Effects of Gibberellins on Nectar Production in *Arabidopsis thaliana*

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Dedication

I dedicate this thesis to all of the inspirational teachers I’ve had over the years, especially in the sciences.

Abstract

Gibberellins (GA) are well known for their roles in regulating stem elongation and seed germination, but less understood is the role of GA in regulating floral maturation. We recently identified *GA 2-OXIDASE 6* (*GA2OX6*, At1g02400) as being highly expressed in the actively secreting nectaries of *Arabidopsis thaliana*, but at low levels in other tissues. *GA2OX6* was previously demonstrated to inactivate bioactive GA. Multiple independent *ga2ox6* mutants displayed decreased nectar production, which suggests that elevated levels of active GA negatively regulate nectar production. Similarly, *spindly* (*spy*) mutants, which also have an increased GA signaling response, displayed decreased nectar production, further supporting the hypothesis that GA negatively regulates nectar production. Wild-type flowers also displayed an intense auxin response in actively secreting nectaries, whereas *ga2ox6* and *spy* mutants had strongly reduced DR5-dependent signal in nectaries. This suggests significant crosstalk occurs between GA and auxin signaling pathways in the regulation of nectar production.

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CHAPTER 1: Introduction

Nectar

Nectar is a sugar-rich liquid that plants secrete to reward animal pollinators and to attract protectors against herbivores (Baker and Baker, 1983; Heil et al., 2001). The nectar involved in pollination is floral nectar, since evolution has structured flowers such that when insects or other animal pollinators consume floral nectar they also pick up pollen, which they transfer to other flowers. Some plants also produce extra-floral nectar, which does not involve pollination, but rather it attracts insects that attack herbivores (Bentley, 1976). Herbivory has been shown to trigger the secretion of extra-floral nectar (Heil, 2001). Extra-floral nectar is even present in some species that predate angiosperms (flowering plants). For example, nectar secretion has been described in several genera of ferns (Koptur et al., 1982). Thus, it appears that nectar evolved first as a defense mechanism. Although nectar did not originate in angiosperms, it is now most common in angiosperms. It is estimated that 87.5% of angiosperms are pollinated by animals (Ollerton 2011).

Nectar is composed of 8-80% sugars (w/w), which are mainly glucose, fructose, and sucrose, though other sugars can be present (Baker and Baker, 1983). The ratio of sugars varies between species, but it tends to be consistent within a species. In *Arabidopsis thaliana*, the model system used in this study, nectar is hexose-dominant and contains roughly an equal amount of glucose and fructose

(Davis et al., 1994). In addition to sugars, there are many other components in nectar. Nectar is often rich in amino acids, which provide a source of nutrition for pollinators (Baker and Baker, 1973). Flowers pollinated by insects that have alternative sources of amino acids, such as pollen or insect prey, tend to have nectar that is lower in amino acids (Baker and Baker, 1975). In addition to sugars and amino acids, other constituents of nectar include organic acids (Baker and Baker, 1975), terpenes (Ecroyd et al.,1995), alkaloids (Deinzer et al., 1977), flavonoids (Ferreres et al., 1996), glycosides (Roshchina and Roshchina, 1993), vitamins (Griebel and Hess, 1990), phenolics (Ferreres et al., 1996), metal ions (Heinrich, 1989), oils (Vogel, 1969), free fatty acids (Kram et al., 2008), and proteins (Carter and Thornburg, 2004). Collectively, these molecules attract pollinators, provide nutrition for pollinators, prevent microbial growth, and sometimes deter visitation by nectar robbers (Adler and Irwin, 2005).

Arabidopsis as a model system

Arabidopsis thaliana is a model plant commonly used in research. Arabidopsis is in the Brassicaceae family, which means it is closely related to many agricultural crops including cabbage, kale, broccoli, brussel sprouts, and cauliflower (Arias et al., 2014). Arabidopsis is highly self-fertile, so it may seem surprising that the plant invests energy and resources into producing nectar. However, in the wild, Arabidopsis undergoes some outcrossing (less than 5%), and it is thought that outcrossing contributes enough to population fitness for the trait of nectar production to be retained (Abbott and Gomes, 1989).

Despite the fact that Arabidopsis produces small nectaries and very little nectar, Arabidopsis was a useful model system for this study, for several reasons: First, the genome of Arabidopsis has been fully sequenced (Kaul et al., 2000), thus many of the genes have been characterized, and many mutants are readily available; secondly, Arabidopsis can be easily transformed via Agrobacterium using the floral dip method (Zhang et al., 2006); third, Arabidopsis nectaries, while small, produce enough nectar to facilitate collection and quantification (Kram and Carter, 2009); finally, Arabidopsis has a short life cycle, and as previously mentioned, it is self-fertile, which makes it easy to grow many isogenic generations of a given line.

Nectaries

Nectar is produced in organs called nectaries. Across species, nectaries exist in a variety of forms. As shown in the model in Figure 1, Arabidopsis nectaries are small, roughly spherical glands located inside the sepals at the base of stamens. Arabidopsis flowers have a pair of lateral nectaries and a pair of median nectaries. The lateral nectaries are larger, better supplied with phloem, and they secrete 95% of the nectar (Davis et al., 1998). Lateral nectaries are the focus of this study. Many aspects of floral development are regulated by the so-called "ABC" genes (Weigel and Meyerowitz, 1994). For example, the ABC genes regulate the arrangement of main floral organs including sepals, petals, stamens and carpels, and this arrangement is conserved in angiosperms (Weigel and Meyerowitz, 1994). Unlike many floral organs, however, the development of

nectaries is not regulated by the ABC genes (Baum et al., 2001). Arabidopsis mutants for various ABC genes still develop nectaries. The only gene known to be essential for nectary formation is *CRABS CLAW* (*CRC*) (Bowman and Smyth, 1999).

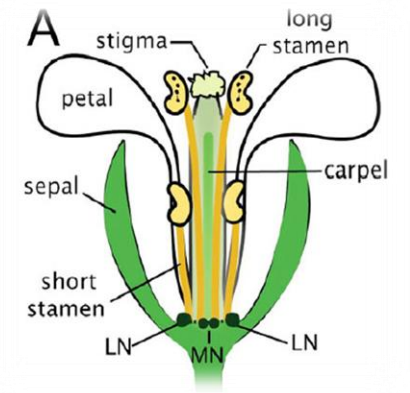

Figure 1. *Arabidopsis thaliana* floral arrangement. Side view of the flower shows two sepal, two short stamen, two long stamen, two petals, two lateral (LN) and two median nectaries (MN).

Nectar secretion

Nectar secretion is a complex, regulated process. It appears the main source of sugar for most nectaries is the phloem sap, though some sugars can also come from photosynthesis occurring in the parenchyma cells of the nectary itself, or in other parts of the flower (Pacini et al., 2003). However, nectar is often hypertonic to the phloem, which suggests that there is modification of phloem sap within the nectaries. Moreover, the major sugar in phloem is sucrose, and nectar in many species, including *Arabidopsis thaliana*, is hexose-dominant (Davis et al., 1994), which further indicates modification of the phloem sap within nectaries. As shown in Figure 2 [taken from Kram and Carter, 2009; adapted by Jia and

Klinkenberg], nectaries have an outer epidermal layer and inner layers of parenchyma cells. The nectaries are supplied with phloem (and, in some species, xylem), but the vasculature does not come into contact with the secretory cells in the parenchyma in Arabidopsis. Vasculature tissue is separated from the secretory cells by layers of parenchyma cells. This means that sugars from the phloem must travel through the parenchyma cells to eventually be secreted. Figure 2 diagrams proposed models of nectar synthesis. Sugars can travel via the symplastic route (through the parenchyma cells, connected via plasmodesmata) or via an apoplastic route (moving between cells, in the extracellular space).

Symplastic route for nectar secretion

Sucrose might be transported into the nectary cells directly, or sucrose might be broken down into hexoses before entering the nectary. There is strong evidence that once inside the nectary, sugars are stored as starch (Horner et al., 2007). Previous data from our lab indicate that at the time of nectar secretion, starch is broken down, and then sucrose is synthesized via sucrose phosphate synthase (Lin et al., 2014). Next, sucrose is transported out of the secretory cells by SWEET9. Once outside of the nectary, sucrose is cleaved into its hexose monomers by CELL WALL INVERTASE, and water follows via osmosis, forming the nectar droplet. This is known as eccrine secretion. Although there is strong evidence for this mode of nectar secretion in Arabidopsis, this does not exclude the possibility that some sugars are exported in vesicles which fuse with the

plasma membrane. That method of secretion is known as granulocrine secretion.

Apoplastic route for nectar secretion

Previous data from our lab strongly indicate that sugars are stored within nectary cells as starch, which means that they travel through the nectary via the symplastic route. However, that does not exclude the possibility that some sugars follow the apoplastic route. In the apoplastic route, sucrose could travel through the extracellular space until leaving the nectary through the permanently open stomata. Outside of the nectary, sucrose would be cleaved by CELL WALL INVERTASE into hexoses. Alternatively, sucrose could be converted to hexoses prior to traveling through the apoplastic space.

In summary, some details of nectar secretion are still unknown. For example, methods of nectar secretion appear vary between species. In *Arabidopsis thaliana*, however, there is strong evidence suggesting that *SUCROSE PHOSPHATE SYNTHASE* is involved in the synthesis of sucrose following the breakdown of starch at the time of nectar secretion, *SWEET9* transports sucrose out of the nectary, and that *CELL WALL INVERTASE 4* cleaves the sucrose in the extracellular space.

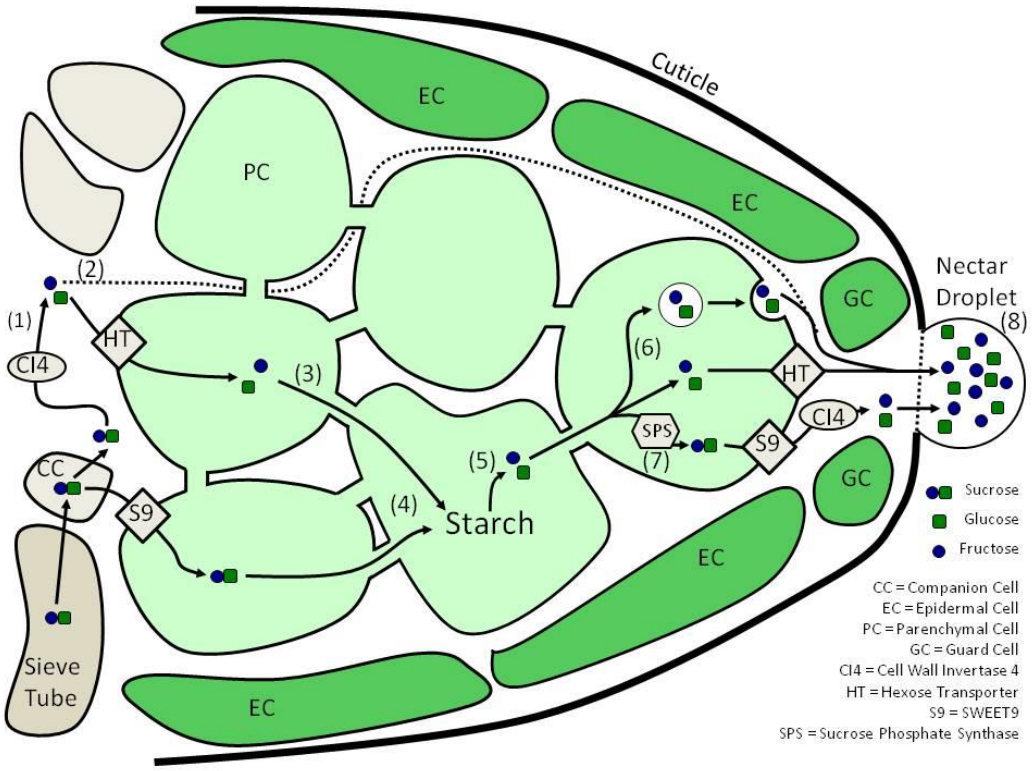

Figure 2: Nectary ultrastructure and proposed model of nectar synthesis. (Figure from Kram and Carter, 2009; adapted by Jia and Klinkenberg) In the symplastic route, sugars enter the nectary parenchyma cells from the phloem, where they are deposited as starch. At the time of nectar secretion, starch is broken down, and sucrose is synthesized by sucrose phosphate synthase. Sucrose is transported out of the nectary parenchyma cells via SWEET9, where the sucrose is then cleaved into hexoses by CELL WALL INVERTASE 4. Water follows via osmosis, forming nectar, which eventually leaves the nectary through permanently open stomates. In the apoplastic route, sugars travel through the nectary in the extracellular space.

Co-evolution of plants and pollinators

In many species, nectar composition and volume are tailored to the needs of pollinators, which suggests that co-evolution has occurred between the plant and its main pollinator(s). There is a general trend of increasing nectar volumes as pollinator size increases. Also, in general, plants pollinated by animals that

depend on nectar for all of their nutrition have amino-acid rich nectar. In contrast, plants pollinated by animals that get amino acids from protein or from insect prey have nectar that is less rich in amino acids (Baker and Baker, 1975, 1982; Baker, 1978). Pollinators have preferences for different nectar compositions, based on their nutritional needs. For example, honey bees prefer concentrated nectar (Heinrich1 975, Corbet 1978). Bees need to expend energy evaporating the water in nectar in the process of making honey, so if the nectar is too dilute they do not profit, energetically. Hummingbirds tend to prefer nectar that is high in sucrose (Baker and Baker, 1982), but many passerines prefer hexose dominant nectar because they lack the enzymes necessary to assimilate sucrose. Needs and preferences of pollinators have shaped the evolution of nectar in terms of volume and composition.

Hormonal regulation of nectar

Several aspects of nectar production are regulated. For example, nectar production begins at anthesis, the stage when the flower opens (Ren et al., 2007). This makes sense, as it would not be beneficial for the plant to expend resources producing nectar before the flower opens. Also, once pollination occurs, nectar production ceases. Nectar production is also dependent on the time of day. For Arabidopsis, nectar production peaks between 4 and 8 hours after dawn (h.a.d) (Búrquez and Corbet, 1991). It makes sense for a plant to secrete nectar only at times its pollinators are active. Regulation of nectar production depends, in part, on hormones (Bender et al., 2013; Heil, 2001). The

major plant hormones are abscisic acid, gibberellins, auxins, ethylene, cytokinins, brassinosteroids, jasmonates, salicylic acid and nitric oxide (Santner and Estelle, 2009). Although not all of the hormones have been studied in relation to nectar production, some hormones are known to be involved. Auxin, for example, is known to be strongly involved. The application of exogenous auxin has been shown to increase nectar production 10-fold, and the application of an auxin transport inhibitor reduced nectar production more than 2-fold (Bender et al., 2013). Jasmonic acid is known to stimulate the production of extrafloral nectar (Heil, 2001), but less is known about the role of jasmonic acid in the regulation of floral nectar. In Arabidopsis flowers, mutants for JA biosynthesis and signaling genes display reduced nectar production (unpublished data). The present study focuses primarily on the role of gibberellins (GA) and GA-related genes in regulating nectar secretion. Although GA is known for its role in seed germination and stem elongation, very little is known about its role in nectar production. Further details on GA and other hormones are provided in the following chapters.

Study impact

Nectar production is strongly correlated to pollination, which, in turn, affects crop yields. Plants that are not well pollinated produce low fruit yields (Nye and Anderson, 1974). Despite the importance of nectar in agriculture, the genes involved in nectar production are not well studied. This study will increase general knowledge of nectar regulation in an agriculturally relevant plant family.

A better understanding of nectar production might lead to the development of crops that produce more nectar or nectar that is more attractive to pollinators. It is also worth noting that managed bee populations, which are main pollinators for many important crops, are currently threatened. Populations of wild pollinators too, such as social bumblebees, solitary bees, hoverflies, wasps and butterflies, are also declining (Bailes et al., 2015). The decline in pollinators is potentially detrimental for agricultural crops, since 75% of the 115 highest producing crops, worldwide, depend on pollinators in order to produce maximum yields (Klein et al., 2007). In the development of crops that are more attractive to pollinators, nectar is a potential target for modification. If plants are bred or developed to make more nectar or nectar higher in nutritive value, pollination might be enhanced (Bailes et al., 2015).

Ch 2: GA regulates nectar production in Arabidopsis

Introduction

GIBBERELLIN 2-OXIDASE6 (GA2OX6)

In 2009, microarray data from our lab revealed nectary-enriched expression of gibberellin 2-oxidase-6 (*GA2OX6*), a gene that converts bioactive GA and its immediate precursors to inactive forms through 2β-hydroxylation (Rieu et al., 2008). Non-reversible deactivation by GA-2-oxidases is one of the main ways in which cellular levels of GA are regulated. Other means of maintaining GA homeostasis include regulation at the biosynthesis level, regulation of the expression of DELLA repressor proteins, and methylation of gibberellins (Daviere, 2008). The inactivation of gibberellins via methylation has only been shown in seeds, so far (Rieu et al., 2008). *GA2OX6* is one of five known GA 2-oxidases in Arabidopsis, all of which have been shown to inactivate bioactive GA's and/or their immediate precursors *in vitro*. There are two additional GA-2-oxidases, *GA2OX7* and *GA2OX8*, which are active against C_{20}-GAs rather than the bioactive C_{19}-GAs, so they are not always counted as part of the core GA-2-oxidases. The expression of each GA2oxidase depends on the tissue and the developmental stage. Of the five core GA2oxidases, *GA2OX2* and *GA2OX6* are the two most highly expressed throughout the whole plant (Rieu et al., 2008). *GA2OX6,* however, is the only one of the GA 2-oxidases that has been shown to

have nectary-enriched expression (Kram and Carter, 2009). Given the apparent up-regulation of *GA2OX6* in nectaries, the goal of this project was to further characterize the gene and its role in nectar production.

GA-signaling

The finding that *GA2OX6* has nectary-enriched expression led our lab to a broader interest in the overall role of GA in nectar production. The plant hormone gibberellin-A (GA) is well known for its role in stem elongation and seed germination (Kahn, 1957; Cosgrove and Sovonick-Dunford, 1989), but little is understood about the role of GA in nectar production and secretion. GA signaling is mediated through DELLA proteins, which are transcriptional repressors (Willige et al., 2007). DELLA proteins are members of the GRAS family of proteins, and they are defined by their N-terminal DELLA domain, which is absent from other GRAS proteins. As shown in Figure 3, when GA binds to the *GID1* receptor, the GA-receptor complex interacts with the DELLA protein. This interaction depends on the DELLA domain of the DELLA protein. The interaction of DELLA with the GA-GID1 complex increases the affinity of the DELLA for the SCF^{sly} ubiquitin ligase. Specifically, the F-box protein SLY is the subunit of the SCF^{sly} ubiquitin ligase that binds to the DELLA, leading to the ubiquitination and subsequent proteasomal degradation of the DELLA. DELLAs are localized to the nucleus, and they are transcriptional regulators, but they are not thought to bind to DNA directly (Daviere et al., 2008). It is thought that DELLAs interact with transcription factors, and that interaction either increases or

decreases the affinity of the transcription factor for its target promoter. For example, in Arabidopsis DELLA has been shown to interact with PIF3 and PIF4 (transcription factors), blocking their ability to bind to their target promoters (Daviere et al., 2008). In summary, the downstream effects of GA are carried out through the degradation of DELLA proteins.

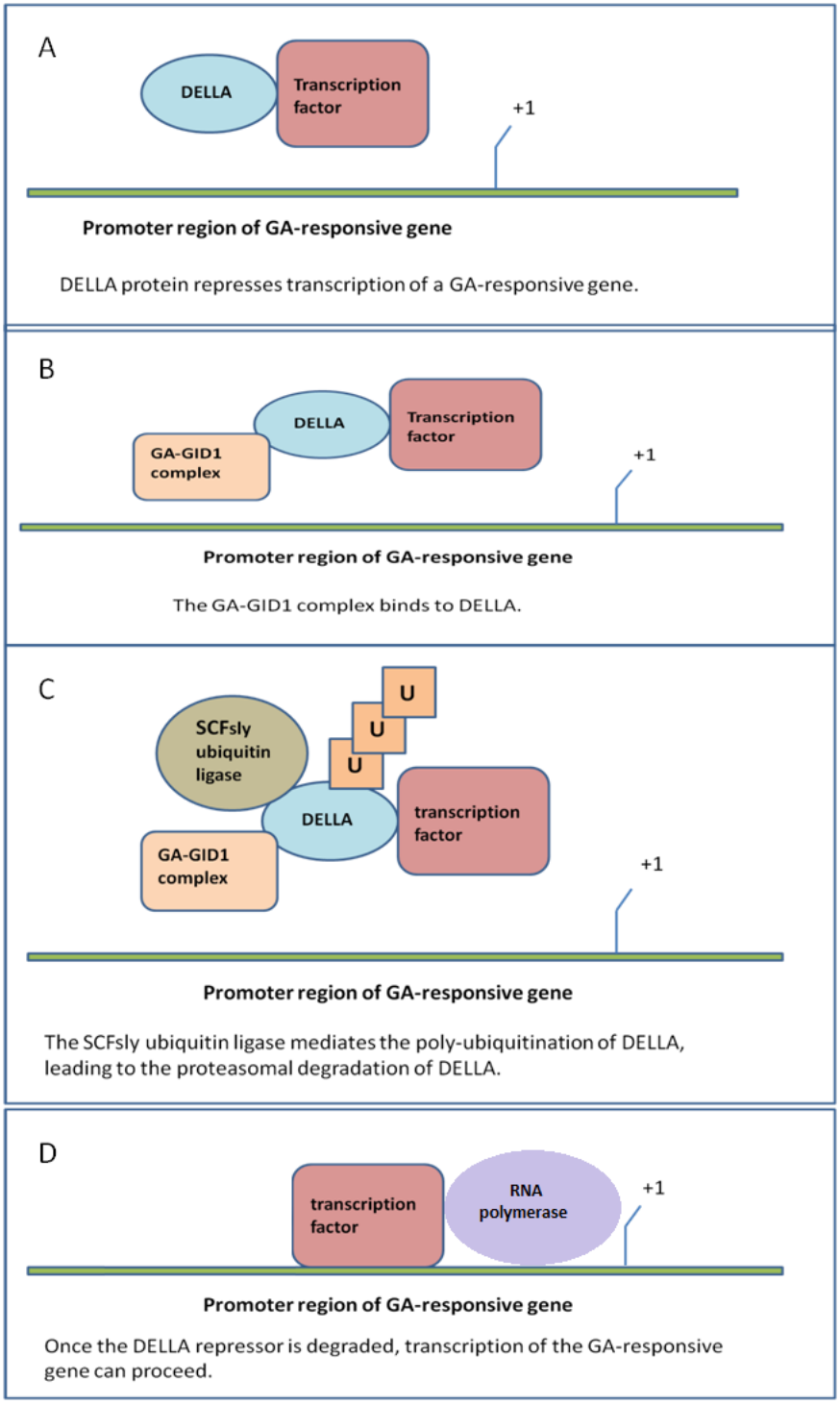

Figure 3: GA signaling is mediated through DELLAs. When GA binds to its receptor, GID1, there is a conformational change in GID1 that exposes a hydrophobic surface which interacts with the DELLA repressor protein. The binding of the GA-GID1 complex to DELLA increases the affinity of DELLA for the SCF^{sly} ubiquitin ligase, leading to the ubiquitination and subsequent proteasomal degradation of the DELLA. The degradation of the DELLA repressor allows for transcription of GA-responsive genes.

Targeted genes in this study

In the present investigation into the role of GA in nectar production, several genes involved in either GA regulation or GA biosynthesis were targeted. In addition to *GA2OX6*, described above, other targeted genes include *SPY*, *RGA*, *GAI, GA1, RGL, RGL2, and RGL3*. The roles of these genes in regulating GA responses are described below.

SPINDLY (SPY)

The "SPY" (SPINDLY) protein is an O-GlcNAc transferase, which catalyzes the transfer of N-acetylgucosamine to other proteins at serine or threonine residues (Scott et al., 2006.) Many proteins in plants and animals are O-GlcNAcylated as a post-translational modification, which can affect the localization of proteins, their stability, and/or their activity (Love and Hanover, 2005). Phosphorylation is another post-translational modification for some proteins, and O-GlcNAcylation can occur at the same site as phosphorylation, acting in competition with phosphorylation. In Arabidopsis, *SPY* is known to be involved in gametogenesis, circadian responses, light responses, and, most relevant to this project, *SPY* has been identified as a negative regulator of GA signaling (Jacobsen and Olszewski, 1993). Thus, in *spy* mutants there is increased GA signaling. The mechanism by which *SPY* negatively regulates GA signaling may involve *SPY* activating the DELLAs via GlcNAc modification (Silverstone et al, 2007). There is strong evidence that DELLA proteins are O-GlcNAcylated (personal communication with Neil Olszewski, unpublished data). In this study, three different *spy* mutants were investigated: *spy-3*, *spy-4*, and *spy-8*. These *spy* mutants, as expected,

have some phenotypes that resemble WT plants treated with GA. For example, shown in Figure 4, *spy-3* is tall, elongated, and spindly.

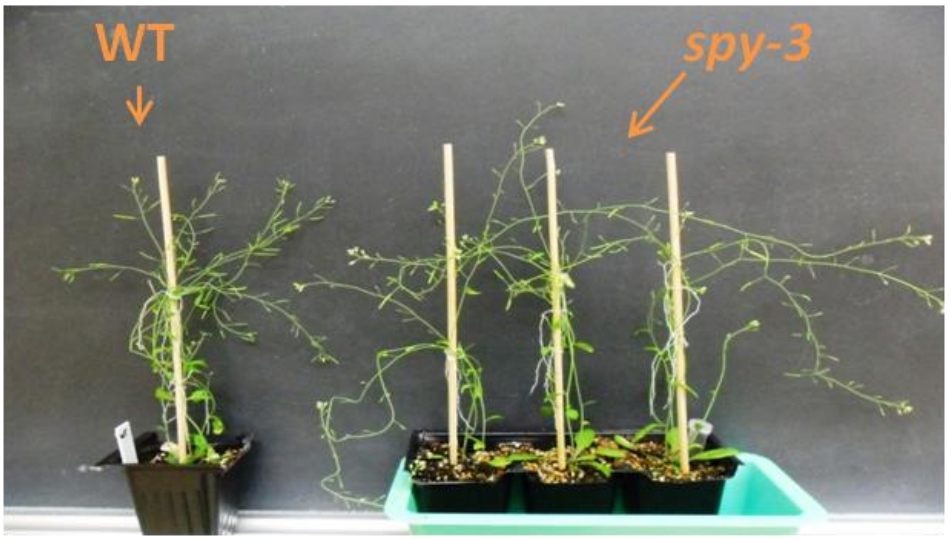

Figure 4: *spy-3* mutants exhibit a tall, elongated phenotype. In *spy-3* there is an increased GA response due to the decreased expression of *SPY*, an O-GlcNAc transferase that represses GA response. The mechanism through which *SPY* represses GA responses likely involves the post-translational modification of DELLA repressors.

RGA, GAI, RGL, RGL2, RGL3

The five DELLA repressor proteins in Arabidopsis (RGA, GAI, RGL, RGL2, and RGL3) are highly homologous (Daviere et al., 2008). As previously described (Figure 3), DELLAs mediate GA signaling, because DELLAs are degraded in response to GA in a ubiquitin-proteasome dependent manner. In the present study, a DELLA quintuple mutant, lacking expression of all five DELLAs was included to examine the effects of increased GA signaling.

GAI, and GA1

In the previously described DELLA quintuple mutant, *GAI* is knocked out along with the other four DELLA proteins. An additional line, *gai*, has a mutation in the DELLA domain, preventing the degradation of GAI, thereby causing a GA-deficient phenotype throughout the whole plant. That line was included to observe the effects of decreased GA signaling. Another GA-related gene, *GA1,* encodes a key enzyme in the GA biosynthesis pathway, which converts geranylgeranyl pyrophosphate to copalyl pyrophosphate (Sun and Kamiya, 1994). Therefore, *ga1* mutants are severely deficient in GA, and they require supplemental GA in order to germinate and grow. *ga1* was included in this study to further examine the effects of GA deficiency on nectar production.

This chapter focuses on the effects of GA-related mutations, as well as GA treatments and GA-inhibitor treatments, on nectar production in Arabidopsis.

Results

In order to examine the effects of GA on nectar secretion, several approaches were used. In particular, the results presented below represent an examination of mutant lines predicted to have an increased GA response.

Spatial and temporal expression of *GA2OX6*

Microarray data from Kram et *al*. (2009) indicated that *GA2OX6 (*AT1G02400) is highly expressed in nectaries relative to other tissues (Figure 5A). To verify that

finding, the GUS reporter system was used to visualize *GA2OX6* expression in Arabidopsis flowers. 2 kb of the promoter region of *GA2OX6* was cloned upstream of the GUS reporter gene in the vector pORE-R2. As shown in Figures 5B and 5C, *GA2OX6* is highly expressed in nectaries. This finding confirmed the nectary-enriched expression of *GA2OX6* seen in the microarray data. Subsequently, expression of *GA2OX6* was characterized in pre-anthesis flowers and post-anthesis flowers. As shown in Figure 6, expression of *GA2OX6* is much higher in post-anthesis flowers, during the stage in which nectar is produced. Expression of *GA2OX6* is low when flowers are in the pre-anthesis stage, the stage prior to the onset of nectar secretion. Collectively, these results support the hypothesis that *GA2OX6* is involved in the regulation of nectar production.

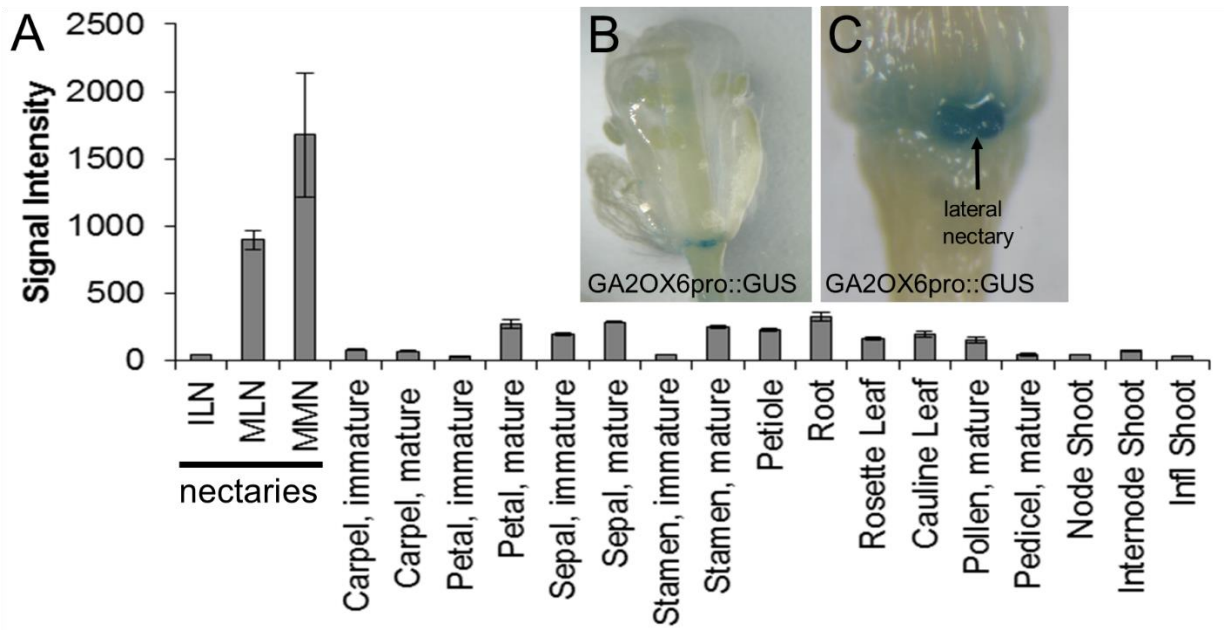

Figure 5: *GA2OX6* displays nectary-enriched expression. (a) Normalized mean ATH1 GeneChip probe set signal intensity for Arabidopsis *GA2OX6*. Original array data for all tissues were described in Kram et al. (2009). ILN = immature lateral nectaries; MLN = mature lateral nectaries, MMN = mature median nectaries. Inset: staining of GUS activity in the nectaries of Stage 14-15 *GA2OX6pro:GUS* flowers. (b) GUS staining in whole flower. (c) Close-up image of GUS staining in a lateral nectary.

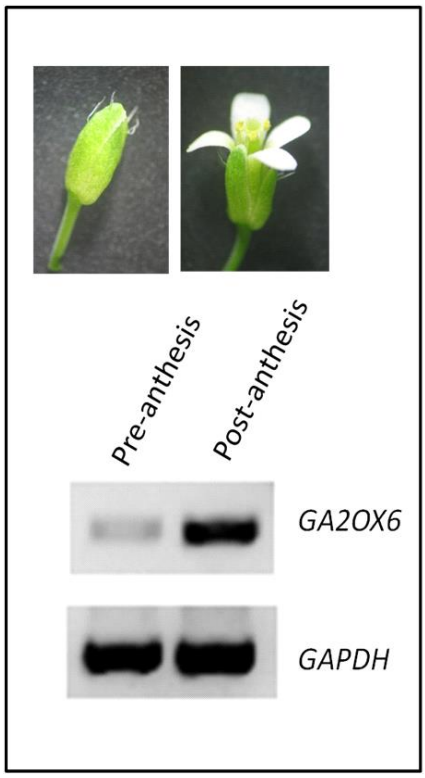

Figure 6: Temporal expression of *GA2OX6*. The temporal expression of *GA2OX6* was examined by semi-quantitative PCR. This demonstrated that *GA2OX6* expression is higher after anthesis, which is also when nectar production occurs.

Effect of increased GA response on nectar production

To determine the potential role of *GA2OX6* nectar production, two independent T-DNA mutants were identified in *Arabidopsis thaliana*. The T-DNA insertion site for *ga2ox6-1* (SALK_044189C) is located in the promoter region 22 bp from the transcriptional start site, and the T-DNA insertion site for *ga2ox6-2* is in the first intron 1,037 bp downstream from the transcriptional start site (Figure 7). Mutants were confirmed to be homozygous by PCR and previously demonstrated to have decreased expression levels compared to wild-type.

Figure 7: T-DNA insertion sites for *ga2ox6-1* and *ga2ox6-2*. The insertion site for *ga2ox6-1* is in the promoter region (22 bp upstream from the transcriptional start site), and the insertion site for *ga2ox6-2* is in the first intron, 1,037 bp downstream from the transcriptional start site. Gray shading represents the 5' and 3' untranslated regions, whereas the black shading represents coding regions.

In addition to the *ga2ox6* mutants, three mutants for *SPINDLY* (*SPY*) (*spy-3*, *spy-4*, and *spy-8*) and a DELLA quintuple mutant [*gai*, *rgl1*, *rgl2*, *rgl3*, *rga*] (Feng et al., 2008) were characterized. All of the above-mentioned mutants have an increased GA response, which is similar to what is hypothesized to be occurring in *ga2ox6* mutants. The *spy-3* mutant has a point mutation at G593 (G to S); *spy-4* has a T-DNA insertion in the promoter, and *spy-8* has a large deletion from M354 to Q376 (Silverstone, 2006). Nectar was quantified with an enzymatic assay in *ga2ox6-1*, *ga2ox6-2*, *spy-8*, *spy-3*, and the DELLA quintuple mutant. As shown in Figures 8 through 12, nectar production was decreased significantly in all five mutant lines. The ratio of nectar production (mutant/ wild-type) was 0.65 for *ga2ox6-1*(Figure 8), 0.83 for *ga2ox6-2* (Figure 9), 0.61 for *spy-3* (Figure 10), 0.53 for *spy-8* (Figure 11), and 0.75 for the DELLA quintuple mutant (Figure 12).

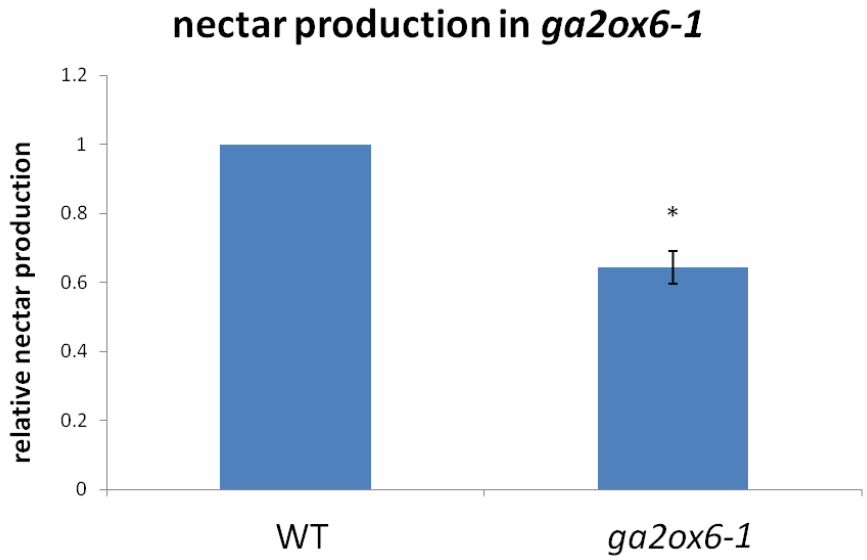

Figure 8: Nectar production in *ga2ox6-1*. Nectar production in *ga2ox6-1* is 0.65 relative to nectar production in wild-type. ***p <0.005 by paired t-test**

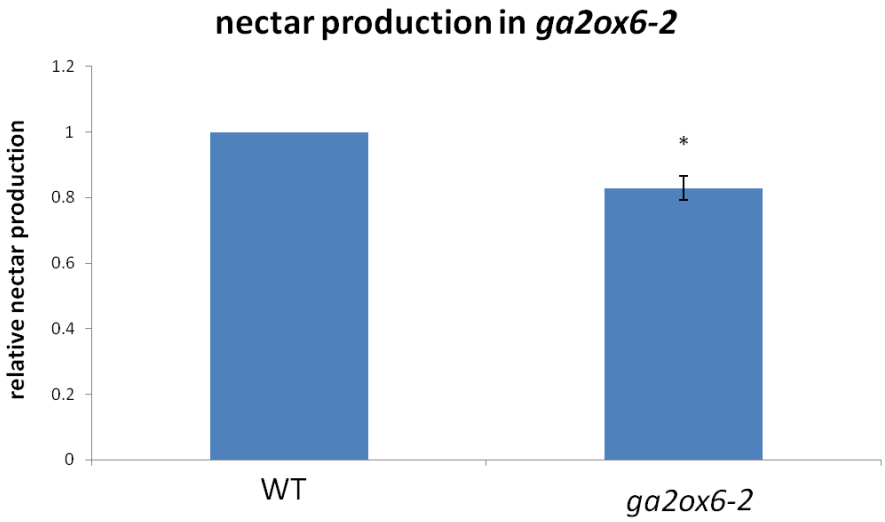

Figure 9: Nectar production in *ga2ox6-2*. Nectar production in *ga2ox6-2* is 0.83 relative to nectar production in wild-type. ***p <0.05 by paired t-test**

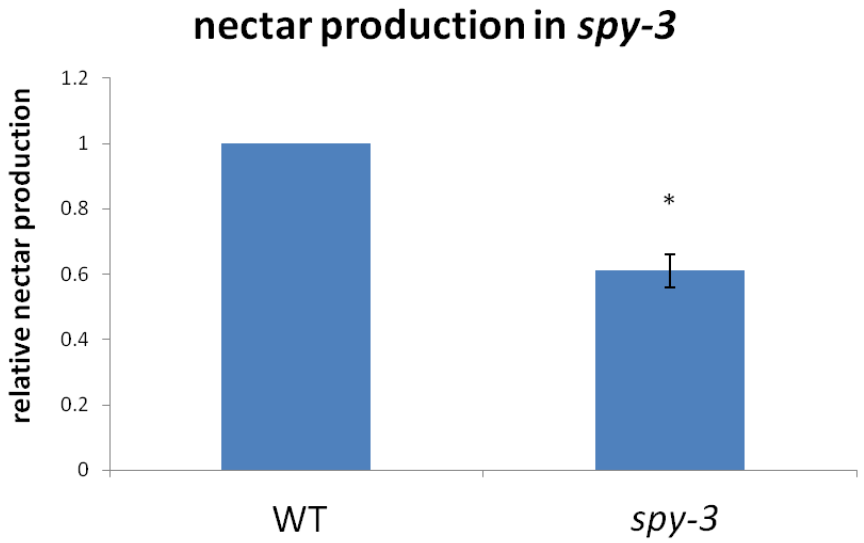

Figure 10: nectar production in *spy-3*. Nectar production in *spy-3* is 0.61 relative to nectar production in wild-type. ***p <0.01 by paired t-test**

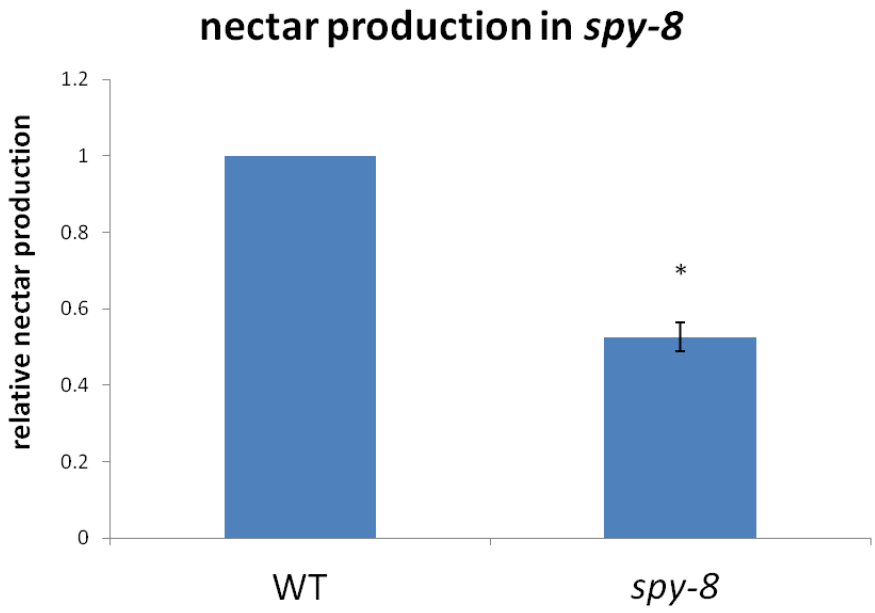

Figure 11: Nectar production in *spy-8*. Nectar production in *spy-8* is 0.53 relative to nectar production in wild-type. ***p <0.005 by paired t-test**

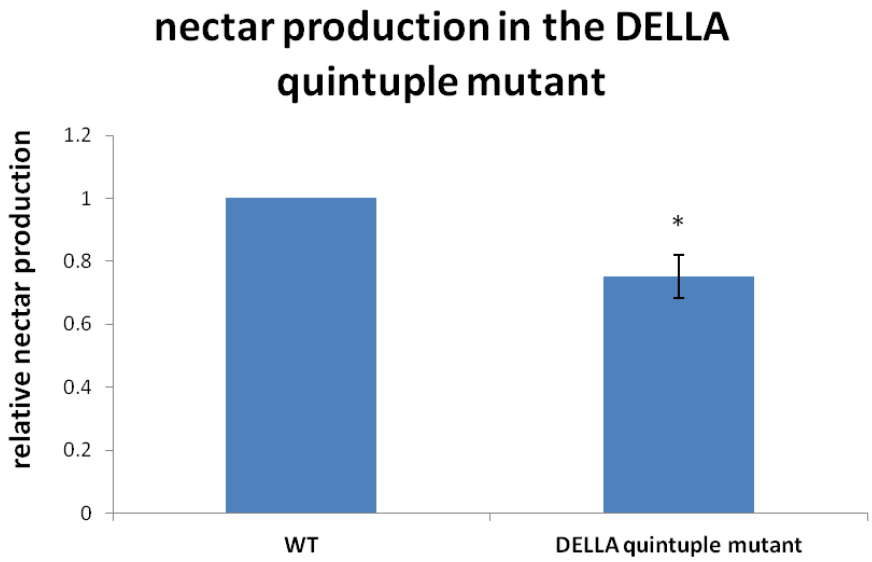

Figure 12: Nectar production in the DELLA quintuple mutant. Nectar production in the DELLA quintuple mutant is 0.75 relative to nectar production in wild-type. ***p <0.01 by paired t-test**

The reduction in nectar production in *ga2ox6-1* indicated that GA negatively regulates nectar production. To further test this hypothesis, *ga2ox6-1* was treated with paclobutrazol, a GA synthesis inhibitor. In the paclobutrazol treated plants, nectar production was restored almost to wild-type levels (Figure 13).

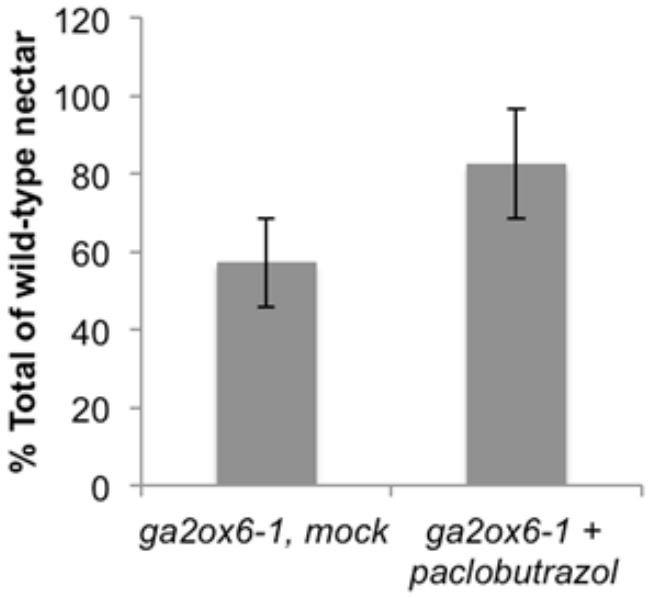

Figure 13: Treating *ga2ox6-1* with paclobutrazol, a GA synthesis inhibitor, partially restored nectar production. In *ga2ox6-1*, a mutant line with increased GA response and decreased nectar, nectar production was partially restored with paclobutrazol treatment. This supports the hypothesis that GA negatively regulates nectar production. Moreover, this finding confirms that GA signaling is increased in *ga2ox6-1*.

Expression of other genes essential for nectar production

Given the reduction in nectar observed in mutants for *GA2OX6*, *SPY*, and in the DELLA quintuple mutant, the expression of previously characterized genes involved in nectar secretion was examined. *AtCWINV4*, a nectary specific invertase required for nectar secretion (Ruhlmann et al., 2010), had significantly decreased transcript levels in *ga2ox6-1* relative to wild-type (Figure 14A). Similarly, the expression of *MYB21*, a transcription factor known to be necessary

for the full expression of several genes required for nectar production, was significantly decreased (Figure 14B).

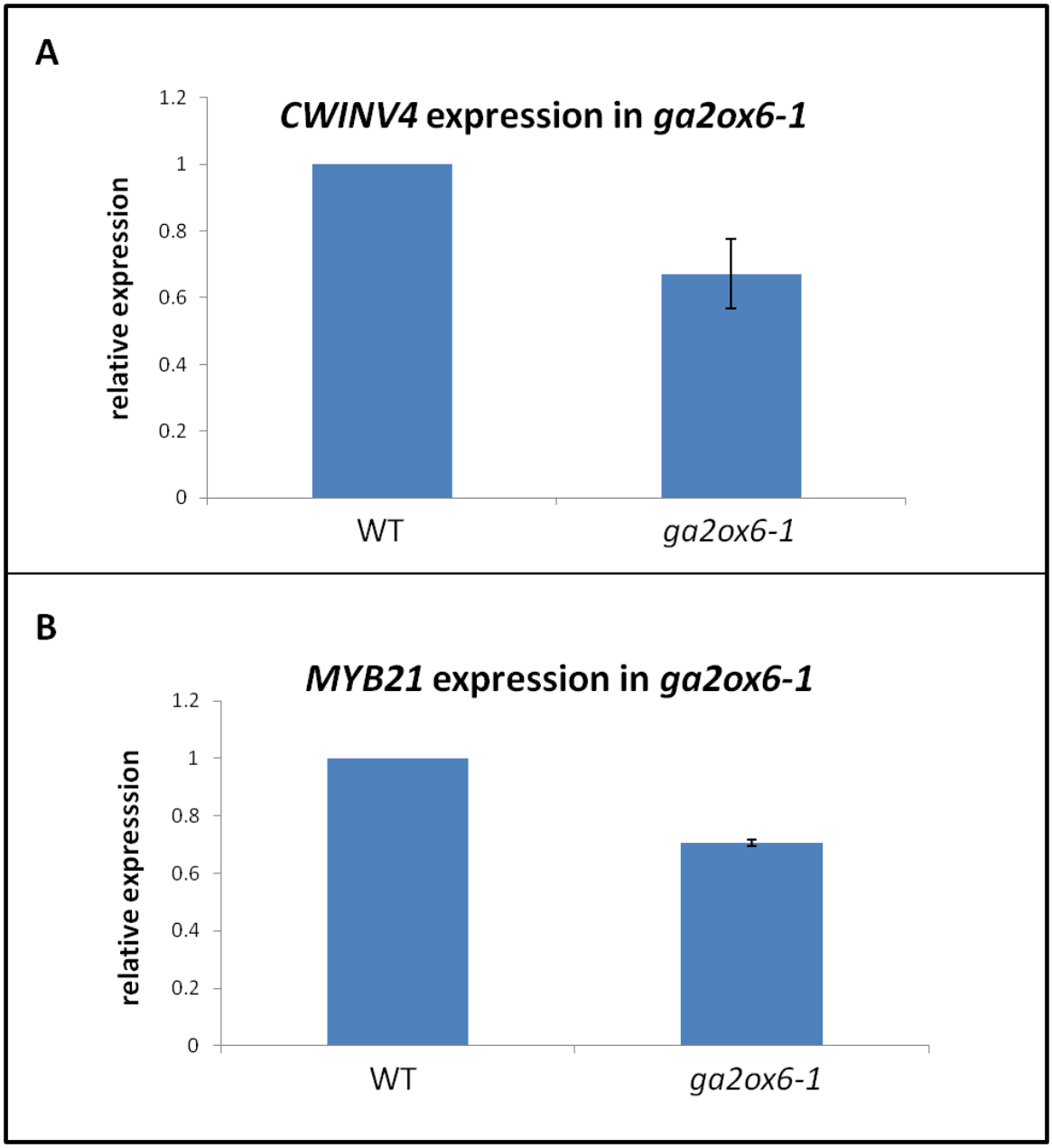

Figure 14: Expression of A) *CWINV4* and B) *MYB21* in *ga2ox6-1*. CWINV4 is an invertase required for nectar production and MYB21 is a transcription factor required for the expression of many genes in nectaries. The decrease in *CWIN4* and *MYB21* expression in *ga2ox6-1* may partially explain the mechanism through which GA negatively regulates nectar production.

DELLA proteins are degraded in response to elevated GA levels. Thus, it was hypothesized that mutants with elevated GA signaling may have decreased DELLA accumulation in nectaries. To test this hypothesis, GA signaling was characterized in *spy-8* via a RGA-GFP reporter (RGA is a DELLA protein). Additionally, qRT-PCR was used to examine the expression of *GA2OX6* in *spy-8*. Contrary to the hypothesis, RGA, the DELLA protein expressed most highly in nectaries, was present at higher amounts in *spy-8* nectaries than in wild-type (Figure 15). GFP fluorescent signal intensity in *spy-8* was over 3-fold higher than in wild-type nectaries (Figure 16). Further, *GA2OX6* expression was more than 3-fold higher in *spy-8* flowers than in wild-type (Figure 19). Accumulation of RGA was not observed in *ga2ox6-1* (Figures 17 and 18).

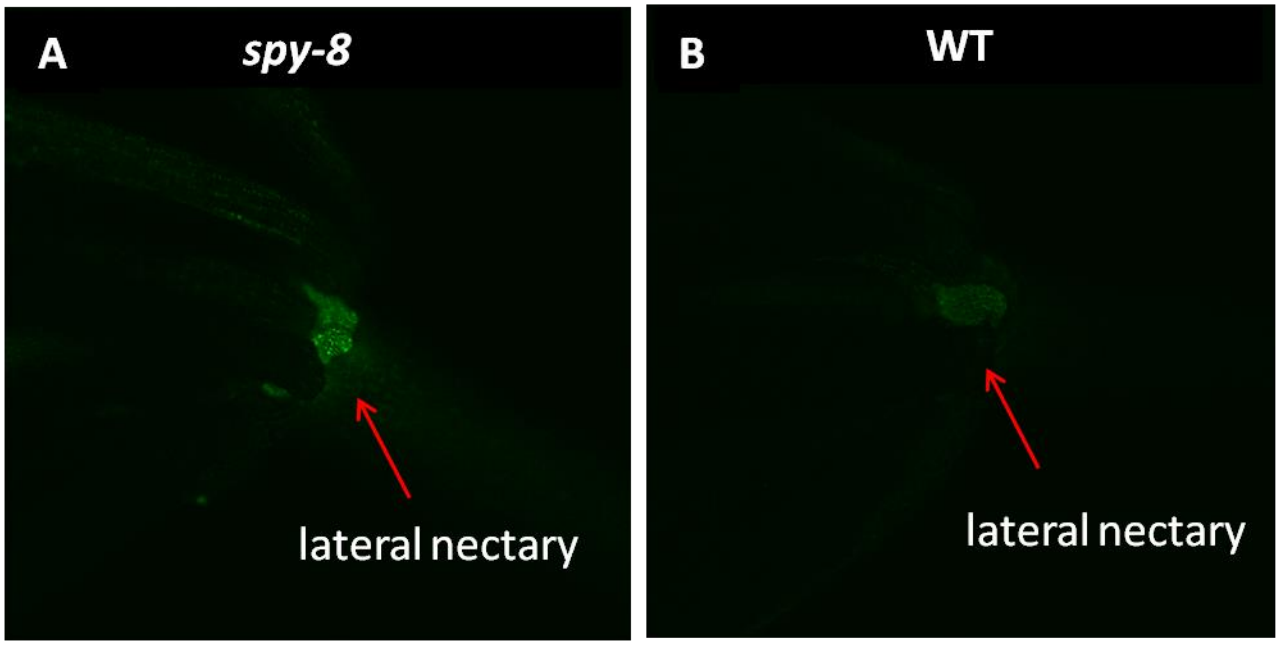

Figure 15: Confocal imaging of RGA::GFP in lateral nectaries. Fluorescent signal is 3.23 times higher in *spy-8* (A) relative to WT (B), indicating that RGA is present in *spy-8* in higher amounts than in wild-type.

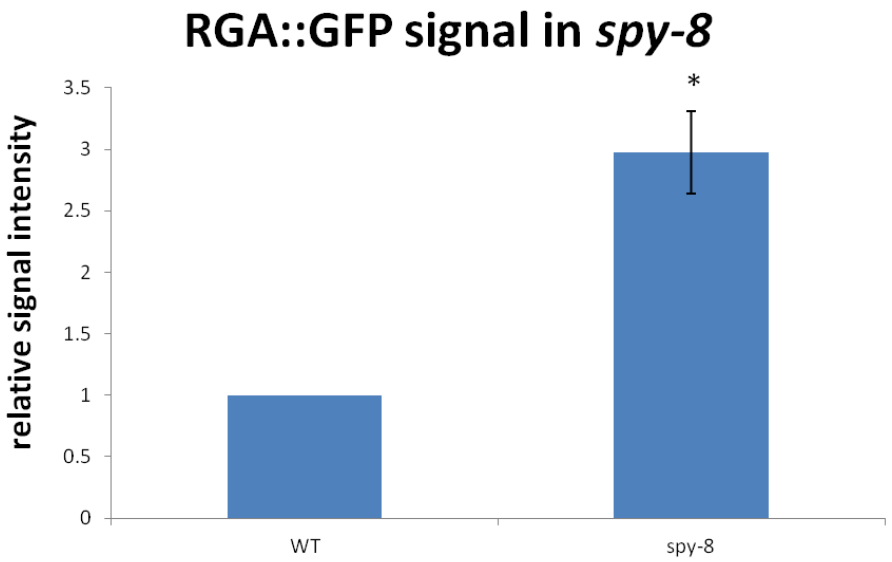

Figure 16: RGA::GFP signal in *spy-8*. Signal intensity is 3.23 higher in *spy-8,* relative to wild-type, indicating that RGA accumulates in *spy-8*. ***p <0.01 by paired t-test**

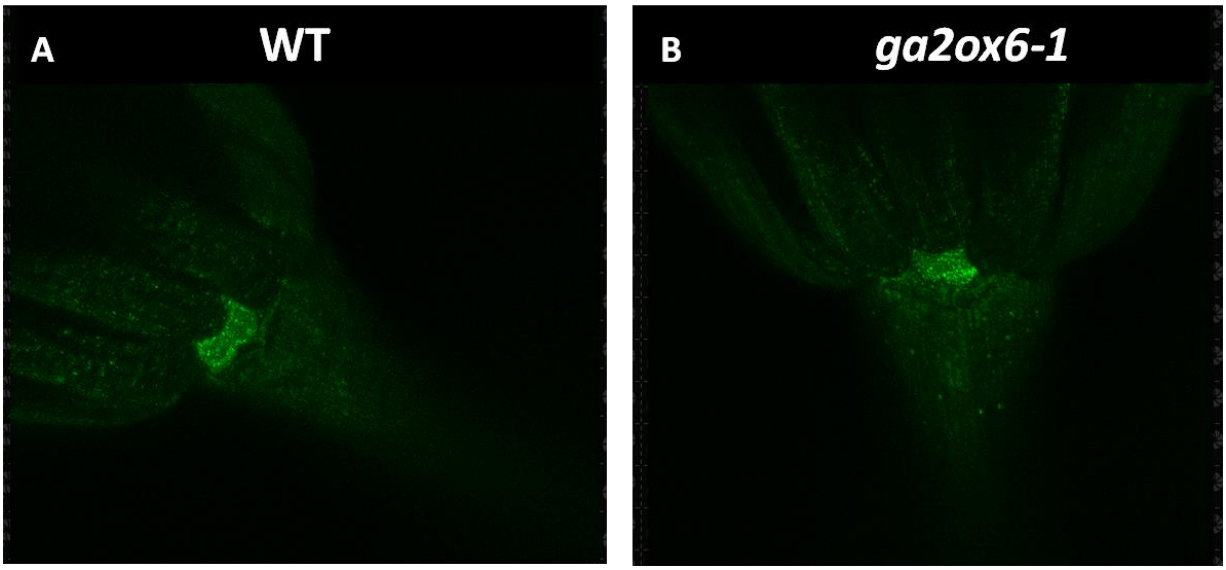

Figure 17: RGA::GFP in *ga2ox6-1*. GFP signal intensity from RGA::GFP is not significantly different in A) wild-type nectaries from B) ga2ox6-1 nectaries. This indicates that RGA does not accumulate in *ga2ox6-1*.

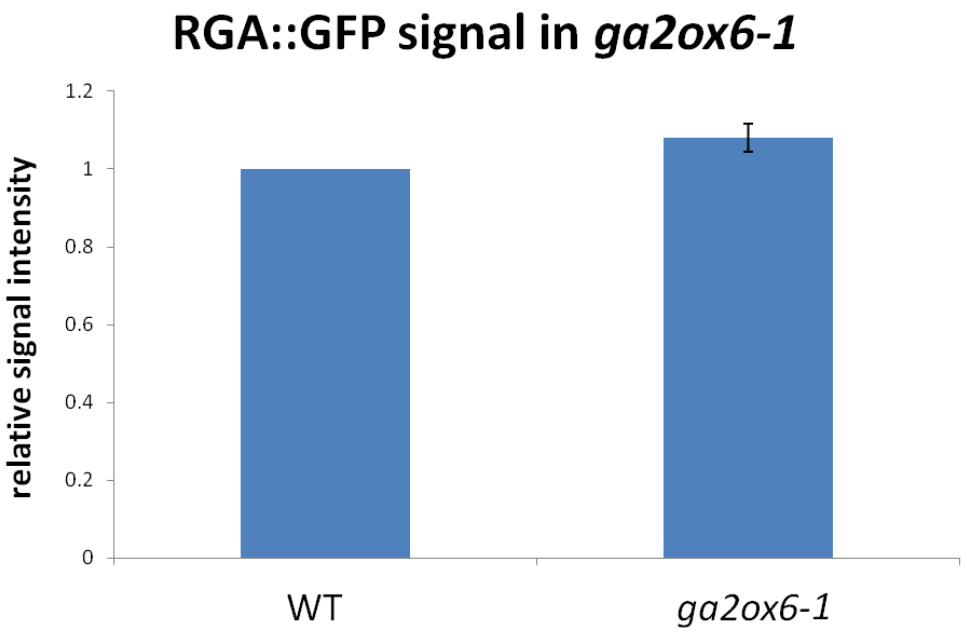

Figure 18: RGA::GFP signal intensity in *ga2ox6-1*. There is no significant difference in signal in signal intensity between RGA::GFP in wild-type and RGA::GFP in *ga2ox6-1*, indicating that RGA does not accumulate in *ga2ox6-1* like it does in *spy-8*.

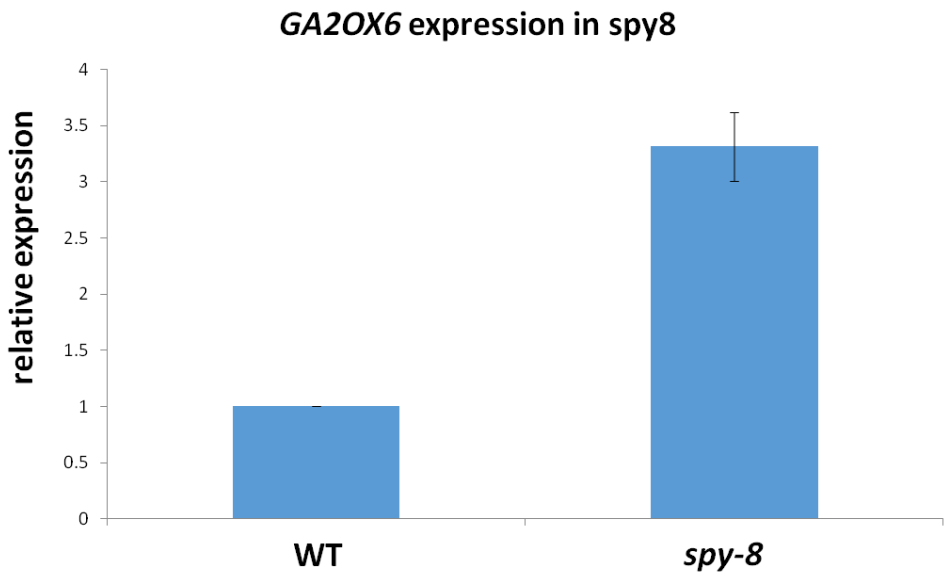

Figure 19: *GA2OX6* expression in *spy-8*. *GA2OX6* is expressed 3.3-fold higher in *spy-8* relative to wild-type. The up-regulation of *GA2OX6*, an inactivator of GA, is likely a feedback mechanism in response to the increased GA signaling in *spy-8*.

Effects of decreased GA response on nectar production

To observe the effects of decreased GA signaling on nectar production, *gai*, *ga1, and GA2OX6* mutants were examined. *gai* has a mutation in the DELLA domain, which renders the DELLA-protein GAI unable to be degraded in a GA-dependent manner. *ga1* mutants are GA biosynthesis mutants, so they are severely deficient in GA. GA2OX6, as previously described, is a negative regulator of GA, so *GA2OX6* mutants have a decreased GA response. As shown in Figure 20, *gai* mutants produce wild-type levels of nectar. In contrast, *ga1* mutants make almost no nectar (not shown). The *GA2OX6* over-expression mutants produced significantly more nectar than wild-type (Figure 21).

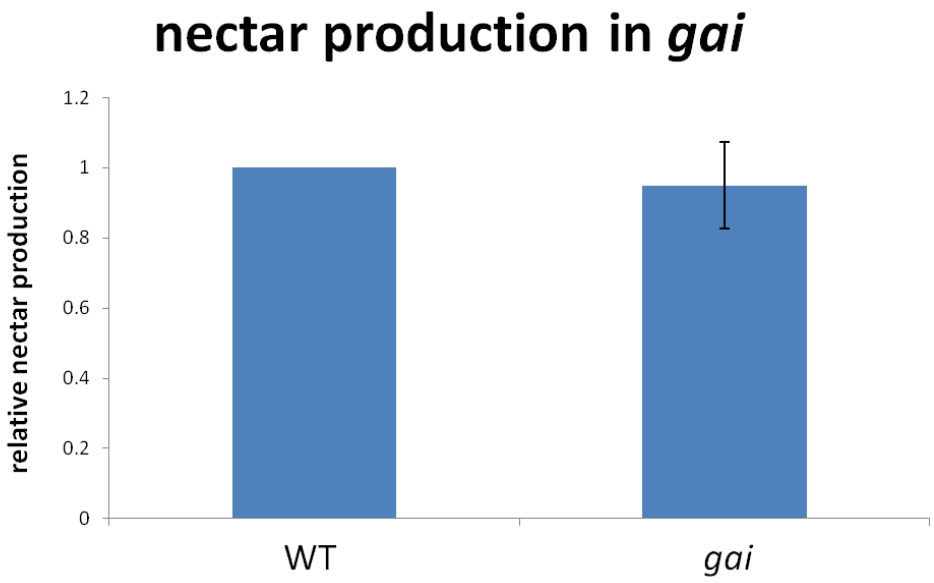

Figure 20: Nectar production in *gai*. *gai* mutants have a mutation in the DELLA domain of DELLA repressor proteins which renders the DELLAs unable to be degraded in a GA-dependent manner. This decreases overall GA signaling. Nectar production in *gai* is not significantly different from nectar production in wild-type.

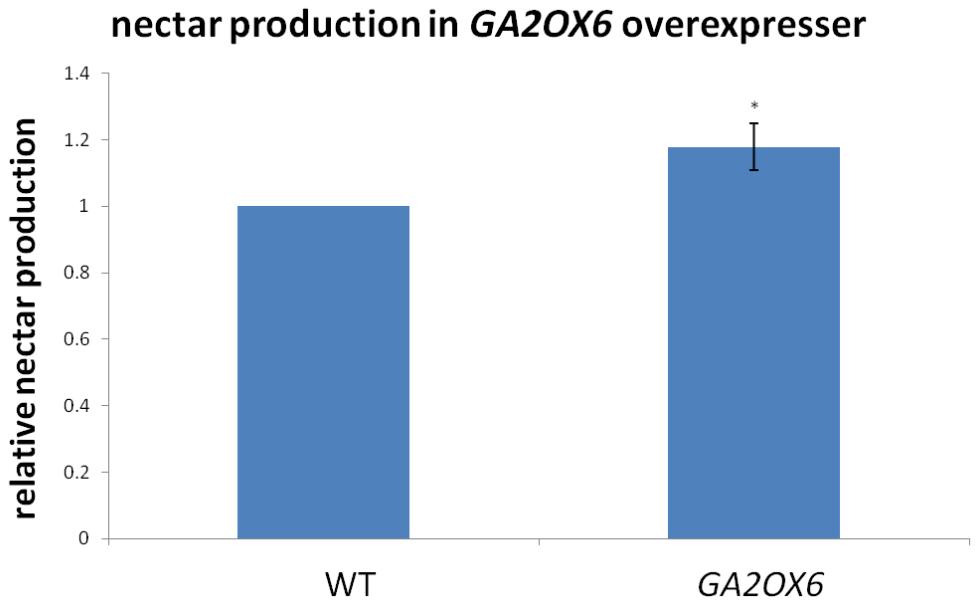

Figure 21: Nectar production in GA2OX6 overexpressers. *GA2OX6* overexpressers produce 1.2 times more nectar than wild-type. ***p <0.05 by paired t-test**

Discussion

Spatial and temporal expression of GA2OX6

GA2OX6 is an oxidase that catalyzes the irreversible inactivation of bioactive GA and its immediate precursors (Rieu et al., 2008). Inactivation of GA and GA precursors by *GA2OX6* and other GA-2-oxidases is one of the main ways in which cells maintain GA levels. Microarray data from Kram et al. 2009, indicated that *GA2OX6* is highly expressed in nectaries relative to other tissues. GUS histochemical staining of *GA2OX6pro::GUS* flowers confirmed this prediction (figure 5B and 5C). GUS staining was not always consistent, and sometimes *GA2OX6* expression was shown to be localized to the anthers, the stigma, and

sepals (data not shown). This is not completely unexpected, since *GA2OX6* is known to be expressed in other tissues (Rieu et al., 2008). To determine if *GA2OX6* has circadian expression patterns in flowers, wild-type flowers were collected every 4 hours for 32 hours. No circadian pattern was evident (data not shown). Expression of *GA2OX6* was also examined in pre-anthesis flowers and post-anthesis flowers. In pre-anthesis flowers expression was found to be low, while in post-anthesis flowers expression was much higher (Figure 6). This finding supports the hypothesis that *GA2OX6* has a role in regulating nectar production, because nectar production does not begin until anthesis.

Effect of increased GA response on nectar production

To examine the effects of GA on nectar production, several mutants with altered GA responses were studied. *GA2OX6*, as mentioned above, catalyzes the inactivation of GA and its immediate precursors (Rieu et al*.,* 2008). Therefore, *ga2ox6* mutants were predicted to have higher levels of bioactive GA. *SPY* (*SPINDLY*), is an O-GlcNAc transferase that is known to repress GA responses (Jacobsen and Olszewski, 1993). The mechanism through which *SPY* represses GA responses may involve post-translational modification of other proteins involved in GA signaling (Silverstone et al., 2007). Because *SPY* represses GA responses, *spy* mutants have an elevated GA response. DELLA proteins are transcriptional repressors that inhibit the expression of GA-responsive genes (Silverstone et al., 1997). In the DELLA quintuple mutant used in this study, none of the five DELLAS in the Arabidopsis genome are expressed. In their

absence, the expression of GA-responsive genes is uninhibited, so there is a constitutively increased GA response. The phenotype exhibited by the DELLA quintuple mutant, which is noticeably larger than wild-type, is consistent with increased GA signaling, since GA is promotes vegetative growth (Davies, 1995). Nectar was found to be significantly decreased in *ga2ox6-1*, *ga2ox6-2*, *spy-3*, *spy-4*, *spy-8*, and the DELLA quintuple mutant (figures 8-12). Since all of those mutants are predicted to have an increased GA response, the reduction in nectar suggests that GA negatively regulates nectar production in Arabidopsis. This finding is consistent with previous experiments in our lab in which paclobutrazol, a GA synthesis inhibitor, was shown to restore nectar production in *ga2ox6-1* almost to wild-type levels (Figure 13). The effect of paclobutrazol on nectar secretion in *ga2ox6-1* also confirms that GA responses are increased in *ga2ox6-1*.

Expression of other genes essential for nectar production

To investigate the mechanism behind the observed decrease in nectar production due to elevated GA response, the expression of other genes known to be essential for nectar production was examined. *CELL WALL INVERTASE4* (*CWINV4*) is a nectary-enriched invertase, localized to the apoplast (extracellular space), and known to be necessary for nectar production (Ruhlmann et al., 2009). There is strong evidence to suggest that CWINV4 cleaves sucrose into its hexose components after the sucrose is transported out of the nectary parenchyma cells. This creates strong osmotic pressure, which drives the flow of

water out of the nectary, forming the liquid nectar droplet. The expression of CWINV4 was found to be decreased in *ga2ox6-1* (figure 14A). *MYB21* is a transcription factor known to be necessary for the full expression of several genes necessary for nectar production, including PIN6, CWINV4, and SWEET9 (Carter, unpublished data). The role of *PIN6* in regulating nectar production is discussed in Chapter 3, whereas *SWEET9* is a sucrose transporter that exports sucrose from the nectary parenchyma and thus is necessary for nectar production (Lin et al., 2014). The expression of *MYB21* was found to be decreased in *ga2ox6-1* (figure 14B). The decrease in *MYB21* expression in *ga2ox6-1* could also explain the decrease in *CWINV4* observed in that mutant, since *CWINV4* expression depends on *MYB21*. Taken together, these findings suggest a possible mechanism through which GA negatively regulates nectar production.

GA signaling in spy-8 and ga2ox6-1

RGA is the DELLA protein that is most highly expressed in Arabidopsis nectaries (Kram and Carter, 2009). To further characterize GA signaling in *spy-8*, an RGA::GFP gene fusion was used to visualize RGA in nectaries. RGA was found to be present at higher amounts in *spy-8* than in wild-type nectaries (Figure 15). This is consistent with previous findings that RGA accumulation is increased in the root tips of *spy-8* (Silverstone et al., 2007), although nectaries, specifically, have not been investigated before. It is not clear why RGA accumulates to high levels in *spy-8*. This finding is somewhat paradoxical, since DELLA proteins

would be expected to be degraded to a greater extent in the presence of elevated GA signaling. It could be, however, that since SPY may catalyze post-translational modifications of DELLA proteins, leading to their degradation, RGA accumulates in *spy-8* due to a lack of modification by SPY (Silverstone, 2007). RGA was not shown to accumulate in *ga2ox6-1* (figures 17 and 18), which indicates that the accumulation of RGA observed in *spy-8* is not due to the increased GA response in general, but rather it has something to do with SPY, specifically.

qRT-PCR data showed that the expression of *GA2OX6* was over 3-fold higher in *spy-8* flowers than in wild-type (Figure 19). This further confirms that the GA response is increased in *spy-8*, since the increase in *GA2OX6* expression can be seen as a feedback mechanism in response to increased GA signaling. The increase in expression of *GA2OX6* in *spy-8* further illustrates the importance of *GA2OX6* in maintaining GA homeostasis. Although the expression of other GA-2 oxidases was not examined, *GA2OX6* is known to be one of the most highly expressed GA-2 oxidases (along with *GA2OX2*) in Arabidopsis, and it is essential for regulating GA levels in cells (Rieu et al., 2008). Taken together, the RGA-GFP data and the observed increase expression in *GA2OX6* provide further insight into GA signaling in nectaries.

Effect of decreased GA response on nectar production

As described above, increased GA response appears to negatively regulate nectar production. To investigate the effects of *decreased* GA response on nectar production, *gai* and *ga1, and GA2OX6 over-expression* mutants were examined. *gai* mutants have a deletion in the DELLA domain which renders mutant *gai* and *rga* DELLAs unable to be degraded in a GA-dependent manner (Willige et al., 2007). Therefore, GA signaling is constitutively decreased in *gai*. *ga1* mutants are GA biosynthesis mutants, so they are severely GA deficient. Interestingly, *gai* grows slowly and is dwarfed, but nectar production was not affected (Figure 20). In contrast, the overall phenotype of *ga1* is much more severe, such as requiring supplemental GA in order to germinate. The plants are dark green, indicating a deficiency in GA. Nectar production is almost entirely absent in *ga1* (data not shown).

Taken together, results from *gai* and *ga1* seem to contradict the hypothesis that GA negatively regulates nectar production, since according to that hypothesis both *gai* and *ga1* should display an increase in nectar production. For *gai*, this apparent contradiction could be explained by the fact that the mutation only affects two out of the five DELLAs in Arabidopsis (RGA and GAI). RGA is the DELLA most highly expressed in nectaries (Kram and Carter, 2009), so it seems likely that a mutation affecting RGA *would* affect nectar production. It is possible, however, that GA responses affecting nectar production are more dependent on one of the other three DELLAs (RGL1, RGL2, RGL3), so they may be unaffected

in *gai*. Moreover, it is possible that feedback mechanisms in response to decreased GA signaling, such as upregulation of GA biosynthesis genes or downregulation of other DELLAs, could explain the lack of effect on nectar production. Future experiments could examine the expression of genes involved in GA homeostasis to determine what feedback mechanisms are occurring in *gai*.

ga1 mutants, which make almost no nectar, have a severe phenotype in being dark green and having stunted growth. It could be that these plants produce little to no nectar due to the effects of GA deficiency on JA. GA is known to promote JA during floral maturation (Reeves et al, 2012). JA, in turn, promotes the expression of *MYB21* and *MYB24*, two transcription factors necessary for proper nectary function. GA-deficient mutants have been shown to have decreased levels of JA and decreased expression of *MYB21* and *MYB24* (Reeves et al, 2012). Therefore, it could be that the lack of nectar in *ga1* is due to decreased JA and the resulting decrease in *MYB21* and *MYB24* expression. One way to test this hypothesis would be to treat *ga1* mutants with JA to see if nectar production was restored. Additionally, *MYB21* and *MYB24* could be overexpressed in *ga1* to see if that restored nectar production. In summary, although *gai* and *ga1* produce wild-type levels of nectar and no nectar, respectively, more research is needed to understand GA signaling in both mutants.

As shown in Figure 21, the *GA2OX6* over-expression mutants showed a significant decrease in nectar. Since GA2OX6 is a negatively regulator of GA, this result supports the hypothesis that GA negatively regulates nectar production.

Materials and Methods

Plant Growth and Conditions

The ecotypes Columbia-0 and Landsberg *erecta* were the genetic backgrounds of the *Arabidopsis thaliana* plants used in this study. Plants were grown in individual pots in one of two peat-based soil mixtures: Pro-Mix BX (Premier Horticulture); or Pro-Mix Lc8 formula (Sun Gro Horticulture). Some plants were grown in a Percival AR66LX growth chamber set to a 16h day/8h night cycle with a photosynthetic flux of 150µmol m^{-2} sec^{-1} at 23˚ C. Most plants, however, were grown in a growth room with a 16h day/8h night cycle around 22˚ C. Plants used for direct comparison were grown on the same tray in the same type of soil.

GA2OX6 T-DNA mutants

Arabidopsis plants, including wild type (Col-0), *ga2ox6-1* (SALK_044189C) and *ga2ox6-2* (SALK_059724), were acquired from The Arabidopsis Biological Resource Center. Genomic DNA isolated from whole leaf tissue served as a template for amplification with *ATGA2OX6* gene specific primers. Three primers were used in the genotyping PCR reaction: two primers flank the T-DNA

insertion site, and the third primer, a T-DNA specific primer “LBb1.3,” falls within the T-DNA insertion.

To determine *AtGA2OX6* expression in Arabidopsis T-DNA mutants, RNA was isolated from whole flower tissue using Agilent Technologies’ Absolutely RNA Miniprep Kit (Catalog #400800). 500ng of RNA was converted to cDNA using the promega reverse transcription kit. Resulting cDNA transcripts were amplified with the primer pair: “*AtGA2OX6* RT-F and *AtGA2OX6* RT-R.” *GAPDH* was used as an internal standard using the primer pair “*AtGAPDH* RT-F and *AtGAPDH* RT-R.”

spy mutants and DELLA quintuple mutant

Mutants for *SPY* used in this study included *spy-3*, *spy-4* and *spy-8*. In *spy-3* (CS6268) there is a point mutation at G593 (G to S) (Silverstone et al., 2006). In *spy-4* there is a T-DNA insertion in the promoter (Jacobsen and Olszewski, 1993; Jacobsen et al., 1996). In *spy-8* the sequence M354 to Q376 is deleted (Silverstone et al., 1997). The DELLA quintuple mutant (N16298) does not express any of the 5 Arabidopsis DELLAs [*gai, rga, rgl1, rgl2, rgl3*] (Feng et al., 2008).

Over-expression construct (pPMK1)

GA2OX6 was overexpressed with the nectary-specific *SWEET9* promoter in the WT (Col-0) background. Previously, 1.4 kb of the promoter region of *AtSWEET9*

was ligated into the plant transformation vector pORE-O4, which contains the kanamycin resistance gene, and the construct was named pPMK1. For this study, the restriction enzymes XMA1 and SPE1 were used to insert *GA2OX6* into pPMK1 directly after the *SWEET9* promoter. *Agrobacterium tumefaciens* (GV3101) cells were transformed to carry the pPMK1 vector and used to transform Arabidopsis using the floral-dip method described by Clough and Bent (1998). Transformed seedlings were selected on one half Muarshige and Skoog medium plates with 50 µg/ml kanamycin.

Nectar sugar assay

An Amp-red glucose assay (Bender et al., 2012) was used to determine the relative glucose content of nectar between WT and mutant lines of *Arabidopsis thaliana*. Nectar was collected from 10 flowers per line (from lateral nectaries only) onto a small wicks (5 flowers per wick) made from Whatman No. 1 filter paper. Wicks were placed in 300 µL sterile water. 75 µL of the resulting sugar-water was combined with 25 µL of an enzyme mix which included 862.5 mM NaPO4, PH 7.4, 1 unit of horseradish peroxidase (Sigma), 10 units of glucose oxidase (Sigma), and 100 µl of Ampliflu Red (Sigma) at a total volume of 2.6 ml. Samples were incubated for 30 minutes (or slightly longer, but not more than 1 hour) in the dark. Light absorbance was measured at 570 nm.

RNA isolation and reverse transcription PCR

15-20 flowers were collected from each line, and RNA was extracted following the Absolutely RNA Miniprep protocol (Alignet). RNA integrity was assessed by gel electrophoresis and by spectrophotometric analysis. RNA amount was measured with spectrophotometric analysis. Either 500ng or 1 µg of total RNA was converted to cDNA using the Promega reverse transcription kit.

Quantitative Real-Time PCR

Total RNA (500 ng) from *ga2ox6-1* and *spy-8* flowers was used as template in cDNA synthesis via the Promega reverse transcription kit. 2 µl of the resulting cDNA (or diluted cDNA) was added to the real time PCR reaction setup, which included 10.0 µl of 2x KAPA SYBR FAST qPCR Master Mix (KAPA BIOSYSTEMS), 0.4 µl of each forward and reverse primer (10 µmolar stock), 0.4 µl rox dye (high), and 6.8 µl nuclease-free H_2O. Primers were designed using the online primer design tool "QuantPrime." The Applied Biosystems StepOnePlus thermocycler was used for real-time PCR, and results were analyzed with Applied Biosystems StepOne software (v2.3). Three biological replicates were used, and at least two technical replicates were included.

Table 1: Oligonucleotides used in this study.

Name	Sequence (5' to 3')	Purpose
GA2OX6 prom-F XbaI	AAAtctagaAGATGGGCCGGTTATGGGCCT	cloning *GA2OX6* promoter into pORE-R2
GA2OX6 prom-R PstI	AAActgcagGTAGTAAAGAATGAAATCAGGAGG	cloning *GA2OX6* promoter into pORE-R2
ga2ox6-1 geno F	CGTACCTGAACTTGAAAGGATC	*ga2ox6-1* genotyping
ga2ox6-1 geno R	CACCAAATTTCAAACTACCCG	*ga2ox6-1* genotyping
ga2ox6-2 gene F	TGATGATCCTTTCAAGTTCAGG	*ga2ox6-2* genotyping
ga2ox6-2 gene R	AAAGTGAGGACCCCATGATTC	*ga2ox6-2* genotyping
LBb1.3	ATTTTGCCGATTTCGGAAC	T-DNA left border genotyping
GA2OX6 RT-F	AAGGCAGTCACCGACCAATACGAA	*GA2OX6* RT-PCR
GA2OX6 RT-R	TCAGCTCGGCGACGAATGATTACA	*GA2OX6* RT-PCR
RGA RT-F	ATCGGAGATGGCGGAGGTTGCTTT	*RGA* RT-PCR
RGA RT-R	AGTTCACCGCAACAGCTTCCGT	*RGA* RT-PCR
GID1A RT-F	TCGGCGTGCACCAGAGAATCCA	*GID1A* RT-PCR
GID1A RT -R	CTTGAGCCCTTCCGCGTATGCC	*GID1A* RT-PCR
GAPDH RT-F	TTCGGTGAGAAGCCAGTCACTGTT	*GAPDH* RT-PCR
GAPDH RT-R	AAACATTGGAGCGTCTTTGCTGGG	*GAPDH* RT-PCR
pPMK1 seq F	AGCAGTCAACTAGCAAGAGAAAGAGA	screening for inserts in pPMK1 and pPMK19 constructs
RGA geno R	AGTTCACCGCAACAGCTTCCGT	*RGA* genotyping
GID1A geno R	CCGCAGACCGCCACACACTTA	*GID1A* genotyping
spy-3 genotyping F	GGCCTGCAGACCAGCACCG	sequencing *spy-3*
spy-3 genotyping R	CAGCTCCTCGACCTGCCTGC	sequencing *spy-3*
CWINV4 real time F	TGGGCTGGTCTTCAGGTGATTC	qRT-PCR
CWINV4 real time R	TGCGTTGACCCATCTTGATGTC	qRT-PCR
MYB21 real time F	AGCTGCAAGCACGTCGAGCCATAA	qRT-PCR
MYb21 real time R	ACTGCCGCGGCCGAATAGTTACCATA	qRT-PCR
PIN6 real time F	ATCATTTCAGATGCAGGTCTTGGG	qRT-PCR
PIN6 real time R	CAGCGCCATAAACAGGCCTAAAC	qRT-PCR
ACTIN8 real time F	TCAGCACTTTCCAGCCGATG	qRT-PCR
ACTIN8 real time R	CTGTGGACAATGCCTGGAC	qRT-PCR
GA2OX6 real time F	GCTTGTGGATCCCAATCCCATCTG	qRT-PCR
GA2OX6 real time R	GTGGCTTCTTTGCTGTGTTTGC	qRT-PCR
GA2OX6 -F Xma1	AAACCCGGGATCCTTCCTTCTCTTCCCAA	cloning *GA2OX6* into pPMK1 and pPMK19
GA2OX6 -R Spe1	AAAACTAGTTGTGATCGGACGCGTTTCAT	cloning *GA2OX6* into pPMK1 and pPMK19
GID1A-F BamH1	AAAGGATCCCACTTCTCGACTTGCAAATT	cloning *GID1A* into pPMK1 and pPMK19
GID1A-R Kpn1	AAAGGTACCAACGCCTCACTGTTCTTCCA	cloning *GID1A* into pPMK1 and pPMK19
RGA-F BamH1	AAAGGATCCAACCCTAGATCCAAGATCA	cloning *RGA* into pPMK1 and pPMK19
RGA-R Sal1	AAAGTCGACTCGACTCCACCACCGTCGT	cloning *RGA* into pPMK1 and pPMK19

Ch 3: GA affects auxin signaling and *PIN6* expression in nectaries.

Introduction

Chapter 2 presented findings that GA negatively regulates nectar production in *Arabidopsis thaliana*. To look for mechanisms underlying this negative regulation, the effects of GA and GA-related genes on auxin signaling and auxin transport in nectaries were investigated. The rationale for investigating the possible involvement of auxin was as follows: 1) Auxin is involved in almost all major plant functions; 2) Our lab has shown that auxin regulates nectar production in Arabidopsis; and 3) cross-talk between hormone signaling pathways is very common in plants, including between GA and auxin.

Auxin

Auxin (indole-3-acetic-acid, IAA) is one of the main plant signaling molecules. Auxin is known as the universal growth hormone, because it is needed in almost every part of the plant, and it is involved in nearly all developmental processes including embryogenesis, organogenesis, root meristem maintenance, vascular tissue differentiation, hypocotyl and root elongation, apical hook formation, apical dominance, fruit ripening, and growth responses to environmental stimuli (Vieten et al., 2007). Auxin regulates developmental processes by affecting the transcription of many genes. In the cell, auxin binds to its receptor, TIR1, which is part of the SCF^{TIR1} ubiquitin ligase complex (Dharmasiri, 2005). The binding of

auxin stabilizes the interaction between the SCF^{TIR1} complex and AUX/IAA proteins, which are transcriptional repressors. This interaction between the SCF^{TIR1} complex and AUX/IAA proteins leads to the ubiquitination and subsequent proteasomal degredation of the AUX/IAA proteins, which allows for transcription of the genes repressed by AUX/IAA. (Gray 2001, Dharmasiri, 2005). In summary, like GA, the downstream effects of auxin are brought about by the degradation of transcriptional repressors. However, recent studies have suggested rapid auxin responses may also be controlled via a separate non-transcriptional pathway mediated by AUXIN BINDING PROTEIN 1 (ABP1) (Lobler and Klambt, 1985; Shi and Yang, 2011).

Auxin transport and PIN6

Auxin is synthesized mainly in young apical tissue (i.e. shoot apical meristem), and then transported throughout the plant (LJung et al., 2001). Although auxin can be transported in the phloem, it is also transported in a regulated, directional, cell-to-cell manner known as polar auxin transport. This mode of transport establishes auxin gradients within the plant tissues, which regulate the developmental programs of those tissues. Polar auxin transport is carried out, in part, by auxin efflux carriers in the PIN-FORMED (PIN) family. Eight PIN proteins have been identified in *Arabidopsis thaliana*. Most PIN proteins are localized on the plasma membrane, distributed asymmetrically on certain faces of the cell, and thus their polarity determines the flow of auxin between cells (Vieten et al., 2007). Other PIN proteins are predicted to be localized to the ER,

where they regulate intracellular levels of auxin by sequestering auxin in the ER lumen. (Mravec et al., 2009). Although most of the PIN proteins have been characterized, until recently the function of PIN6 (At1g77110) remained unknown. In 2013 it was shown that PIN6 has nectary-enriched expression in *Arabidopsis thaliana* and that PIN6 is necessary for auxin-dependent responses in nectaries. *PIN6* expression was also found to be positively correlated with total nectar production in *Arabidopsis* (Bender et al., 2013).

The effect of auxin on nectar production

Given the wide range of developmental processes that involve auxin, it is not surprising that auxin profoundly affects nectar production in *Arabidopsis*. Exogenous NAA (synthetic auxin) has been shown to cause more than a 10-fold increase in nectar production, whereas NPA (an auxin transport inhibitor) reduced nectar production more than 2-fold (Bender et al., 2013). The mechanisms by which auxin regulates nectar production in *Arabidopsis thaliana* are still unknown, but it is clear that auxin plays an important role.

Examples of cross-talk between GA and Auxin are known.

The major plant hormones are abscisic acid, gibberellins, auxins, ethylene, cytokinins, brassinosteroids, jasmonates, salicylic acid and nitric oxide (Santner and Estelle, 2009). Crosstalk between hormone signaling pathways is common, and it can occur at the hormone response level or at the level of hormone biosynthesis (Weiss and Ori, 2007). Although the effect of GA on auxin signaling

in nectaries has not been well studied, examples of crosstalk between GA and auxin in other plant tissues are known. For example, in hybrid aspen (Populus tremula x tremuloides) it was shown that GA treatment increases auxin in the stem by stimulating polar auxin transport (Björklund et al., 2007). In another example, dwarf pea plants, normal pea plants, and sunflower plants yielded 3, 2, and 10 times more diffusible auxin in stem apices, respectively, after treatment with gibberellin (Kuraishi and Muir, 1962). In *coleus* plants, the application of GA increased the levels of endogenous auxin in stems (Muir and Valdovinos, 1970). In tomato flowers, treatment with GA induces synthesis of diffusible auxin at anthesis, a stage where diffusible auxin is normally not present (Sastry and Muir, 1963).

The molecular basis for the crosstalk between GA and auxin was largely unknown until 2011 when it was found that Arabidopsis mutants deficient in GA biosynthesis and signaling displayed reduced auxin signaling, correlated with a reduction in the abundance of PIN proteins, which are auxin efflux transporters (Willige et al., 2011). Interestingly, it was found that the effects of GA on PIN protein abundance (PIN2) cannot be explained at the level of transcription; rather, GA deficiency promotes the vacuolar degradation of PIN2 (and possibly other PIN proteins). This chapter describes the impacts of GA on auxin-dependent signaling in relation to nectary function in *Arabidopsis thaliana.* Activation of the auxin-responsive promoter, DR5, was visualized in the nectaries

of GA-related mutants, and the expression of *PIN6*, a nectary-enriched auxin efflux transporter, was also examined.

Results

Because auxin is known to be strongly involved in the regulation of nectar production in Arabidopsis, potential crosstalk between GA signaling pathways and auxin signaling pathways in nectaries was investigated. The DR5::GFP reporter was used to visualize auxin signaling in nectaries. As shown in Figure 22, auxin signaling is decreased in *ga2ox6-1* nectaries relative to wild-type nectaries. Quantitatively, the signal intensity in *ga2ox6-1* was reduced to 64 percent that of wild-type (Figure 23). Given the apparent decrease in auxin signaling observed in *ga2ox6-1*, the expression of *PIN6*, a nectary-enriched auxin transporter known to be essential for nectar production, was examined in *spy-8* and *ga2ox6-1*. The expression of *PIN6* was decreased ~25% in *spy-8* and 45% in *ga2ox6-1* relative to wild-type (Figure 24).

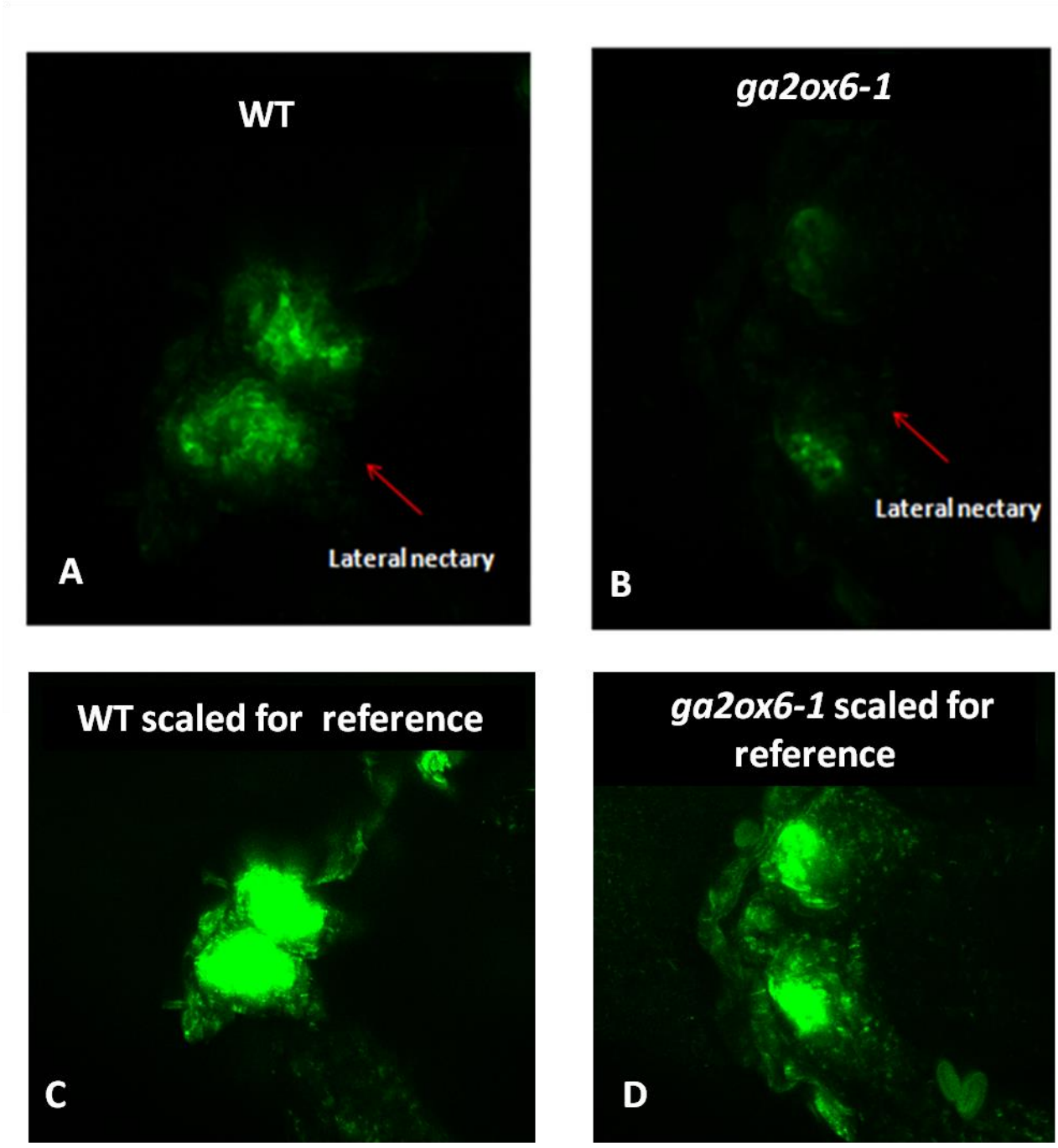

Figure 22: Auxin signaling in *ga2ox6-1*. A) In *ga2ox6-1* nectaries, fluorescent signal is 0.64 that of B) wild-type nectaries. Panels C and D are digitally scaled images of the nectaries. DR5::GFP was expressed in wild-type and *ga2ox6-1*. The reduction in fluorescent signal in *ga2ox6-1* indicates a decrease in auxin signaling.

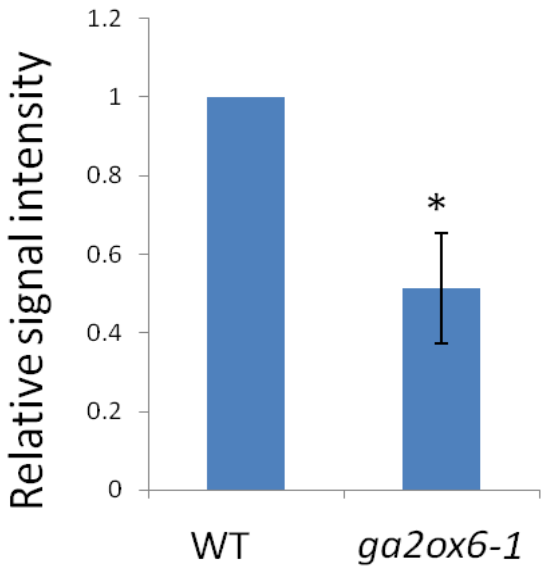

Figure 23: DR5::GFP signal intensity in *ga2ox6-1*. In *ga2ox6-1* nectaries, fluorescent signal is 0.64 that of wild-type nectaries. The reduction in fluorescent signal in *ga2ox6-1 3* indicates a decrease in auxin signaling in *ga2ox6-1*. ***p <0.05 by paired t-test**

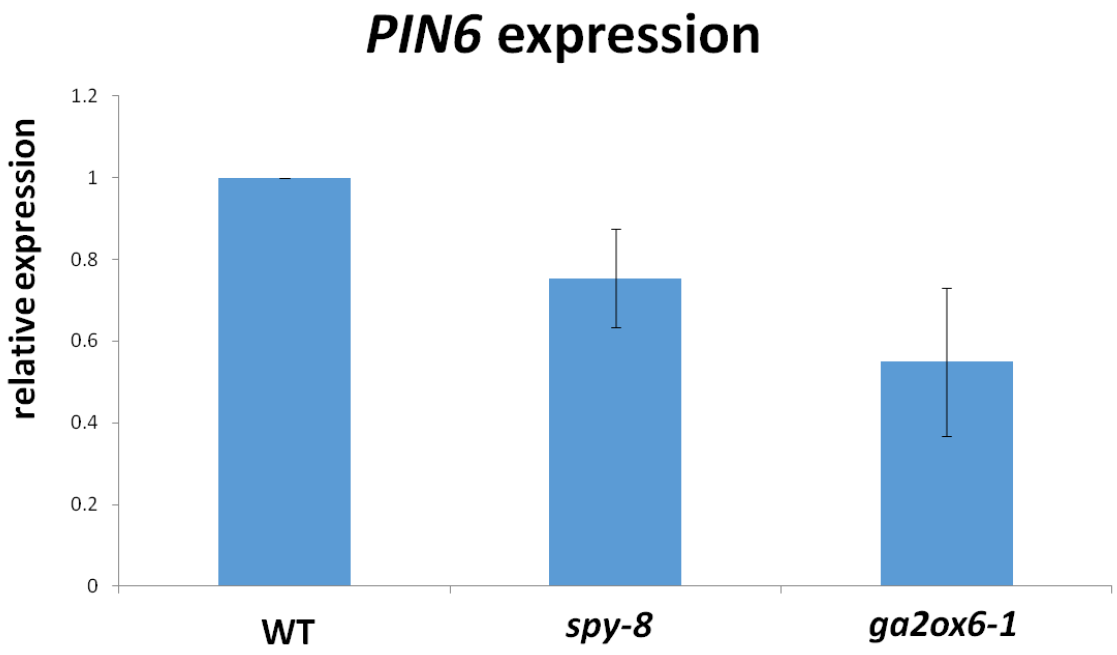

Figure 24: *PIN6* expression in *spy-8* and *ga2ox6-1*. Relative to wild-type, *PIN6* expression is 0.75 in *spy-8* and 0.55 in *ga2ox6-1*. PIN6 is an auxin efflux transporter thought to be located on the ER membrane where it may sequester auxin within the ER. The reduction in *PIN6* expression in *spy-8* and *ga2ox6-1* may partially explain the reduction in auxin signaling observed in *spy-3* and *ga2ox6-1*.

Discussion

Auxin signaling in ga2ox6-1 and spy-3

As described in Chapter 2, elevated GA signaling was found to negatively regulate nectar production in Arabidopsis. The mechanism through which GA affects nectar production, in part, involves altered expression of genes known to be essential for nectar production. To further investigate the mechanism through which GA regulates nectar production, potential crosstalk between GA signaling pathways and auxin signaling pathways was investigated. The rationale for

investigating auxin signaling in GA-related mutants was based on previous data showing that auxin is strongly involved in the regulation of nectar production in Arabidopsis (Bender et *al*., 2013). The DR5::GFP reporter was used to visualize auxin signaling in nectaries. Auxin signaling was found to be decreased in *ga2ox6-1* (Figures 22 and 23). The decrease in auxin signaling observed in *ga2ox6-1* could partly explain the decrease in nectar observed in that mutant, given the known importance of auxin in nectar production.

To probe the mechanism behind the decrease in auxin signaling observed in *ga2ox6-1*, the expression of *PIN6* was examined in *ga2ox6-1.* *PIN6* expression was also examined in *spy-8*. *PIN6* is an auxin efflux transporter, highly expressed in nectaries (Feraru and Friml, 2008; Kram et al, 2009). *PIN6* is predicted to be localized to the ER membrane where it might sequester auxin within the ER (Mravec *et al*., 2009). The expression of *PIN6* was found to be decreased in both *ga2ox6-1* and *spy-8* (Figure 24). This may be a result of the decrease in expression of *MYB21,* discussed in Chapter 2, since *MYB21* is a transcription factor that is necessary for full expression of *PIN6*. Taken together, the confocal imaging data and the *PIN6* expression data suggest a possible mechanism through which GA regulates nectar production. As shown below in Figure 25, multiple mutations that cause an increase in GA signaling correlate with a reduction in several genes known to be essential for nectar production, and they also correlate with a decrease in auxin signaling as well as a decrease in nectar production. Notably, this is consistent with previous findings from our

lab in which a *pin6* null mutant was found to have a strong decrease in both auxin signaling and nectar production (Bender et al., 2013). However, these results are inconsistent with previous data showing that GA affects the abundance of PIN proteins independent of transcription (Willige et al., 2011). Previously it was shown that GA promoted the abundance of PIN proteins, whereas the present study showed that GA negatively regulates *PIN6*. This difference could be explained by the fact that the previous study was investigating roots and cotyledons - not nectaries. Furthermore, the previous study addressed PIN proteins that transport auxin between cells, whereas *PIN6*, examined in the present study, involves auxin transport *within* a cell.

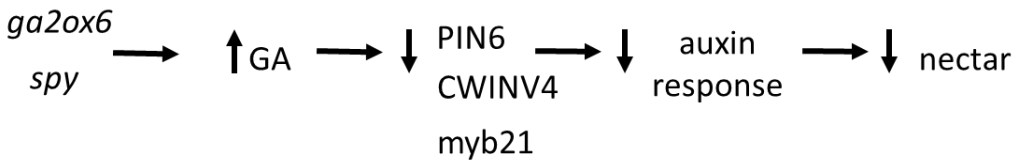

Figure 25: proposed model showing the effects of GA on nectar production, auxin response and related genes. Multiple mutants that have increased GA signaling also have decreased nectar production which also correlates to a reduction in auxin response and a reduction in several genes necessary for nectar production.

Materials and methods

Plant Growth and Conditions

Columbia-0 was the genetic backgrounds of the *Arabidopsis thaliana* plants used in this study. Plants were grown in individual pots in one of two peat-based soil mixtures: Pro-Mix BX (Premier Horticulture); or Pro-Mix Lc8 formula (Sun Gro

Horticulture). Some plants were grown in a Percival AR66LX growth chamber set to a 16h day/8h night cycle with a photosynthetic flux of 150µmol m^{-2} sec^{-1} at 23˚ C. Most plants, however, were grown in a growth room with a 16h day/8h night cycle around 22˚ C. Plants used for direct comparison were grown on the same tray in the same type of soil.

GA2OX6 T-DNA mutant

Arabidopsis plants, including wild type (Col-0) and *ga2ox6-1* (SALK_044189C) were acquired from The Arabidopsis Biological Resource Center. Genomic DNA isolated from whole leaf tissue served as a template for amplification with *ATGA2OX6* gene specific primers. Three primers were used in the genotyping PCR reaction: two primers flank the T-DNA insertion site, and the third primer, a T-DNA specific primer "LBb1.3," falls within the T-DNA insertion.

spy-3

Seed stock for *spy-3* was obtained from Dr. Neil Olszewski's lab in St. Paul, MN. In *spy-3* (CS6268) there is a point mutation at G593 (G to S) (Silverstone et al., 2006). Mutants were confirmed to be homozygous via gene sequencing.

Confocal microscopy

GFP imaging was performed with the Nikon AZ100 C1si Spectral Confocal Macroscope at the UMN imaging center. Freshly picked whole flowers, with sepals removed, were oriented with one of the lateral nectaries facing upward

toward the objective. A 3-dimensional z-stack image of the whole nectary was taken. The software used for image analysis and quantification was “Nikon Elements.” To analyze the images, first the z-stack images were compressed. Then a maximum intensity projection was created from each compressed z-stack image. Circular regions of interest were designated on nectaries that were being compared. Regions of interest were of equal size on their respective images. Mean intensity of each region of interest was measured, and that was the number used for reporting signal intensity.

Quantitative Real-Time PCR

Total RNA (500 ng) from *ga2ox6-1* and *spy-8* and flowers was used as template in cDNA synthesis via the Promega reverse transcription kit. 2 µl of the resulting cDNA (or diluted cDNA) was added to the real time PCR reaction setup, which included 10.0 µl of 2x KAPA SYBR FAST qPCR Master Mix (KAPA BIOSYSTEMS), 0.4 µl of each forward and reverse primer (10 µmolar stock), 0.4 µl rox dye (high), and 6.8 µl nuclease-free H_2O. Primers were designed using the online primer design tool “QuantPrime.” The Applied Biosystems StepOnePlus thermocycler was used for real-time PCR, and results were analyzed with Applied Biosystems StepOne software (v2.3). Three biological replicates were used, and at least two technical replicates were included.

RNA isolation and reverse transcription PCR

15-20 flowers were collected from each line, and RNA was extracted following the Absolutely RNA Miniprep protocol (Alignet). RNA integrity was assessed by gel electrophoresis and by spectrophotometric analysis. RNA amount was measured with spectrophotometric analysis. Either 500ng or 1 µg of total RNA was converted to cDNA using the Promega reverse transcription kit.

Primers

For a reference of all primers used in this study, refer to Table 1 in Chapter 2.

Chapter 4: Concluding remarks and future directions

Concluding remarks

Effects of GA on nectar production

This study has shown that GA is involved in regulating nectar production in *Arabidopsis thaliana*. Several mutant lines that have elevated GA signaling also have a decrease in nectar, indicating that GA negatively regulates nectar production. However, the bigger picture of how GA affects nectar production appears to be more complicated. In *gai*, a GA insensitive mutant, thus constitutively repressing GA responses, nectar production does not differ significantly from wild-type. In *ga1*, a GA biosynthesis mutant, little to no nectar is present, which contradicts the hypothesis that GA negatively regulates nectar production. These apparently contradictory results could be due to feedback mechanisms in the case of *gai*, and possible effects on JA in *ga1*.

From this study we can conclude that GA affects nectar production, but the effects depend on GA concentration and location.

Effects of GA on the expression of CWINV4 and MYB21

An increase in GA response was shown to negatively regulate expression of *CWINV4* and *MYB21*, two genes essential for nectar production. The altered

expression of *CWINV4* and *MYB21* may partially explain the mechanism through which GA negatively regulates nectar production.

Effects of GA on auxin and PIN6

This study has shown that an increased GA response causes a reduction in auxin signaling in nectaries, correlated with a decrease in the expression of *PIN6*. The reduction in *PIN6* expression may be due, in part, to the decrease in *MYB21* expression observed in *ga2ox6-1*.

Future directions

GA2OX6

Two independent mutants for *GA2OX6* were examined in this study. Although *GA2OX6* is the GA2-oxidase most highly expressed in nectaries (Kram and Carter, 2009) and it is one of the two GA2-oxidases most highly expressed in the whole plant (Rieu et al., 2008), there are four other GA2-oxidases that potentially affect the GA response in nectaries. It is also possible that one or more of the other GA2-oxidases are upregulated in the *ga2ox6* mutant as a feedback mechanism. It has been shown that GA-related phenotypes are much more pronounced in the GA2OX quintuple mutant, which lacks all five GA2-oxidases capable of inactivating bioactive GA (Rieu et al., 2008). Examining nectar production in the GA2oxidase quintuple mutant would be beneficial for this study in the future.

Measuring endogenous GA

Another approach that would strengthen this study, going forward, is the measurement of endogenous bioactive GA in the mutant lines. There is strong evidence for increased GA response in the *ga2ox6* mutants, *spy* mutants and the DELLA quintuple mutant. However, plant cells employ several feedback mechanisms to maintain GA levels, so it is difficult to know how much bioactive GA is present in the mutant lines relative to wild-type. Measuring endogenous levels of GA and perhaps some key intermediates would provide a clearer picture of GA signaling in the Ga-related mutants. This would be especially useful in the over-expression lines, since sometimes plant cells can respond to an over-expressed gene by silencing it. Measuring endogenous GA in those lines would verify that GA signaling is altered in the predicted manner.

RNA sequencing

In this study, the expression of several key genes known to be involved in regulating nectar was examined in GA-related mutants. A broader approach of RNA sequencing could show global changes in the transcriptomes of the various mutants, which might reveal genes affected by GA that were not examined in this study. Although this study showed that GA affects the expression of *PIN6*, *CWINV4*, and *MYB21*, the mechanism of how GA affects those genes is unclear. RNA sequencing might elucidate more about these pathways.

Crosstalk between GA and auxin

This study showed that there is crosstalk between GA and auxin in the regulation of nectar production. The details of this interaction are still unclear. To further study the effects of GA on auxin and vice versa, it would be beneficial to treat GA signaling and biosynthesis mutants with auxin, and to treat auxin signaling and biosynthesis mutants with GA. Furthermore, more details about the mechanism of *PIN6* in nectaries are needed. This study showed that GA negatively regulates the transcription of *PIN6*, which, in turn, correlates with a decrease in auxin signaling and a decrease in nectar. A previous study showed that *PIN2* is affected by GA independent of transcription (Willige, 2011). Deficiency in GA appears to promote the vacuolar degradation of PIN2. Based on that finding, it would be beneficial to investigate the abundance of *PIN6* proteins in nectaries. In this study *PIN6* expression, but not PIN6 protein abundance, was investigated.

Crosstalk between GA and other hormones

In this study, crosstalk between GA and auxin, in the regulation of nectar production, was examined. It would be interesting to investigate crosstalk between GA and other hormones. For example, it is known that in Arabidopsis GA acts through JA to regulate expression of *MYB21* and other transcription factors (Cheng et al., 2009). This interaction likely affects nectar production, since *MYB21* is necessary for the full expression of genes essential for nectar production (Carter, unpublished data), and, moreover, JA is known to regulate nectar production as well (Carter, unpublished data). It would be interesting to

apply exogenous JA to *ga1* mutants, which produce little to no nectar. It seems likely that the lack of nectar in *ga1* involves decreased JA levels, leading to non-functional nectaries. This could be tested with exogenous JA treatment at various developmental time points.

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