of nectar production, *coi1-1* was crossed into DR5::GUS plant. GUS histochemical staining was done on *coi1-1* x DR5::GUS plants and no signal was detected in the nectaries of either young flowers or the nectar-producing old flowers (Figure 3) while wild-type DR5::GUS showed GUS signal in both median and lateral nectaries. However, DR5::GUS signal was detected in anthers and stamens of some *coi1-1* x DR5::GUS flowers (data not shown).

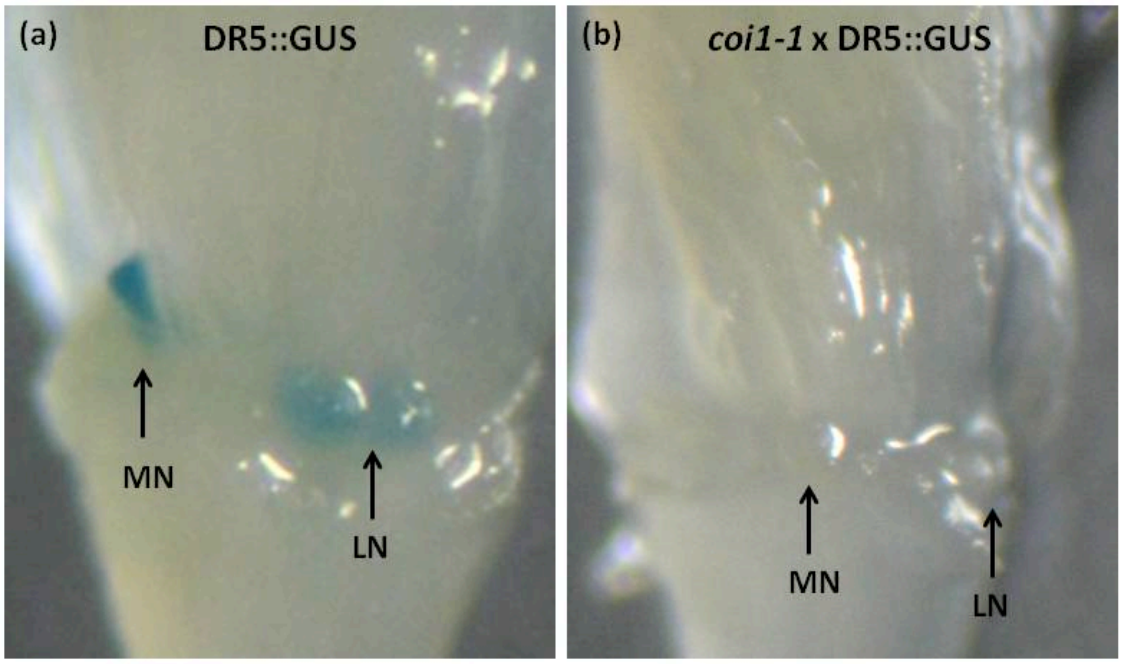

Figure 3: Preliminary GUS histochemical staining on *coi1-1*x DR5::GUS flowers. Blue staining was found on both median and later nectaries of GUS histochemical stained wild-type DR5::GUS flowers. No blue staining was found in nectaries of *coi1-1* x DR5::GUS plants in both young and old flowers. Image (b) here is a representative image of an old coi1-1 x DR5::GUS

Key gene expression in JA mutants

Previous result showed that *SWEET9* and *CWINV4* expression was not altered in JA synthesis mutant *aos-2* or *dad1* but was down-regulated in *myb21-4*. *PIN6* expression,

however, was down-regulated in all three mutants with restoration of its expression in MeJA treated *aos-2* (Klinkenberg and Carter, unpublished data). To further investigate the expression of key genes involved in nectar production in JA mutants, *MNC1* expression was examined in JA synthesis mutants, *MYB21* mutants (before and after exogenous MeJA treatment), and in the *coi1-1* background (Figure 4, 5).

MNC1 expression level was down-regulated in JA biosynthesis mutant *aos-2* and *dad1*, and was knocked out in *myb21*. *MNC1* expression was restored in both *aos-2* and *myb21* after exogenous MeJA treatment directly onto flowers (Figure 4).

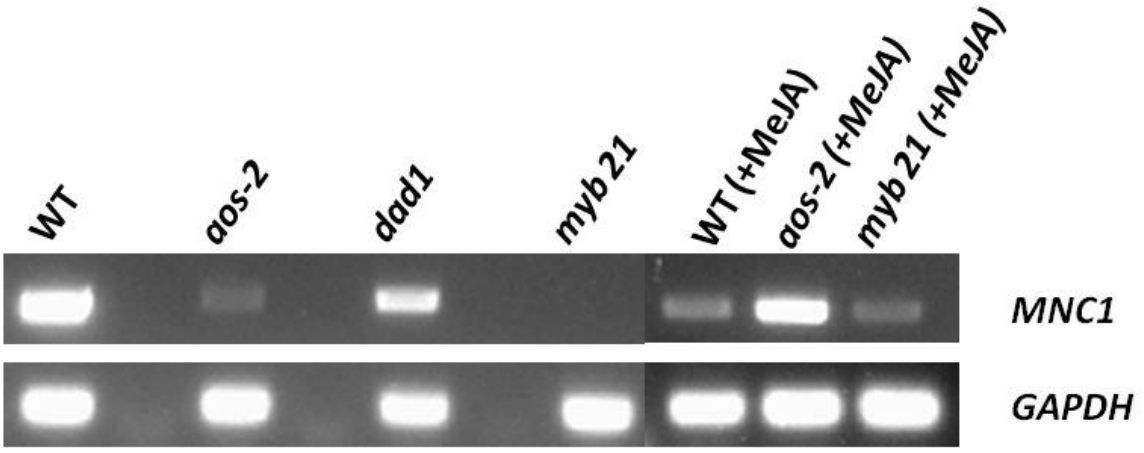

Figure 4: RT-PCR expression of *MNC1* in JA mutant plants and in MeJA treated JA mutants. Ten flowers were used for each line for the RNA isolation and RT-PCR. *MNC1* expression level was down-regulated in JA biosynthesis mutant *aos-2* and *dad1*. *MNC1* expression was knocked out in *myb21* (knockout mutant of JA induced gene *MYB21*). *MNC1* expression was restored in both aos-2 and myb21 after exogenous MeJA treatment. *GAPDH* was used as internal standard.

Expression of the key genes involved in nectar production and regulation were also examined in the COI1 loss-of-function mutant *coi1-1* flowers. Young *coi1-1* flowers that do not produce nectar and old *coi1-1* flowers that do produce nectar were collected separately for gene expression examination. *MYB21* expression was at similar levels in

coi1-1 young (at or after stage 13) and old flowers (at or after stage 13). *MNC1* expression level was down-regulated in both young and old flowers when comparing to wild-type. *CWINV4* expression, which is required for nectar production was up-regulated in *coi1-1* old flowers. *SWEET9* expression, which is positively correlated with nectar production, was down-regulated in *coi1-1* young flowers but upregulated in *coi1-1* old flowers compared to wild-type expression (Figure 5).

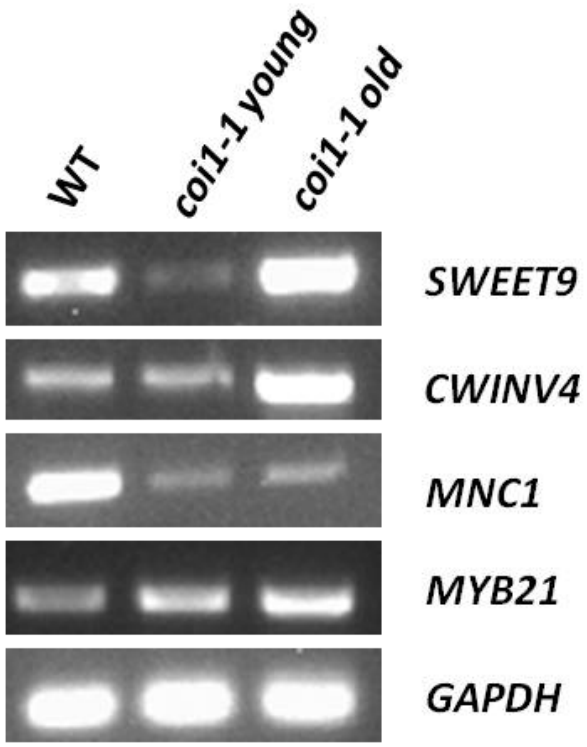

Figure 5: RT-PCR expression of nectary-specific genes in JA receptor COI1 loss-of-function mutant (*coi1-1*). Ten flowers were used in each line for the RNA isolation and RT-PCR. *MYB21* expression was at similar levels in *coi1*-1 young and old flowers (both at or after stage 13). *MNC1* expression level was down-regulated in both young and old flowers when comparing to wild-type. *CWINV4* expression was up-regulated in *coi1-1* old flowers. *SWEET9* expression was down-regulated in *coi1-1* young flowers but upregulated in *coi1-1* old flowers compared to wild-type expression. *GAPDH* was used as internal standard.

Discussion and Future Directions

JA dependent *COI1*-independent nectar production pathway

As stated earlier, JA was suggested to be required for nectar production (Klinkenberg and Carter, unpublished result). However, our recent result suggested that JA regulation of nectar production is independent of the well established COI1-dependent JA regulation pathway because nectar production was found to be significantly increased in *coi1-1* loss-of-function mutant plants compared to wild-type (Figure 2). It is remarkable that young *coi1-1* flowers produced no nectar even at stage 13 when nectar secretion normally begins, indicating the COI1-independent pathway might require a time lag in responding to JA signaling in the regulation of nectar production. Our results also showed that anther dehiscence was not required for nectar production because anther dehiscence was not observed in old *coi1-1* flowers despite the fact that nectar production was restored and even significantly increased in these flowers (Figure 2).

Auxin response in JA mutants

A previous study showed that the JA synthesis mutant *dad1* did not have nectar production or auxin activity response in young flowers but both nectar and auxin activity response were restored in old flowers (Klinkenberg and Carter, unpublished data). Given that *coi1-1* phenocopied *dad1* in restoration of nectar production in old flowers, auxin response activity in *coi1-1* mutant was also examined. However, auxin response activity GUS signal was not observed in *coi1-1* x DR5::GUS nectaries of either young flowers with no nectar or nectar-producing old flowers (Figure 3). However, additional GUS staining should be done on whole crowns of *coi1-1* x DR5::GUS flowers containing 4-5

opened flowers on the same peduncle to look for possible auxin response after nectar secretion began in old flowers for more definite conclusion (Figure 2).

Both auxin and JA has been suggested to play a role in anther dehiscence and filament elongation involving potential crosstalk (Sanders et al., 2000; Ishiguro et al., 2001; Nagpal et al., 2005; Reeves et al., 2012). Interestingly, auxin response activity was found in some *coi1-1 x DR5::GUS* stamens and anthers at stage 13 and later which is not normally seen in wild-type DR5::GUS plants, indicating auxin response activity was abnormal in stamens and anthers when JA was not perceived through the COI1-dependent pathway.

Model of auxin and JA crosstalk in regulating nectar production

SWEET9 and *CWINV4* expression were both up-regulated in *coi1-1* old flowers while *SWEET9* expression was down-regulated in *coi1-1* young flowers (Figure 5). This is consistent with the nectar production result in *coi1-1* young vs. old flowers (Figure 2) because a recent study showed that *SWEET9* is a nectary-specific sugar transporter that is required for nectar production and secretion. Knockout mutants of *SWEET9* did not produce nectar and an overexpresser of *SWEET9* produced more nectar when compare to wild-type (Lin et al., 2014). Additionally, CWINV4 knockout and knock down mutants also showed no nectar secretion (Ruhlmann et al., 2010). The up-regulation of SWEET9 and CWINV4 was a result of the loss-of-function mutation on *COI1*, indicating although the delayed COI1-independent pathway could signal for nectar production, it is not the main or regular pathway used in nectar production since gene expression of key nectary-specific genes were not under a fine control as wild-type.

MNC1 expression was down-regulated in both *coi1-1* young and old flowers (Figure 5). MNC1 knock down mutant *mnc1* produced 11.5% more nectar glucose than wild-type (Chapter 2, Figure 6). In the *coi1-1* old flowers, more nectar production and decreased *MNC1* expression level are consistent with the negative regulating role of MNC1 in nectar production. However *coi1-1* young flowers did have nectar secretion but *MNC1* expression level was still down-regulated. Unfortunately, *PIN6* expression examination in coi1-1 young vs. old flower was not successful due to primer malfunctioning. Future expression examination of *PIN6* in this mutant line should shine more light on the *coi1-1* nectar regulation pathway with the relationship of *MNC1-PIN6* co-regulation of auxin.

It was hypothesized that since the JA induced *MYB21* further induces *SWEET9*, *CWINV4*, *PIN6* expression, it will also induce the expression of *MNC1*. Our result showed that *MNC1* expression was strongly decreased in the *myb21* knockout mutant and exogenous MeJA treatment on *myb21* restored *MNC1* expression to wild-type level (Figure 4). However, when *MYB21* was expressed normally in *coi1-1*, *MNC1* expression was down-regulated (Figure 5). It can also be inferred from Figure 5 that *MYB21* can be induced with a COI1-independent pathway, possibly the same pathway that nectar production is regulated in *coi1-1* mutant. Result in Figure 5 indicates that *MYB21* does not directly determine *MNC1* expression level. Nonetheless, knock out of *MNC1* expression in JA synthesis mutant *aos2* and JA induced MYB21 mutant *myb21* and the restoration of its expression after exogenous MeJA treatment on these mutants showed

that *MNC1* expression can be induced by JA directly or indirectly. Future experiments can be done to determine if JA or precursors of JA could induce *MNC1* expression.

Reeves et al. (2012) proposed a positive feedback loop of auxin-JA crosstalk in flower maturation involves MYB21, MYB24 induced by JA and serve as negative regulator of JA biosynthesis pathway, and that ARF6, ARF8 induced by auxin can promote JA production which forms a positive feedback loop through COI1 (Reeves et a., 2012, Nagpal et al., 2005).This could serve as a base model for the construction of the auxin-JA crosstalk (Figure 1) in nectar regulation through both COI1-dependent and COI1-independent pathways. In Figure 1, the possible crosstalk between auxin and JA was hypothesized to be through an OPDA-dependent COI1-independent pathway and/or through auxin or JA induced transcription of nectary-specific genes such as *MNC1* and *PIN6*. There are still many unknowns and uncertain factors in this crosstalk, but what is obvious is that both auxin and JA are essential in nectar production of Arabidopsis. Future directions to decipher the auxin-JA crosstalk mechanism are proposed as follows.

Future directions

Identification of potential Auxin Response Factors involved in JA and auxin crosstalk is an essential step in deciphering the mechanism of the crosstalk-mediated nectar production and secretion. A recent study suggested ARF6 and ARF8 might be involved in nectary formation and function (Reeves et al., 2012). Additional study has also implicated that ARF6 and ARF6 can target OPR3 which is a biosynthesis gene of JA to regulation level of JA production (Nagpal et al., 2005). ARF3 and ARF4 are also

candidate genes for the reasons stated previously in introduction of this chapter. Expression level examination of ARF3, 4, 6 and 8 in *PIN6*, *MNC1*, *TIR1* and *COI1* mutant lines can provide important information for identifying potential ARF involved in this crosstalk.

TIR1 mutant (*tir1-1*) showed increased nectar production and decreased *MNC1* expression. DR5::GUS plants could be crossed into *tir1-1* to get tir1-1 x DR5::GUS plants and GUS Histochemical staining or MUG assays should be done on these flowers to elucidate the role of auxin in regulating nectar production.

AUXIN RESISTANT 1 (*AXR1*) is a gene required for auxin response. AXR1 protein is localized primarily in nucleus and is a subunit of RUB1-activating enzyme which regulates the protein degradation of SCF complex (Del Pozo et al., 1998). Both TIR1-dependent auxin response pathway and COI1-dependent JA response pathway utilize the SCF complex to degrade proteins. Research has also found that a mutant of *AXR1* (*axr-24*) did not respond to MeJA treatment suggesting that *AXR1* plays an important role in auxin-JA crosstalk in *Arabidopsis thaliana* (Tiryaki and Staswick, 2002). Relavent nectary-specific gene expression examination in this particular mutant might be informative.

Our results showed that JA synthesis mutants *aos2* and *dad1* did not produce nectar but *coi1-1* old flowers produced nectar, which led us to question if precursors of JA such as OPDA could play a role in the COI1-independent pathway of nectar production. In the biosynthesis pathway of JA, OPDA is a product of AOC and it is further catalyzed by OPR3/DDE1 to form OPC-8:0 (Figure 1). As stated earlier, OPR3

was suggested to be a potential target of ARF6 and ARF8 in regulation of JA level (Nagpal et al., 2005), indicating its important role in auxin-JA crosstalk. *DDE1/OPR3* (*dde1/opr3*) double mutants showed incapability of converting OPDA into OPC-8:0 resulting in accumulation of OPDA with no JA production (Sanders et al., 2000). If nectar production in Arabidopsis is indeed regulated through an OPDA-dependent pathway, nectar production should be expected in the *dde1/opr3* mutant line. What could also be done is to apply exogenous OPDA to *aos2*, *dad1* and *myb21* mutant lines and search for rescued nectar phenotype.

Conclusion

The auxin and JA crosstalk in regulation of nectar production involves complex hormone regulation mechanism and multi-gene regulation. A JA response pathway feedback loop and *auxin-PIN6-MNC1* feedback loop is intertwined in this cross-talk as shown in Figure 1. Nonetheless, it is important to decipher the mechanism of auxin-JA crosstalk in regulation of nectar so that researchers could have better understanding of phytohormone-mediated nectar regulation when studying the gene regulation and biochemistry of nectar production.

Material and Method

Plant growth condition

All plants were growing in the same condition as described in Chapter 2.

Mutant lines

Dr. Jason Reed at the University of North Carolina at Chapel Hill provided seeds for mutant lines of *aos2* and *dad1*.

Exogenous MeJA treatment

Flowers were directly dipped in a 500μM Methyl Jasmonate in a 0.05% Aqueous Tween 20 solution. RNA isolation was performed as described below.

GUS Histochemical staining

GUS staining was done following the protocol by Jefferson et al., 1987.

RNA isolation and Reverse Transcription

RNA isolation and reverse transcription was done in the same manner as described in Chapter 2.

Expression examination

Expression examination of different genes were done in the same manner as described in Chapter 2 with primers listed in Table 1.

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Table 1: Oligonucleotides used in this chapter**.**

Oligo Name	**Sequence**	**Purpose**
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
AtMYB21 RT F	TTCGGGATCATCAGCAGAAGCAGA	*AtMYB21* RT-PCR
AtMYB21 RT R	CGTGCTTGCAGCTTGATCGTTGAT	*AtMYB21* RT-PCR
AtMNC1 RT F	TCGAACCCAAAAGGTATTGCCGC	*AtMNC1* RT-PCR
AtMNC1 RT R	AGCAGTAAACTTTGACCTGAGCATGTT	*AtMNC1* RT-PCR
GAPDH RT F	TTCGGTGAGAAGCCAGTCACTGTT	*AtGAPDH* RT-PCR
GAPDH RT R	AAACATTGGAGCGTCTTTGCTGGG	*AtGAPDH* RT-PCR

Chapter 4: Concluding Remarks

This study has shown that the cupin-family protein MEDIAN NECTARY CUPIN 1 (*MNC1*) is highly expressed and mostly secreted from cells in median nectaries of *Arabidopsis thaliana* with a circadian expression pattern coincided with nectar production. *MNC1* knockout mutant (*mnc1*) showed more nectar production and more auxin response activity while *MNC1* overexpresser mutant (MNC1 T6) showed significantly less nectar production and less auxin response activity in nectaries. Taken together, we concluded that MNC1 is a negative regulator of nectar production in controlling maximum production of nectar.

MNC1 has a conserved zinc binding domain found in Auxin Binding Protein 1 and Auxin Binding Protein 19. Computational analysis showed that AtMNC1 is more closely related to PpABP19 than PpABP19 is to ZmABP1. Thus, we hypothesized that MNC1 negatively regulates nectar production, likely through an auxin dependent pathway. *PIN6* (At1g77110), an auxin transporter family protein, has been reported to be an auxin transporter localized to the ER and may modulate cytoplasmic free auxin concentration in nectaries (Bender et al., 2013). *MNC1* expression level in *pin6* mutants were upregulated and *PIN6* expression was also upregulated in *mnc1* mutant plants indicating *MNC1* and *PIN6* might work together to control the homeostasis of cellular auxin level in lateral and median nectary cells by negatively regulating auxin. *MNC1* expression was also partially auxin-induced through the TIR1-dependent pathway which was not the auxin-response pathway required for nectar production.

Transformed *Escherichia coli* and *Pichia pastoris* expressing MNC1 protein were made for studying the MNC1 biochemical nature, including auxin binding activity. However, the auxin affinity column results in this study were inconclusive. Future experiments using heterologous expression system requires more preliminary testing to ensure that the protein is properly folded and/or enzymatically active.

Jasmonic acid (JA) was also suggested to be required for nectar production with possible crosstalk to auxin in regulation of nectar production (Radhika et al., 2010; Klinkenberg, 2013). In this study, COI1-independent JA response pathway was suggested to regulate nectar production by altering the expression of nectary-specific genes such as *SWEET9*, *CWINV4* and *MNC1*. *MNC1* expression was found to be dependent on JA response since its expression in JA synthesis mutants *aos2* and *dad1* and JA induced gene *MYB21* mutant (*myb21*) were down-regulated and application of MeJA exogenously rescued *MNC1* expression in *aos2*. MNC1 expression was also down-regulated in COI1-1 loss-of-function mutant which had increased nectar production in old flowers, suggesting MNC1 expression can be regulated through both COI1-dependent and COI1-independent JA response pathway to regulate nectar production. The basis for a auxin-JA crosstalk mechanism was created based on results in this study and previous studies along with future directions suggested in other genes and molecules possibly involved in this crosstalk including *ARF*s, *TIR1*, *AXR1* and JA precursor OPDA.

It is important to decipher the mechanism of auxin-JA crosstalk in the regulation of nectar production so researchers can have better understanding of phytohormone-

mediated nectar regulation when studying the gene regulation and biochemistry of nectar production, which is important in many agricultural applications including the honey industry and crop yield.

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