PIN6 is required for nectary auxin response and short stamen development

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SUMMARY

The PIN family of proteins is best known for its involvement in polar auxin transport and tropic responses. PIN6 (At1g77110) is one of the remaining PIN family members in Arabidopsis thaliana to which a biological function has not yet been ascribed. Here we report that PIN6 is a nectary-enriched gene whose expression level is positively correlated with total nectar production in Arabidopsis, and whose function is required for the proper development of short stamens. PIN6 accumulates in internal membranes consistent with the ER, and multiple lines of evidence demonstrate that PIN6 is required for auxin-dependent responses in nectaries. Wild-type plants expressing auxin-responsive DR5:GFP or DR5:GUS reporters displayed intense signal in lateral nectaries, but pin6 lateral nectaries showed little or no signal for these reporters. Further, exogenous auxin treatment increased nectar production more than tenfold in wild-type plants, but nectar production was not increased in pin6 mutants when treated with auxin. Conversely, the auxin transport inhibitor N–1–naphthylphthalamic acid (NPA) reduced nectar production in wild-type plants by more than twofold, but had no significant effect on pin6 lines. Interestingly, a MYB57 transcription factor mutant, myb57–2, closely phenocopied the loss-of-function mutant pin6–2. However, PIN6 expression was not dependent on MYB57, and RNA-seq analyses of pin6–2 and myb57–2 mutant nectaries showed little overlap in terms of differentially expressed genes. Cumulatively, these results demonstrate that PIN6 is required for proper auxin response and nectary function in Arabidopsis. These results also identify auxin as an important factor in the regulation of nectar production, and implicate short stamens in the maturation of lateral nectaries.

Keywords: nectar, nectaries, nectary, PIN6, MYB57, Arabidopsis.

INTRODUCTION

Despite its central importance in plant–animal interactions, the molecular and genetic basis of nectar synthesis and secretion is largely unknown. Floral nectar is offered to increase pollinator visitation, while extra-floral nectar is used to attract mutualistic insects that provide protection from herbivory (Heil, 2011). Interestingly, although Arabidopsis thaliana is highly self-fertile, it has maintained functional nectaries, which have been implicated in facilitating out-crossing events (Chen et al., 2003; Hoffmann et al., 2003; Tholl et al., 2005; Kram and Carter, 2009). Thus, Arabidopsis may be used as a model for functional nectary analysis (Kram and Carter, 2009).

Arabidopsis flowers produce two types of nectaries: median and lateral. Lateral nectaries are located at the base of short stamens and produce >99% of total nectar, whereas median nectaries occur at the base of long stamens and petals and produce little or no nectar (Davis et al., 1998; Kram and Carter, 2009). Nectar production by both median and lateral nectaries is developmentally regulated. Immature lateral nectaries (to stage 12) accumulate starch, which is broken down at anthesis and the released sugars are secreted in mature flowers (stages 13–15; pollen shed and nectar secretion coincide) (Ren et al., 2007; Kram and Carter, 2009). In Arabidopsis and most Brassicaceae
species, the sugars in the secreted nectar are nearly all hexoses (glucose and fructose) and accumulate in approximately equal concentrations (Davis et al., 1998).

In recent years, several genes and molecular processes have been implicated in nectar production in multiple plant species. For example, Arabidopsis CELL WALL INVERTASE 4 is required for maintaining sink status and ultimately nectar secretion (Ruhlmann et al., 2010), and the transcription factor MYB305 regulates starch accumulation and hydrolysis in tobacco nectaries (Liu et al., 2009; Liu and Thornburg, 2012). The role of invertases in generating hexose-rich nectars via post-secretory action has also been reported (Heil et al., 2005; Nepi et al., 2012; Shenoy et al., 2012). In addition, the transcription factors AtMYB21 and AtMYB24 are required for nectary maturation via jasmonic acid-dependent pathways (Reeves et al., 2012). Jasmonic acid also induces nectar secretion in the extra-floral nectaries of lima bean (Phaseolus lunatus) (Heil, 2004) and the floral nectaries of Brassica sp. (Radhika et al., 2010). Other aspects of nectary development and function have also been reviewed (Kram and Carter, 2009; Heil, 2011).

Recent transcriptomic analyses identified a large number of genes whose expression is enriched in the nectaries of Arabidopsis and Brassica rapa (Kram et al., 2009; Hampton et al., 2010). One such nectary-enriched gene was PIN6 (At1g77110), a member of a family of auxin-efflux carriers. Here we demonstrate a role for PIN6 and auxin, as well as the transcription factor MYB57, in the regulation of nectar synthesis and secretion in Arabidopsis thaliana.

RESULTS

PIN6 and MYB57 have nectary-enriched expression profiles

Previous Affymetrix ATH1 microarray analyses identified a large number of genes with enriched expression in Arabidopsis nectaries (Kram et al., 2009). One gene displaying extreme up-regulation in nectaries is PIN6 (At1g77110) (Figure 1a). Use of PIN6pro:GUS lines demonstrated enrichment in both post-secretory and lateral nectaries (Figure 1a), and also showed that PIN6 is expressed in immature (stage 8) stamen (Figure 1b,c,d). PIN6 expression was not observed in other floral organs or at other developmental time points. The expression profiles of all PIN family genes in mature lateral nectaries and other reference tissues were also examined. Analysis of previous microarray data indicated that PIN6 was the only PIN family member displaying significant expression in nectaries (Table S1); this result was later supported by RNA-seq analyses of mature lateral nectaries (Table 1 and Table S2). Another gene examined in this study, MYB57 (At3g01530), displayed nectary enrichment, as demonstrated by microarray and RT–PCR analysis, although it is also expressed at lower levels in other floral tissues (Figure 1e).

pin6 and myb57 mutants have altered nectar and nectary phenotypes

To identify biological roles for PIN6 and MYB57, multiple T-DNA mutant alleles were identified (Figure 2a). Three independent homozygous pin6 mutants (all with T-DNA insertions in introns) were examined for altered expression level via quantitative RT–PCR, whereas myb57-2 expression was examined by end-point RT–PCR and Illumina-based RNA-seq (Figure S1 and Table S2). pin6-1, pin6-2 and pin6-3 were identified as knock-up, knock-out and knock-down mutants, respectively (Figure 2b, black bars), whereas myb57-2 was identified as a strong knock-down mutant due to a T-DNA insertion near the transcriptional start site (Figure S1 and Table S2).

The reason for the observed increase in PIN6 expression in pin6-1 is not clear; however, the quantitative RT–PCR primers used for PIN6 spanned the T-DNA insertion site for pin6-1, and were located 3’ to the insertion sites for pin6-2 and pin6-3. Further, 3’ RACE demonstrated that full-length transcript is produced in pin6-1 (Figure S2). The presence of a truncated PIN6 transcript in pin6-2 was confirmed by RNA-seq in pin6-2 lateral nectaries (Figure S3).

To demonstrate a role for PIN6 and MYB57 in nectar function, mutant lines were analyzed for total nectar glucose, as the vast majority (>99%) of sugars in Arabidopsis nectar are glucose and fructose in an approximately equal amounts (Davis et al., 1998). Significantly, PIN6 expression level was positively correlated with total nectar glucose (Figure 2b, gray bars), with nectar production being increased approximately 30% in the pin6-1 knock-up mutant, but significantly reduced in pin6-2 (knock-out) and pin6-3 (knock-down). Metabolite profiling of pin6-2 nectar (Table S3) confirmed a significant reduction in nectar sugar, with total glucose being approximately 75% lower than in wild-type Col-0, consistent with the results obtained from the enzymatic assays shown in Figure 2. Other nectar metabolites displaying significant differences between pin6-2 and Col-0 are highlighted in Table S3. Although not fully penetrant, pin6-1 flowers also often displayed large nectar droplets, which were not observed in wild-type Col-0 (Figure 2b). Total nectar glucose was also significantly reduced in myb57-2 (Figure 2). Finally, the putative involvement of PIN6 in auxin-dependent processes led us to examine nectar production in the auxin co-receptor mutant tir1-1 (Ruegger et al., 1998; Dharmasiri et al., 2005). Total nectar glucose was significantly increased in tir1–1, phenocopying the pin6–1 knock-up mutant.

pin6 and myb57 mutants have altered floral morphology

To identify potential reasons for the altered nectar secretion observed in pin6 and myb57 lines, mutant flowers were subjected to gross morphological analyses. pin6–1 and pin6–3 showed no observable differences in overall
floral morphology or nectary size compared with wild-type (Figure 3); however, pin6–2 flowers had petals that failed to fully expand, often had reduced nectary size, and lacked one or both short stamens significantly more frequently than wild-type (Figure 3 and Table 2). A wild-type Arabidopsis flower usually has four long stamens and two short stamens. The abnormal developmental phenotype of pin6–2 was complemented using a PIN6pro:PIN6-GFP construct (Figure S4), producing plants with petals that fully expanded, showed restored nectary size, and for which 86.6/4.5% of flowers had both short stamens present, compared to 84.3/3.2% of wild-type.

myb57–2 partially phenocopied the pin6–2 knockout mutant, having smaller lateral nectaries and missing one or both short stamens significantly more often than Col–0 (Figure 3 and Table 2). Further, when present, the short stamens of myb57–2 displayed a petaloid phenotype (Figure 4b,c). Interestingly, myb57–2 in the DR5:GUS background displayed extensive staining in the anther portion of the petal/ther fusion. RT–PCR on RNA isolated from myb57–2 anther/petal fusions also demonstrated a large

Table 1 Normalized RNAseq counts for PIN-family gene expression in mature lateral nectaries

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*Full read counts are presented in Table S2.

No reads were identified after the 3’ end of the T–DNA insertion site in pin6–2 (see Figure S3).

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increase in PIN6 expression compared with RNA isolated from Col-0 short stamen anthers. It should be noted that the petaloid stamen phenotype reverted to wild-type when complemented with a full-length genomic clone of MYB57 (Figure S4); however, the total number of short stamens present in complemented lines was only partially restored, with 72 ± 9.8% of flowers having both short stamens present, compared to 39.3 ± 16.2% in myb57-2 and 84.3 ± 3.2% in wild-type. The total amount of nectar in complemented myb57-2 lines was also partially restored, to 72 ± 19% of wild-type levels, whereas the amount of nectar in myb57-2 was 45 ± 9% of wild-type levels. The reason for the partial complementation phenotype of myb57-2 is unknown, although it is possible that MYB57 is mis-expressed in the myb57-2 mutant background due to the T-DNA insertion being located in the promoter region, and is thus not a true null allele.

RNA profiling of lateral nectaries from pin6-2 and myb57-2

pin6-2 and myb57-2 flowers partially phenocopied each other, displaying smaller lateral nectaries and reduced nectar production (Figures 2 and 3), as well as often lacking one or both short stamens (Figure 3 and Table 2). Further, PIN6 was mis-expressed in myb57-2 petaloid stamens (Figure 4). These results suggest that expression of these two genes may be dependent on the other for expression. RT-PCR analysis of gene expression in stage 14–15 (post-anthesis) flowers indicated that PIN6 is expressed at normal levels in myb57-2, and that MYB57 is also expressed normally in pin6-2 (Figure S1). Expression of a strong nectary-specific gene required for nectar production, AtSWEET9 (Lin IW, Chen L-Q, Sosso D, Gase K, Kim S-G, Kessler D, Klinkenberg P, Qu X-Q, Hou B-H, Carter C, Baldwin IT, Frommer WB, submitted), was also not altered in either myb57-2 or pin6-2. These results were later confirmed by transcriptome analysis of RNA isolated from the mature lateral nectaries of pin6-2 and myb57-2, as counts for both of these genes in each other’s backgrounds were similar to those of the wild-type (Table S2, first column). Comparative analyses of the pin6-2 and myb57-2 mature lateral nectary transcriptomes identified many genes that were differentially expressed compared with wild-type in one or the other mutant background, but few that were differentially expressed in both pin6-2 and myb57-2 (Table S2). Genes with counts twofold higher or lower in both pin6-2 and myb57-2 versus wild-type are listed in the second and third columns of Table S2, respectively, with the vast majority being expressed at relatively low levels. No significant enrichment in gene ontologies was identified for genes that are differentially expressed in both pin6-2 and myb57-2 versus wild-type.

It should be noted that we also examined the previously described myb57-1 mutant (SALK_065776), and did not observe any noticeable floral phenotype, consistent with previous findings (Cheng et al., 2009). MYB57 activity was previously implicated in stamen development through
redundant action with MYB21 and MYB24; however, only in higher-order mutants of these genes did stamens fail to properly elongate (Cheng et al., 2009). Cheng et al. (2009) did not report missing short stamens or petaloid stamen phenotypes in the myb57–1 or higher-order myb mutants. The T-DNA insertion in myb57–1 occurs toward the 3′ end of the third and final exon of this mutant, thus it is possible some functional MYB57 is produced, resulting in no visible change in phenotype.

Auxin responses are altered in pin6 nectaries

To further examine a role for PIN6 and auxin in nectar production, pin6 alleles were crossed into the auxin-responsive DR5::GFP reporter line and examined by confocal laser scanning microscopy. In the wild-type background, the DR5::GFP reporter displayed extensive signal in the distal portion of both lateral and median nectaries (Figure 5a), which co-localized with expression of a PIN6pro:PIN6::GFP reporter gene (Figure 5d–f). However, the DR5::GFP signal was greatly reduced in the lateral nectaries of both the pin6–1 (knock-up) and pin6–2 (knock-out) backgrounds, even when the nectary morphology was normal (Figure 5b, c). Interestingly, DR5-dependent signal did not appear to be reduced in the median nectaries of pin6–1 or pin6–2. Similar observations were made for DR5::GUS for both the pin6–1 and pin6–2 alleles (Figure S5). Significantly, plant lines expressing PIN6pro:PIN6::GFP demonstrated that PIN6 expression overlaps with that of auxin response in Arabidopsis nectaries.

As DR5 reporter genes were expressed at a lower level in both knock-out (pin6–2) and knock-up (pin6–1) mutants in the lateral nectaries, the mutants’ responses to exogenous synthetic auxin [α-naphthaleneacetic acid (NAA)] and auxin transport inhibitor N-1-naphthylphthalamic acid (NPA) were examined. We observed a positive correlation between NAA concentration and nectar production in feeding experiments with up to 100 μM NAA in 10% sucrose solutions, with a sharp decline at higher concentrations (Peter M. Klinkenberg and Clay J. Carter, unpublished data); therefore 100 μM NAA and NPA were used in these studies. Exogenous NAA significantly increased nectar production in wild-type Col-0 (>10-fold), whereas NPA caused a >2-fold reduction (Figure 6). Conversely, in pin6–1, NAA significantly reduced nectar production and NPA had no significant effect (Figure 6). No differences in nectar production were observed when pin6–2 was treated with NAA or NPA. While not statistically significant, nectar production in pin6–3 (knockdown) displayed responses to NAA and NPA that were similar to those for Col-0, but with smaller magnitude. These results may be due to pin6–3 being a knock-down rather than a knock-out mutant.

DISCUSSION

We previously identified a large number of genes in Arabidopsis with nectary-enriched expression profiles (Kram et al., 2009), and have subsequently used a large-scale reverse genetics approach to identify factors...
controlling nectar production in the Brassicaceae. This report describes initial efforts to characterize the involvement of PIN6 and MYB57 in Arabidopsis nectary function.

The canonical function of the PIN family of proteins is polar auxin transport, which controls differential growth and cellular response via establishment of auxin gradients (Feraru and Friml, 2008; Kleine-Vehn and Friml, 2008; Krecek et al., 2009; Petrasek and Friml, 2009; Robert and Friml, 2008; Vanneste and Friml, 2009; Friml, 2010; Grunewald and Friml, 2010; Wabnik et al., 2011a). PIN6 has been characterized as an irregular PIN protein that does not follow the typical intron-exon motif of most characterized PINs. Specifically, PIN6 has a shorter hydrophilic loop in the middle of its protein structure; however, like PIN1 and PIN4, it has ten transmembrane domains (Paponov et al., 2005) and demonstrates in vitro auxin transporter activity (Petrasek et al., 2006). Nonetheless, a biological function has not yet been ascribed to PIN6.

**The role of PIN6 in the nectary auxin response**

Microarray and reporter assays demonstrated that PIN6 is highly up-regulated in nectaries (Figures 1 and 5), with other PIN genes being expressed at very low levels (Table 1 and Table S1). This specificity of expression suggested a very particular role for PIN6 and auxin in nectary function, which was supported by several findings in this study as discussed below.

Three independent pin6 T-DNA mutant alleles with differing PIN6 expression levels were examined here. Interestingly, the level of PIN6 expression positively correlated with nectar production (Figure 2). To obtain an explanation for this phenotype, we first examined floral and nectary morphology. An attractive initial hypothesis was that PIN6 activity may affect nectary size, which in turn would affect nectar production. Indeed, pin6-2 often did have smaller nectaries and petals that failed to fully expand; however, no noticeable differences in nectary size or morphology were observed for pin6-1 (knock-up mutant) or pin6-3 (knock-down), even though they had contrasting nectar secretion phenotypes.

These results suggest that PIN6 and auxin may play a direct role in the regulation of nectar production rather than singularly affecting nectary development, a conclusion supported by several findings in this study. For example, a very strong DR5::GFP signal was observed in post-anthesis nectaries of the Col-0 background, which co-localized with PIN6::GFP at nectary tips (Figure 5). This observation is consistent with previous studies suggesting that the floral nectaries of multiple plant species produce indoleacetic acid (IAA) immediately prior to anthesis (Endress, 1994; Aloni et al., 2006). Aloni et al. (2006) used DR5::GUS expression analyses as a proxy to follow the initiation and progression of free auxin production in floral organs throughout development, including nectaries. The authors suggested that free IAA has two primary functions in flower development: (i) promotion of growth within the organs that produce auxin, and (ii) repression of development in adjacent organs that do not produce auxin (Aloni et al., 2006). One conclusion of their study was that auxin production shifts from anthers to nectaries at anthesis. It was suggested that IAA derived from anthers in pre-anthesis Arabidopsis flowers prevents nectar secretion until anthesis, whereupon nectaries become the primary sites of auxin synthesis in flowers (Aloni et al., 2006). However, it is important to note that DR5-based reporters are only a proxy for the auxin response, not auxin synthesis itself. Thus, nectaries may sequester auxin from surrounding tissues instead of directly synthesizing auxin, thereby controlling auxin homeostasis and response. Indeed, an analysis of the RNA-seq data in Table S2 indicates that genes involved in IAA biosynthesis (Mano and Nemoto, 2012) are expressed at very low levels (Table S4, most of these genes are near or in the lowest quartile of all genes for total RNA-seq counts). This analysis suggests that nectaries may not produce large amounts of free IAA; however, more studies are required...
to determine whether or not nectaries are active sites of auxin synthesis.

Another piece of evidence for a role for PIN6 and auxin in nectary function was that the auxin-dependent DR5:GFP signal in both the pin6-1 (knock-up) and pin6-2 (knock-out) alleles was significantly decreased in mature lateral nectaries (even when the nectary morphology was normal in pin6-2), whereas median nectaries appeared unaffected (Figure 5). This result was unexpected because: (i) pin6-1 and pin6-2 have opposite expression levels and nectar secretion phenotypes relative to wild-type Col-0, and (ii) PIN6 is highly expressed in both median and lateral nectaries, so it is expected that both nectary types would be affected equally. PIN6 is most closely related to PIN5, which was previously localized to the ER membrane (Mravec et al., 2009), thus differing from the plasma membrane localization of other described PINs. Mravec et al. (2009) also showed via transient expression assays that PIN6 and PIN8 appear to be located in the ER of tobacco BY-2 cells.

The observed localization patterns of PIN6pro:PIN6-GFP (Figure 5e,f) are consistent with the previously suggested ER localization; unfortunately, attempts to observe co-localization of PIN6-GFP with ER-specific dyes (e.g. ER-Tracker Red) and even diamidino-2-phenylindole (DAPI; nuclear stain) were unsuccessful because the thick cuticle covering the nectaries prevented staining in sub-epidermal cells.

The presence of PIN6 on the ER membrane suggests that it may play a role in intracellular auxin homeostasis,

Figure 5. PIN6 is required for proper auxin responses in nectaries.
(a) Confocal laser scanning microscopy of the DR5:GFP auxin-responsive reporter in the Col-0 background displayed strong signal in the distal portions of both lateral and median nectaries from stage 14 flowers (post-anthesis, secretory nectary).
(b,c) The auxin-responsive signal was significantly reduced in the lateral nectaries of both pin6-2 (b) and pin6-1 (c). Interestingly, DR5:GFP signal was still observed in the median nectaries of both pin6-1 and pin6-2.
(d-f) Wild-type plants expressing a PIN6pro:PIN6-GFP fusion showed that PIN6 is also expressed in the distal nectary (d,e) (overlaps with DR5:GFP expression), and that it accumulates in internal membranes (f) (arrowheads indicate modified open stomata of a lateral nectary).
LN, lateral nectary; MN, median nectary. Scale bars = 100 µm (a-e) and 20 µm (f). DAPI was used as a counter-stain in (a)–(c), (e) and (f); endogenous autofluorescence provided the background in (d).

Figure 6. Exogenous auxin increases and auxin transport inhibitor (NPA) decreases total nectar sugar in wild-type Arabidopsis flowers.
The peduncles of freshly cut inflorescences were placed in 10% buffered sucrose solutions containing either 100 µM NAA (synthetic auxin) or NPA (auxin transport inhibitor) in microcentrifuge tubes covered in Parafilm (Pechiney Plastic Packaging Company, Chicago, IL, USA). Peduncles were chosen based on the number of inflorescences nearing stage 13 (anthesis), which averaged between four and five for every three peduncles treated. Following 20 h incubation in a dark, humid environment, nectar was collected from the lateral nectaries of five stage 15 flowers (post-anthesis, secretory) using paper wicks, and assayed for total glucose. Data are presented as the percentage increase or decrease in total nectar glucose relative to mock treatments for each individual line (n = 3 biological replicates of nectar collected from five flowers each, **P < 0.005 relative to mock treated flowers).
as demonstrated for PIN5 (Mravec et al., 2009) and later expanded upon from a mechanistic perspective by Wabnik et al. (2011b). Thus, the function of PIN6 may be to sequester auxin from the cytosol into the lumen of the ER, thereby modulating cytosolic auxin concentrations and its cellular availability for auxin-dependent processes such as the SCFTIR1/AFB signaling pathway (Gray et al., 2001; Dharmasiri et al., 2005; Kepinski and Leyser, 2005). This idea is supported by the finding that, in the pin6–1 mutant, which has nearly a twofold increase in PIN6 transcript (Figure 2), the DR5:GFP signal is significantly reduced in lateral nectaries (Figure 5), and did not respond to exogenously applied auxin (Figure 6). Thus, the increased PIN6 expression in pin6–1 may result in decreased cytosolically available auxin and a concomitant reduction in SCFTIR1/AFB signaling. This notion is further supported by the finding that the auxin co-receptor mutant tir1–1 (Ruegger et al., 1998) phenocopied pin6–1 with regard to nectar secretion, secreting significantly higher amounts of nectar than Col–0 (Figure 2).

Additionally, one may expect auxin-responsive genes to be down-regulated in pin6–2 nectaries (due to decreased DR5:GFP signal in pin6–2 nectaries, Figure 5); indeed, seven of 271 genes with more than twofold higher counts in Col–0 versus pin6–2 nectaries were annotated as being auxin-responsive via GO annotation at http://arabidopsis.org/tools/bulk/go/index.jsp (data from Table S2; only genes with counts in the top half of all genes expressed in nectaries were analyzed due to low counts in remaining genes). These genes included At4g30080 (auxin response factor 16), At4g36740 (homeobox protein 40), At3g11820 (syntaxin of plants 121), At4g23570 (phosphatase-related), At4g37390 (auxin-responsive GH3 family protein), At5g35735 (auxin-responsive family protein) and At2g33830 (dormancy/auxin-associated family protein). However, enrichment of auxin-responsive genes was not statistically significant, which suggests that at least part of the auxin response in nectaries may be independent of transcriptional processes.

As mentioned above, whereas exogenously applied auxin (via peduncles placed in 100 μM NAA in 10% sucrose solutions) resulted in a large increase in nectar production in Col–0, it caused a significant reduction in pin6–1, no change in pin6–2, and a small but statistically insignificant increase in pin6–3 (Figure 6). The results we observed with wild-type flowers were consistent with a previous study in which exogenous auxin and gibberellic acid (GA3) (applied by spraying, not in cultured flowers) caused significant increases in nectar volume, nectar sugar concentration, dry nectar sugar mass, insect visitation abundance and seed yield in two species closely related to Arabidopsis (Brassica campestris and Brassica oleracea) (Mishra and Sharma, 1988). Other studies on excised flowers of snapdragon (Antirrhinum majus) supported a conflicting role for auxin in inhibiting nectar secretion (Shuel, 1959, 1964, 1978); however, under some conditions, exogenous IAA resulted in an increase in nectar production, suggesting a dual role for auxin in nectar production (Shuel, 1964). Whether these conflicting observations result from species differences (Brassicaceae versus snapdragon) or experimental design (the snapdragon studies used 500 μM IAA, whereas this study used 100 μM NAA) are unclear; however, we observed a positive correlation between NAA concentration and nectar production in our sucrose feeding experiments up to 100 μM NAA in 10% sucrose solutions, with a sharp decline at higher NAA concentrations (Peter M. Klinkenberg and Clay J. Carter, unpublished data). Significantly, through 14C-labeled IAA and sucrose experiments, Shuel (1978) concluded that exogenously applied auxin affects the secretory process itself within nectaries, rather than the movement of sugars to nectaries.

In contrast to NAA application, the auxin transport inhibitor NPA caused a significant decrease in nectar production in Col–0, but not in any of the pin6 mutants (Figure 6). The accumulation of PIN6 and auxin at nectary tips was also intriguing, and suggests that PIN6 may also play a role in defining nectary polarity. The reason for PIN6 expression in the distal nectary is uncertain; however, other nectary-enriched genes, such as CELL WALL INVERTASE 4 and a sesquiterpine synthase (At5g44630), appear to be expressed throughout the entire nectary (Tholl et al., 2005; Ruhlmann et al., 2010), suggesting that sub-domains exist within the Arabidopsis nectary. Cumulatively, these results indicate that fine-tuned control of auxin concentration in the sub-epidermal nectary parenchyma is essential for proper nectary function.

As to why median nectaries still have a strong DR5:GFP signal in pin6 mutant backgrounds, it should be noted that lateral nectaries secrete >99% of nectar in Arabidopsis, and there are also clear differences in gene expression and development between median and lateral nectaries (Davis et al., 1998; Kram and Carter, 2009; Kram et al., 2009). For example, a cupin-family gene, At1g74820, was previously found to be highly up-regulated in median versus lateral nectaries (Kram et al., 2009). RNA-seq analysis in this study identified At1g74820 as being expressed at a level that was 24-fold higher in the mature lateral nectaries of pin6–2 compared with Col–0 (Table S2). The cupin family of genes includes the auxin receptor AUXIN BINDING PROTEIN 1 (Shi and Yang, 2011) and other auxin binding proteins (Ohmiya, 2002). Thus, it is possible that At1g74820 may play a role in regulating the auxin response in nectaries.

The role of PIN6 and MYB57 in short stamen development

We also found that the pin6–2 and myb57–2 mutants partially phenocopied one another in having significantly reduced nectar production, nectary size and short stamen presence; however, differences between these two lines...
included that pin6–2 had petals that failed to fully expand (Figure 3), whereas myb57–2 had petaloid short stamens (when short stamens were present). Since PIN6 is expressed at normal levels in both myb57–2 whole flowers and mature lateral nectaries, it is unlikely that MYB57 directly regulates PIN6 expression (Figure S1 and Table S2). However, the anther portion of petaloid stamens in myb57–2 stained strongly in the DR5:GUS background in mature flowers, and PIN6 was also found to be mis-expressed in myb57–2 petaloid stamens instead of only being expressed in the nectaries of wild-type Col-0 flowers. As PIN6 is also expressed in immature stamens of wild-type plants (Figure 1), which coincides with auxin production or response in stamens at this stage (Aloni et al., 2006), these results are not necessarily surprising. It is currently unknown whether PIN6 activity is required for the petaloid short stamen phenotype observed in myb57–2, or whether PIN6 mis-expression in myb57–2 short stamens is a consequence of the petaloid phenotype. Nonetheless, the observed role of MYB57 in the proper development of short stamens is consistent with previous results, as MYB57 involvement in stamen elongation, through redundant action with MYB21 and MYB24, has been demonstrated previously (Cheng et al., 2009). Interestingly, MYB21 and MYB24 are also highly expressed in nectaries and are required for proper floral maturation (Reeves et al., 2012). Indeed, myb21 null mutants do not secrete nectar (Peter M. Klinkenberg and Clay J. Carter, unpublished data).

Cumulatively, these observations suggest the existence of an indirect link between MYB57 and PIN6, as well as a general role for PIN6 in the auxin response in mature flowers. The fact that small under-developed nectaries occur when short stamens are absent is consistent with previous results. Most floral jasmonic acid appears to be produced in stamen filaments (Ishiguro et al., 2001), and is not only required for floral maturation as a whole, including nectaries (Cheng et al., 2009; Reeves et al., 2012), but may also induce floral nectar secretion (Radhika et al., 2010). Indeed, when short stamens are present in pin6–2 and myb57–2, nectary morphology occasionally appears normal (e.g. Figure 5b). The link between stamen presence and nectary development and maturation is unclear; however, one previously proposed model is that jasmonic acid is transported downwards from filaments to the rest of the flower for maturation and expansion of other organs, such as petals (Ishiguro et al., 2001). Regardless, PIN6 activity is clearly required for proper nectary function, even when nectary morphology is normal, as demonstrated by the reduced nectar secretion phenotype of pin6–3 (Figure 2).

CONCLUSIONS

To conclude, we have demonstrated a clear role for PIN6, auxin and MYB57 in nectary development and function. These results indicate a crucial role for auxin homeostasis in nectaries for proper nectar secretion and floral development. Future studies will focus on the involvement of endogenous nectar auxin production in flower and nectary maturation, the precise role of PIN6 in regulating the auxin response in nectaries, and how PIN6 expression is regulated.

EXPERIMENTAL PROCEDURES

Plant materials and growth

The background for all plant materials was Arabidopsis thaliana cv Col-0. T-DNA mutant lines for PIN6 (AT1g77110: pin6–1, SALK_082098; pin6–2SALK_046393; pin6–3, SALK_095124C) and MYB57 (At3g01530: myb57–2, SALK_030969) were obtained from the Arabidopsis Biological Resource Center (Columbus, OH) (Alonso et al., 2003), and genotyped to obtain homozygous mutants as described at http://signal.salk.edu/tdnaprimers2.html. PIN6pro:GUS seed was also obtained from the Arabidopsis Biological Resource Center (CS9371), and DR5:GUS, DR5:GFP and tir1-1 were previously described (Ulmasov et al., 1997; Ruegger et al., 1998; Benkova et al., 2003). PIN6pro:PIN6-GFP lines were created by amplifying a 6662 bp PIN6 genomic fragment from Col-0 DNA using the PIN6-FULL-F and PIN6-GFP-R primers (Table S5) and Phusion polymerase (New England Biolabs, http://www.neb.com), and directly cloned into the XhoI and Xmal sites of the GFP-containing binary vector pORE–R4 (Coutu et al., 2007). This fragment contained 3051 bp of sequence upstream of the PIN6 start codon, together with the full PIN6 coding region, minus the stop codon, and was cloned in-frame with the GFP coding region found in pORE–R4. The sequence of the resulting construct, pCC15, was confirmed via dideoxy sequencing at the University of Minnesota DNA Sequencing and Analysis Facility (St Paul, MN), and transformed into Arabidopsis using Agrobac-

rrium tumefaciens (GV3101) by the floral-dip method (Clough and Bent, 1998). Transformed seedlings were selected on solid Murashige and Skoog (MS) medium with kanamycin (50 μg ml). For myb57–2 complementation, the full-length gene (promoter and coding region of MYB57) was PCR-amplified using the primer pair MYB57 comp–F and MYB57 comp–R (Table S5), and then ligated into the EcoRI and SpeI sites of the plant transformation vector pORE_O3 (Coutu et al., 2007), generating the construct pPMK23. myb57–2 was transformed with pPMK23 using Agrobacterium-mediated transformation by the floral-dip method (Clough and Bent, 1998). Transformed plants were selected on half-strength Murashige & Skoog medium with 50 μm phosphinothricin. Healthy seedlings were transplanted into soil and genotyped for the presence of pPMK23. All plants were grown in individual pots on a peat-based growth medium with vermiculite and perlite (Pro-Mix BX; Premier Horticulture, http://www.premierhorticulture.com/) in Percival AR66LX environmental chambers (http://www.percival-scientific.com/) under standard conditions: 16 h day/8 h night cycle, with a photosynthetic flux of 150 μmol m–2 sec–1 and a temperature of 23°C.

Chemicals and reagents

Unless noted otherwise, all chemicals were obtained from Sigma-Aldrich Chemical Co. (http://www.sigmaaldrich.com/) or Thermo Fisher Scientific (http://www.thermofisher.com/).

Microscopic analyses

Scanning electron microscopy and confocal analyses were performed as previously described (Ruhlmann et al., 2010), except for...
that DAPI, which is usually used as a nuclear stain, was found to extensively label the thick cuticle covering the nectaries. The cuticle prevented extensive staining of nectary parenchyma nuclei, even with extended periods of incubation. Thus, DAPI was used primarily as a counter-stain to observe the nectary surface. Flowers with sepals removed were briefly placed in 300 nM DAPI, and rinsed in ddH₂O just prior to imaging.

**Gene expression analyses**

Promoter-GUS assays were performed as previously described (Jefferson et al., 1987). For both end-point and quantitative RT-PCR, RNA was isolated from tissues using an Absolutely RNA Miniprep kit according to the manufacturer’s instructions (Agilent, http://www.agilent.com). RNA quality was confirmed by spectrophotometric analysis and agarose gel electrophoresis. For end-point RT-PCR analyses, total RNA (200 ng) was used as a template in the Promega reverse transcription system (http://www.promega.com/). The resulting cDNA (1 µl) was PCR-amplified using GoTaq Green Master Mix (Promega) according to the manufacturer’s instructions. Expression was analyzed on 1% agarose gels using SybrSafe (Invitrogen, http://www.invitrogen.com/). A minimum of three biological replicates and corresponding PCR reactions were used to confirm results. The sequences of the primers designed for gene expression analyses are given in Table S5. For 3′ RACE in pin6 mutants, total RNA was reverse-transcribed using the Promega reverse transcription system with the 3′ adapter + the oligo (dT) oligonucleotide primer (Table S5), and subsequently subjected to PCR amplification using the PIN6 qPCR F and 3′ RACE adapter oligonucleotide primers. RACE PCR products were subjected to DNA sequencing at the University of Minnesota DNA Sequencing and Analysis Facility (St Paul, MN).

For quantitative real-time PCR analyses, total RNA (100 ng) from pin6-1, pin6-2, pin6-3 and wild-type Arabidopsis flowers was used as template for cDNA synthesis via the QuantiTech reverse transcription system with the 3′ oligo (dT) oligonucleotide primer (Table S5), and subsequently subjected to PCR amplification using the PIN6 qPCR F and 3′ RACE adapter oligonucleotide primers. RACE PCR products were subjected to DNA sequencing at the University of Minnesota DNA Sequencing and Analysis Facility (St Paul, MN).

**Hormone treatment assays**

The peduncles of freshly cut inflorescences were placed in 10% w/v sucrose solutions in MES-buffered MS medium (pH 5.8) containing 100 µM of either the synthetic auxin α-naphthalene-acetic acid (NAA; MP Biomedicals, http://www.mpbio.com/) or the auxin transport inhibitor N-1-naphthalphthalamic acid (NPA; Supelco, www.sigmaaldrich.com/Supelco). Peduncles were chosen based on the number of inflorescences nearing stage 13 (anthesis), which averaged between four and five for every three peduncles treated. Following a 20 h incubation in a dark, humid environment, nectar was collected from the lateral nectaries of five stage 14–15 flowers (post-anthesis, secretory) using paper wicks and assayed for total glucose. Data were presented as the percentage increase or decrease in total nectar glucose relative to mock treatments for each individual line (n = 3 biological replicates of nectar collected from five flowers each; **P < 0.005 relative to mock treatments via Student’s paired two-tailed t-test).

**Microarray data mining**

The mean probe set signal intensities for all PIN family genes expressed in Arabidopsis nectaries, as identified by an Affymetrix ATH1 GeneChip microarray (http://www.affymetrix.com/), were compared to those in 13 tissues at multiple developmental stages, and are presented in Table S1. The raw normalized microarray data used for the analyses presented here were originally presented by Kram et al. (2009).

**RNA-seq analyses**

Collection of mature lateral nectaries from stage 14–15 flowers (Col-0, pin6-2 and myb57-2), and subsequent RNA isolation and quality controls were performed as previously described (Kram et al., 2009). Sequencing libraries were created using TruSeq RNA Sample Prep Kits (Illumina, http://www.illumina.com), and sequenced as 100 bp single end reads via Illumina HiSeq 2000 at the University of Minnesota DNA Sequencing and Analysis Facility (St Paul, MN). The number of reads for the wild-type Col-0, myb57-2 and pin6-2 samples were 24 712 426, 28 031 745 and 17 085 542, respectively. Sequences were compared to the TAIR10 reference (version 2.2.25+ (National Center for Biotechnology Information; Altschul et al., 1997). Quantification of splice variants was performed using BOWTIE (version 0.12.7) and TOPHAT (version 1.3.3) (Langmead et al., 2009; Trapnell et al., 2009). For blastn alignments, an E-value cut-off of 10⁻⁵ and a bit score of at least 75 were required. Counts were normalized using upper-quartile normalization (Bullard et al., 2010). The correction multipliers were 1.05, 0.79 and 1.27 for the wild-type, myb57-2 and pin6-2 samples, respectively. Full RNA-seq count data are presented in Table S2, and corresponding data have been deposited in NCBI Sequence Read Archive as SRA056392.
ACKNOWLEDGEMENTS

The authors thank Bryan Bandli (Research Instrumentation Laboratory, University of Minnesota Duluth) for assistance with scanning electron microscopy imaging, Molly Gorder and Mengyu Jia for technical assistance, and Dr Marci Surpin (Valent Bio-Sciences Corporation, Long Grove, IL, USA) for helpful discussions. We also thank Dr Bill Gray (University of Minnesota) for helpful discussions and providing seed for the DRS reporter lines and tir1-1. This work was funded by a grant from the US National Science Foundation (#0820730) to C.J.C.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. PIN6 expression in mature flowers is not dependent on MYB57.

Figure S2. 3' RACE analysis of PIN6 transcripts in pin6-1.

Figure S3. RNA-seq count distribution for PIN6 in pin6-2 and wild-type mature lateral nectaries.

Figure S4. Complementation phenotypes of pin6-2 and myb57-2.

Figure S5. DR5:GUS expression in pin6 mutant backgrounds.

Table S1. PIN family gene expression in Arabidopsis nectaries and reference tissues.

Table S2. RNA-seq count data for pin6-2 and myb57-2 mature lateral nectaries.

Table S3. Analysis of pin6-2 nectar metabolites.

Table S4. Analysis of gene expression associated with IAA synthesis and inactivation/homeostasis.

Table S5. Oligonucleotide primers used in this study.

REFERENCES


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PIN6 and auxin control nectary function 11


