

FEATURED ARTICLE

PIN6 is required for nectary auxin response and short stamen development

Ricci L. Bender^{1,†}, Megan L. Fekete^{1,†,‡}, Peter M. Klinkenberg¹, Marshall Hampton², Brittany Bauer¹, Matthew Malecha¹, Khrystine Lindgren³, Jennifer A. Maki³, M. Ann D. N. Perera^{4,5}, Basil J. Nikolau^{4,5} and Clay J. Carter^{1,*}

¹Department of Biology, University of Minnesota Duluth, Duluth, MN 55812, USA,

²Department of Mathematics & Statistics, University of Minnesota Duluth, Duluth, MN 55812, USA,

³Department of Chemistry, The College of St Scholastica, Duluth, MN 55811, USA,

⁴Department of Biochemistry, Biophysics & Molecular Biology, Iowa State University, Ames, IA 50011, USA, and

⁵W.M. Keck Metabolomics Research Laboratory, Iowa State University, Ames, IA 50011, USA

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*For correspondence (e-mail cjcarter@d.umn.edu).

†These authors contributed equally to this work.

‡Present address: Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, MI, 48109 USA.

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 (Ruhmann *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 *PIN6*pro:*GUS* lines demonstrated enrichment in both median 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 nectary 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

Figure 1. *PIN6* and *MYB57* have nectary-enriched expression patterns.

(a) Normalized mean ATH1 GeneChip probe set signal intensity for *Arabidopsis PIN6*. Original array data for all tissues were presented by Kram *et al.* (2009). ILN, immature lateral nectaries; MLN, mature lateral nectaries; MMN, mature median nectaries.

(b–d) Staining of GUS activity in the nectaries of stage 14–15 *PIN6::GUS* flowers; staining was not observed in other floral tissues or developmental stages. (b) GUS staining in nectaries. (c, d) GUS staining in a stage 8 stamen; side and top views, respectively.

(e) Normalized mean ATH1 GeneChip probe set signal intensity for *MYB57*. Inset: RT-PCR validation of *MYB57* expression patterns; the nectary lane comprised pooled median and lateral nectaries.

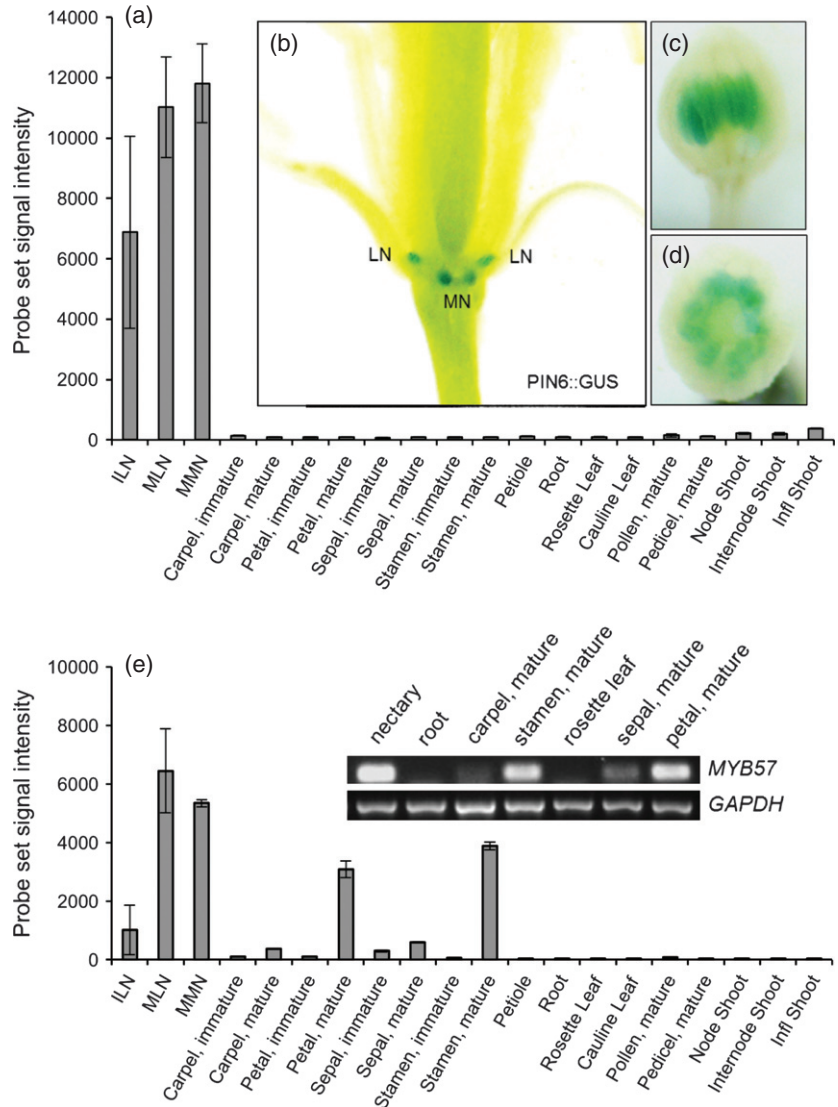


Table 1 Normalized RNAseq counts for *PIN*-family gene expression in mature lateral nectaries^a

	Col-0	<i>myb57-2</i>	<i>pin6-2</i>
<i>PIN1</i>	3	3	11
<i>PIN2</i>	0	1	0
<i>PIN3</i>	346	286	745
<i>PIN4</i>	4	2	5
<i>PIN5</i>	1	0	0
<i>PIN6</i>	26 662	22 601	9900 ^b
<i>PIN7</i>	15	14	8
<i>PIN8</i>	1	1	4

^aFull read counts are presented in Table S2.

^bNo reads were identified after the 3' end of the T-DNA insertion site in *pin6-2* (see Figure S3).

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 $86.6 \pm 4.5\%$ of flowers had both short stamens present, compared to $84.3 \pm 3.2\%$ 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/anther fusion. RT-PCR on RNA isolated from *myb57-2* anther/petal fusions also demonstrated a large

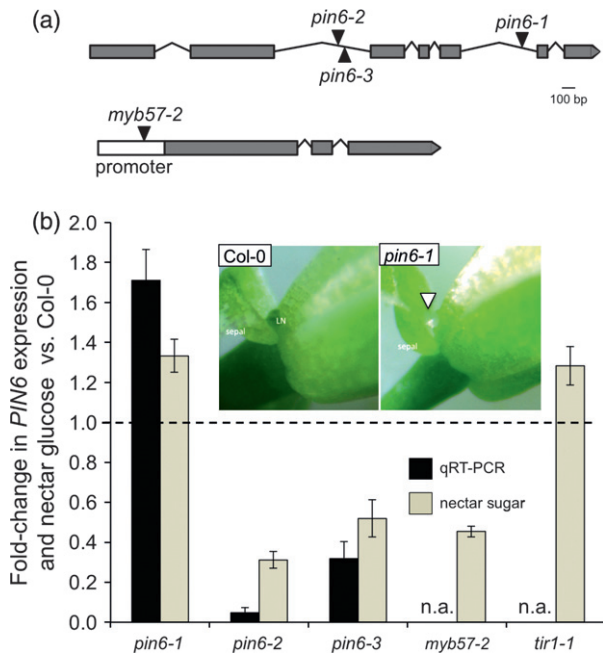


Figure 2. *pin6* and *myb57* T-DNA mutant allele series and associated nectar secretion phenotypes.

(a) Three independent *pin6* T-DNA mutants with altered gene expression levels were identified; the relative position of each mutation is indicated by arrowheads. A single mutant, *myb57-2*, resulted in a strong knockdown for *MYB57*.

(b) Quantitative RT-PCR (black bars) was used to examine changes in *PIN6* expression in whole flowers of each mutant line ($n = 3$, $P < 0.001$ for each mutant line versus wild-type). The mean *PIN6* expression in each mutant relative to wild-type is shown; the mean wild-type expression level is indicated by a dashed line. Total nectar glucose (gray bars) was also evaluated in *pin6* mutants, as well as *myb57-2* and *tir1-1*, and is also shown relative to wild-type nectar glucose ($n = 10$, $P < 0.005$ versus wild-type for each mutant line). Inset: the arrowhead indicates a large nectar droplet that is often present in *pin6-1* (knock-up mutant) but is not observed in Col-0 flowers; LN, lateral nectary. 'n.a.' indicates that quantitative RT-PCR assays were not applicable.

Table 2 Number of short stamens present in *pin6-2* and *myb57-2* flowers

Plant line	Mean percentages of plants with 0, 1 or 2 short stamens per flower ^a		
	0	1	2
Wild-type (Col-0)	1.3 ± 0.6	14.3 ± 3.1	84.3 ± 3.2
<i>pin6-2</i>	50.3 ± 6.4 ($P = 1.8 \times 10^{-4}$)	37.3 ± 7.6 ($P = 8.1 \times 10^{-3}$)	12.3 ± 1.5 ($P = 4.0 \times 10^{-6}$)
<i>myb57-2</i>	19.0 ± 14.8 ($P = 0.11$)	41.7 ± 4.5 ($P = 9.6 \times 10^{-4}$)	39.3 ± 16.2 ($P = 9.1 \times 10^{-3}$)

^a $n = 3$ biological replicates of 100 flowers each. P values versus Col-0 are indicated (paired Student's t test); significant changes are shown in bold. Wild-type Arabidopsis flowers usually have two short stamens and four long stamens.

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 $72 \pm 9.8\%$ of flowers having both short stamens present, compared to $39.3 \pm 16.2\%$ in *myb57-2* and $84.3 \pm 3.2\%$ in wild-type. The total amount of nectar in complemented *myb57-2* lines was also partially restored, to $72 \pm 19\%$ of wild-type levels, whereas the amount of nectar in *myb57-2* was $45 \pm 9\%$ 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

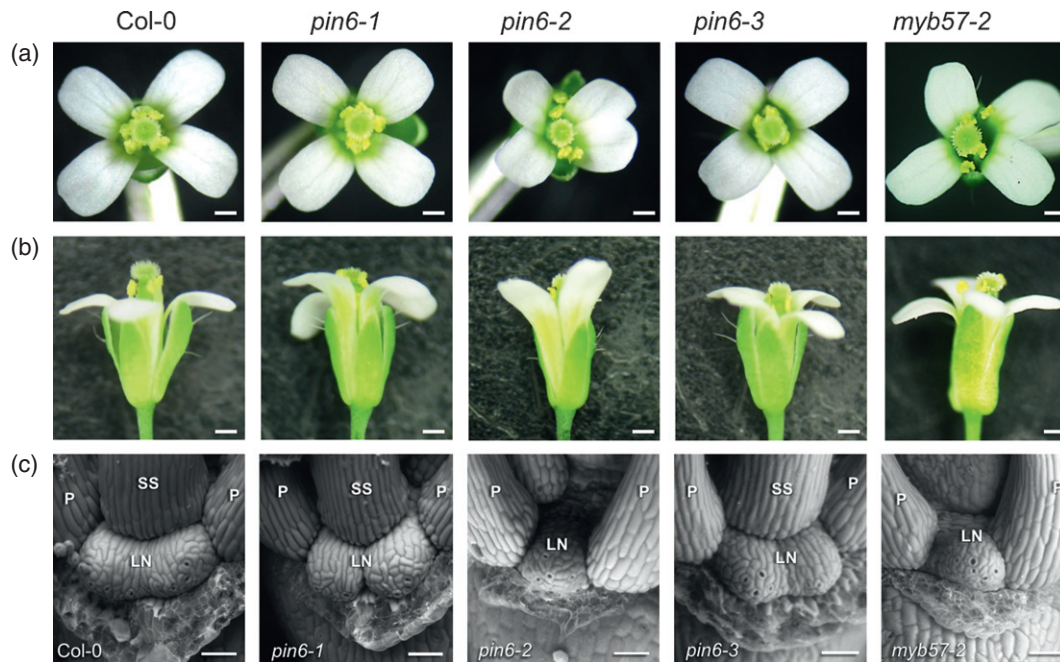


Figure 3. Gross morphology of *pin6* and *myb57* mutant flowers. *pin6-1* and *pin6-3* displayed normal floral morphologies; however, *pin6-2* had petals that failed to expand (a,b), smaller nectaries and missing short stamens (c) (Table 1). In addition, *myb57-2* partially phenocopied *pin6-2* in having reduced nectary size (c), missing short stamens at a significantly increased rate versus Col-0 (c) (Table 1), and less total nectar (see Figure 2). Scale bars = 500 μm (a,b), 50 μm (c). SS, short stamen; P, petal; LN, lateral nectary.

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

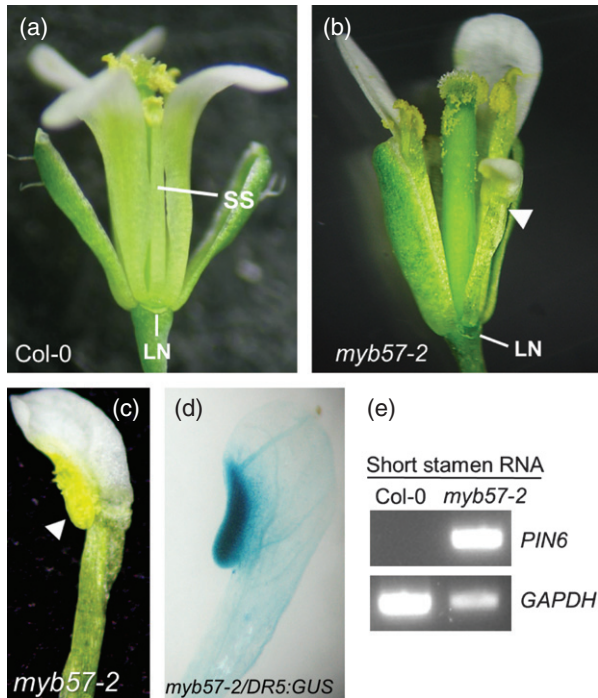


Figure 4. *MYB57* is required for proper development of short stamens. (a) Wild-type Col-0 flower with one sepal removed to reveal the location of a lateral nectary (LN) and a short stamen (SS). (b,c) *myb57-2* flowers. When present, the short stamen in *myb57-2* flowers displayed a petaloid phenotype. (d) *myb57-2* in the *DR5:GUS* background stained intensely in the anther portion of the short stamen, and RNA isolated from the *myb57-2* short stamen showed a significant increase in *PIN6* expression compared with wild-type short stamens.

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, 2009; 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 (Eन्द्रess, 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

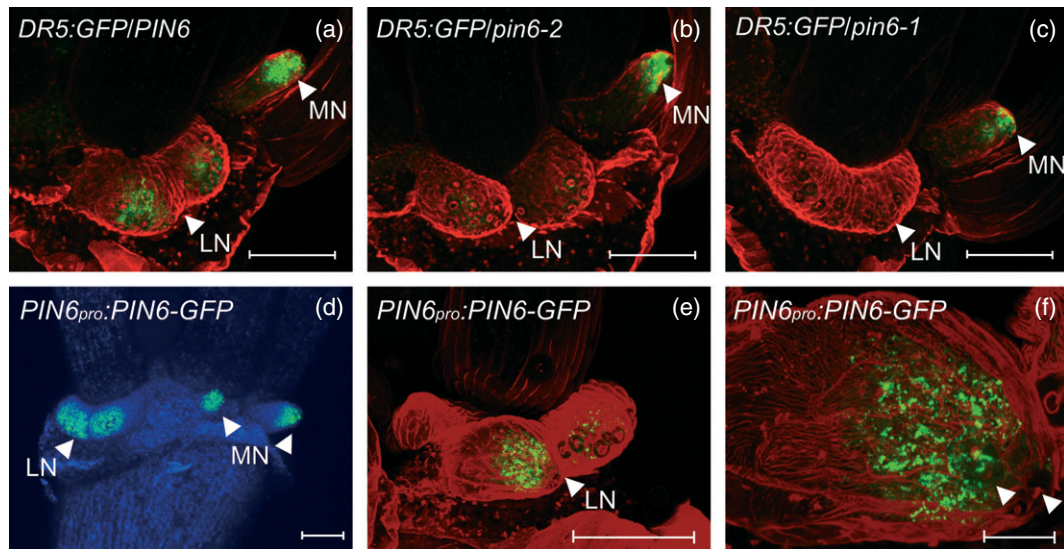


Figure 5. *PIN6* is required for proper auxin responses in nectaries.

(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).

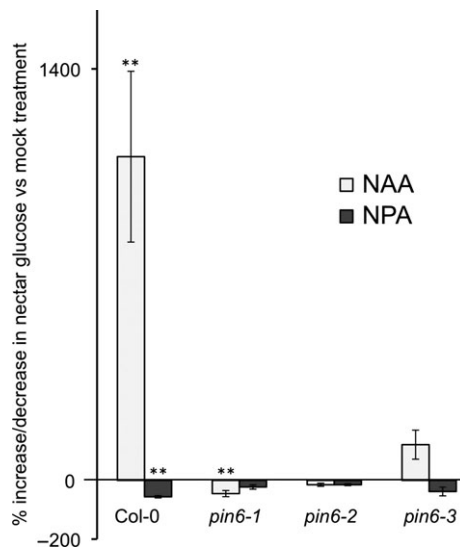


Figure 6. Exogenous auxin increases and auxin transport inhibitor (NPA) decreases total nectar sugar in wild-type *Arabidopsis* flowers.

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).

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,

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 SCF^{TIR1/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 SCF^{TIR1/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 μ M 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 μ M IAA, whereas this study used 100 μ M NAA) are unclear; however, we observed a positive correlation between NAA concentration and nectar production in our sucrose feeding experiments up to 100 μ M 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 sesquiterpene 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 nectary 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; *pin6-2*, SALK_046393; *pin6-3*, SALK_095142C) 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/tdnaprimers.2.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 6602 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 *XmaI* 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 *Agrobacterium 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.pthorticulture.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.fishersci.com/>).

Microscopic analyses

Scanning electron microscopy and confocal analyses were performed as previously described (Ruhlmann *et al.*, 2010), except

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 QuantiTect reverse transcription kit (Qiagen, <http://www.qiagen.com/>). Synthesized cDNA (50 ng) was added to the real-time PCR reaction set-up, which included 12.5 µl of 2 × Rotor-Gene SYBR Green PCR Master Mix (Qiagen), 2.5 µM forward primer and 2.5 µM reverse primer to a final reaction volume of 25 µl. Primers were designed based on the Roche Universal Probe Library (www.roche-applied-science.com) using the accession numbers At1g77110 (*PIN6*) and At1g49240 (*ACTIN8*) (Table S1). The *PIN6* quantitative RT-PCR primers spanned the T-DNA insertion site for *pin6-1*, and were located 3' to the insertion sites for *pin6-2* and *pin6-3*. Standard curve reactions were prepared using serial dilutions of shoot cDNA and *ACTIN8* primers. The standard curve, *pin6* and wild-type reactions were set up in triplicate and incubated at 95°C for 5 min, then underwent 40 cycles of 95°C for 5 sec followed by 60°C for 10 sec in a Corbett Rotor-Gene 3000 Light Cycler (<http://www.corbettlifescience.com/>). SYBR Green fluorescence was detected during the 60°C incubation step. Standard reaction analysis was performed using the ROTOR-GENE 6 software. PCR reactions were performed six times. The three most efficient trials were chosen for final comparison.

Nectar collection and metabolite analysis

Nectar collection was performed as previously described (Bender *et al.*, 2012). Briefly, a single biological replicate for nectar glucose assays consisted of nectar collected from the lateral nectaries of ten stage 14–15 flowers using small, uniform wicks cut from Whatman No. 1 filter paper (<http://www.whatman.com/>). Completed wicks were placed in 500 µl of nuclease-free water for elution, and stored at –20°C until further analysis. Relative glucose

concentration was then analyzed using a modified glucose oxidase assay as previously described (Bethke and Busse, 2008; Ruhlmann *et al.*, 2010; Bender *et al.*, 2012). Nectar collection for full metabolite analyses was performed as for glucose assays, except that 20 flowers were used for a single biological replicate, and the nectar was eluted in 100 µl nuclease-free water. Nectar samples used for metabolomic analyses were flash-frozen and stored at –80°C until analyzed via GC×GC/MS (Agilent, Santa Clara, CA, USA, <http://www.agilent.com>) as previously described (Bender *et al.*, 2012) at the W.M. Metabolomics Research Laboratory at Iowa State University (Ames, IA).

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 -naphthaleneacetic acid (NAA; MP Biomedicals, <http://www.mpbio.com/>) or the auxin transport inhibitor *N*-1-naphthylphthalamic 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 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 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 updated 14 December 2010) using BLASTN 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.

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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.

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