Two GATA Transcription Factors Are Downstream Effectors of Floral Homeotic Gene Action in Arabidopsis[1[W][OA]]

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Floral organogenesis is dependent on the combinatorial action of MADS-box transcription factors, which in turn control the expression of suites of genes required for growth, patterning, and differentiation. In Arabidopsis (*Arabidopsis thaliana*), the specification of petal and stamen identity depends on the action of two MADS-box gene products, APETALA3 (AP3) and PISTILLATA (PI). In a screen for genes whose expression was altered in response to the induction of *AP3* activity, we identified *GNC* (GATA, nitrate-inducible, carbon-metabolism-involved) as being negatively regulated by AP3 and PI. The *GNC* gene encodes a member of the Arabidopsis GATA transcription factor family and has been implicated in the regulation of chlorophyll biosynthesis as well as carbon and nitrogen metabolism. In addition, we found that the *GNC* paralog, *GNL* (*GNC*-like), is also negatively regulated by AP3 and PI. Using chromatin immunoprecipitation, we showed that promoter sequences of both *GNC* and *GNL* are bound by PI protein, suggesting a direct regulatory interaction. Analyses of single and double *gnc* and *gnl* mutants indicated that the two genes share redundant roles in promoting chlorophyll biosynthesis, suggesting that in repressing *GNC* and *GNL*, AP3/PI have roles in negatively regulating this biosynthetic pathway in flowers. In addition, coexpression analyses of genes regulated by AP3, PI, GNC, and GNL indicate a complex regulatory interplay between these transcription factors in regulating a variety of light and nutrient responsive genes. Together, these results provide new insights into the transcriptional cascades controlling the specification of floral organ identities.

The floral homeotic *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) genes encode MADS-box transcription factors that are necessary for specifying petal and stamen identity in Arabidopsis (*Arabidopsis thaliana*; Bowman et al., 1989; Jack et al., 1992, 1994; Krizek and Meyerowitz, 1996). They have been shown to act as obligate heterodimers in binding to DNA, and likely act as components of higher order transcriptional complexes in conjunction with other MADS-box proteins (Goto and Meyerowitz, 1994; McGonigle et al., 1996; Riechmann et al., 1996a; Honma and Goto, 2001). Presumably each distinct MADS-box protein complex directs the development of a specific organ type through regulating the transcription of downstream target genes, although the exact mechanism by which this occurs is unknown (Jack, 2001).

A number of studies have been carried out to identify candidate downstream target genes regulated by MADS-box transcription factors. Global gene expression profiling methods have led to the identification of many genes whose transcription is regulated by AP3 or PI (Sablowski and Meyerowitz, 1998; Zik and Irish, 2003; Wellmer et al., 2004; Sundstrom et al., 2006; Alves-Ferreira et al., 2007). Although understanding the roles of such genes is critical to defining the hierarchy of activities required for appropriate organ specification and differentiation; to date, only a few such target genes have been functionally analyzed. *AP3* and *PI* positively autoregulate their own expression, and AP3/PI heterodimers have been shown to bind to sequences in the *AP3* promoter suggesting that this regulation is direct (Jack et al., 1992; Goto and Meyerowitz, 1994; Hill et al., 1998; Tilly et al., 1998; Sundstrom et al., 2006). By contrast, *PI* autoregulation appears to be indirect, as de novo protein synthesis is required for this regulatory feedback loop to occur (Honma and Goto, 2000). AP3/PI also acts to positively regulate *NAP* (NAC-like, activated by AP3/PI), a gene that is involved in the transition from the cell division to cell expansion phase during the growth of petals and stamens (Sablowski and Meyerowitz, 1998). AP3/PI has also been shown to act as a negative regulator of transcription of the floral homeotic *AP1* gene (Sundstrom et al., 2006). Chromatin immunoprecipitation (ChIP) experiments using *35S::PI-HA* plants demonstrated that PI can directly bind to a 52-bp region in the *AP1* promoter, suggesting that regulation of *AP1* by AP3/PI is direct (Sundstrom et al., 2006). *AP1* expression was also shown to decrease rapidly (within 2 h) after AP3/PI induction, lending further support for the direct regulation of *AP1* by AP3/PI. In turn, AP1 has been

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shown to positively regulate *AP3* and *PI*. Furthermore, AP1, AP3, and PI proteins have been shown to interact in a multimeric protein complex, suggesting that a complex network of regulatory feedback loops is important in establishing individual floral organ identities (Honma and Goto, 2001; Ng and Yanofsky, 2001; Lamb et al., 2002).

In this study we have screened for genes that are targets of AP3/PI action using an inducible *AP3* transgenic construct. One such gene is *At5g56860*, *GNC* (GATA, nitrate-inducible, carbon-metabolism involved), whose expression profile decreased 2.8-fold after AP3 induction. We also found that a paralog of *GNC*, *GNL* (*At4g26150*), is negatively regulated by AP3/PI. These data lend support to the idea that AP3/PI may have important roles in negative as well as positive regulation of downstream target genes.

GNC and *GNL* belong to a family of 29 genes encoding GATA transcription factors in Arabidopsis (Riechmann et al., 2000; Reyes et al., 2004; Bi et al., 2005; Manfield et al., 2007). GATA transcription factors, so-named because they bind to conserved GATA motifs, contain a characteristic type-IV zinc finger DNA-binding domain (Teakle et al., 2002; Reyes et al., 2004; Manfield et al., 2007). The functional roles of many of these transcription factors still need to be elucidated, but some have been implicated in the regulation of light responsive genes (Arguello-Astorga and Herrera-Estrella, 1998; Jeong and Shih, 2003). Consistent with a role in light regulation, an insertional mutation in *GNC* disrupts chlorophyll biosynthesis, as well as having defects in Glc signaling (Bi et al., 2005). *GNC* expression is also nitrate-inducible (Bi et al., 2005). Genes known to be involved in nitrate assimilation such as those encoding nitrite reductase (NiR) and nitrate reductase (NIA) have GATA motifs in their regulatory regions, lending support to the idea that GNC has a role in nitrogen metabolism (Jarai et al., 1992; Bi et al., 2005).

Here we show that GNL is partially redundant with GNC in regulating chlorophyll biosynthesis and in the transcription of a number of GATA-motif-containing target genes. Furthermore, we show that *GNC* and *GNL* are both directly and negatively regulated by AP3/PI in petals and stamens. These observations suggest that AP3/PI function in part to repress *GNC* and *GNL* in these organs, resulting in the down-regulation of chlorophyll biosynthesis in petals and stamens. Furthermore, we examine the regulatory interplay between these MADS-box and GATA transcription factors, and reveal a complex network of regulatory interactions in the control of a variety of light- and nutrient-responsive genes.

RESULTS

Identification of *GNC* and *GNL* as Targets of AP3 and PI

To identify genes regulated by AP3/PI, we carried out microarray experiments using an Arabidopsis whole genome GeneChip array (ATH1 GeneChip; Affymetrix) in conjunction with an inducible AP3-GR system. In this system, the AP3 protein is translationally fused to the rat glucocorticoid receptor; the fusion protein is rendered inactive because it is trapped in the cytoplasm through binding to heat shock protein hsp90 (Sablowski and Meyerowitz, 1998). When the steroid hormone dexamethasone (dex) is applied to transgenic Arabidopsis plants containing this construct, hsp90 is released and the activated AP3-GR protein can enter the nucleus and regulate the expression of downstream target genes. For these experiments, we used *35S::AP3-GR* transgenic plants in a *35S::PI*, *ap3-3* null mutant background for various dex or mock treatments (Sablowski and Meyerowitz, 1998). It has been shown previously that induction of the AP3-GR fusion protein can restore AP3 function in the null *ap3-3* mutant (Sablowski and Meyerowitz, 1998). Thus, plants of the genotype *35S::AP3-GR*, *35S::PI*, *ap3-3* show a *ap3-3* mutant phenotype and, upon induction with dex, display a rescue of the mutant phenotype, as well as partial homeotic conversions of sepals to petals and carpels to stamens, reflecting the combined ectopic expression of *AP3* and *PI* (Sablowski and Meyerowitz, 1998).

RNA was extracted from inflorescences at 0 and 4 h after dex or a mock treatment and used as probes for our microarray experiments. Three biological replicates of each were hybridized to Affymetrix ATH1 arrays. We used the Affymetrix Microarray Suite software (MAS 5.0) to identify genes whose expression profiles changed only after dex treatment and are likely targets of AP3/PI. Thus, we eliminated genes whose expression profiles changed in the same direction after both dex and mock treatments and retained 283 genes that changed only in 0-h versus 4-h dex-treated samples and are potential targets of AP3/PI (Supplemental Tables S1–S3). Of these 283 genes, 100 genes were up or down-regulated at least 2-fold in two out of three replicates (Supplemental Table S4). To verify our microarray data, we chose 34 candidate genes that had a significant P value (<0.05), changed at least 2-fold in all three replicates, and/or belonged to a small gene family (Supplemental Table S4).

For all 34 genes, reverse transcription (RT)-PCR analyses corroborated the direction of fold change in 0-h versus 4-h dex and 0-h versus 4-h mock microarray experiments (data not shown). Because dex treatment should induce AP3 activity, genes that are up-regulated after dex treatment should be positively regulated by AP3/PI and genes that are down-regulated should be repressed. To determine if these 34 genes were potential targets of AP3/PI, we monitored their expression patterns in *ap3-3* and *pi-1* mutant plants compared to wild type by RT-PCR (data not shown). We confirmed 18 genes as putative targets of AP3/PI (Supplemental Table S5). One such gene was *At5g56860* (*GNC*). *GNC* encodes a member of the GATA transcription factor family that has been implicated in regulating carbon and nitrogen metabolism and in promoting chlorophyll biosynthesis (Bi et al., 2005).

Our microarray data indicated that *GNC* expression was down-regulated 2.8-fold after AP3 induction, suggesting that AP3/PI may negatively regulate *GNC*. RT-PCR data corroborate the microarray data such that *GNC* expression decreases 4 h after dex treatment of AP3-GR plants and increases in *ap3-3* and *pi-1* mutant flowers as compared to wild type (Fig. 1). The most closely related paralog of *GNC* is *At4g26150* (Reyes et al., 2004), which we have named *GNL* (*GNC*-like). Because *GNC* and *GNL* arose from a large chromosomal gene duplication (Reyes et al., 2004) and showed similar expression profiles (Manfield et al., 2007), we tested whether AP3/PI also regulates *GNL*. We found that *GNL* expression decreases after AP3 induction and increases in *ap3-3* and *pi-1* mutant plants (Fig. 1). Thus, *GNL* is also negatively regulated by AP3/PI.

We used ChIP to determine if *GNC* and *GNL* are direct targets of AP3/PI. It has been shown that the AP3/PI heterodimer can bind to a 10-bp conserved DNA region called the CArG box ($CC(A/T)_6GG$; Schwarz-Sommer et al., 1992; Riechmann et al., 1996b; Hill et al., 1998). Using RSA tools software we identified a CArG-like box present in each of the promoter regions of *GNC* and *GNL* (Fig. 2A). As a positive control, we assayed for binding to CArG box 3, a known autoregulatory region in the *AP3* promoter (Fig. 2A; Hill et al., 1998). We extracted nuclei from wild-type and *35S::PI-HA* epitope tagged transgenic plants and immunoprecipitated with either anti-HA antibody or normal mouse serum. For ChIP PCR, we used primers designed around each CArG-like box to monitor enrichment. We detected a 3.1-fold enrichment of a 354-bp fragment in the *GNC* promoter region that contains the CArG-like box in *35S::PI-HA* extracts precipitated with anti-HA antibodies as compared to normal serum controls (Fig. 2, B and C). Thus, *GNC* is bound by PI protein, suggesting that GNC is a direct target of AP3/PI. We also detected a specific 2.0-fold enrichment of a 216-bp region spanning the CArG-like box present in the *GNL* promoter, suggesting that *GNL* may also be a direct target of AP3/PI (Fig. 2, B and C). As expected, we detected significant enrichment of the CArG box 3 region of the *AP3* promoter in anti-HA immunoprecipitated DNA from *35S::PI-HA* plants while no enrichment was detected for the promoter regions of our negative controls, *PI* (an indirect target of AP3/PI) or *AST101* (a root-specific gene not regulated by AP3/PI; Fig. 2, B and C).

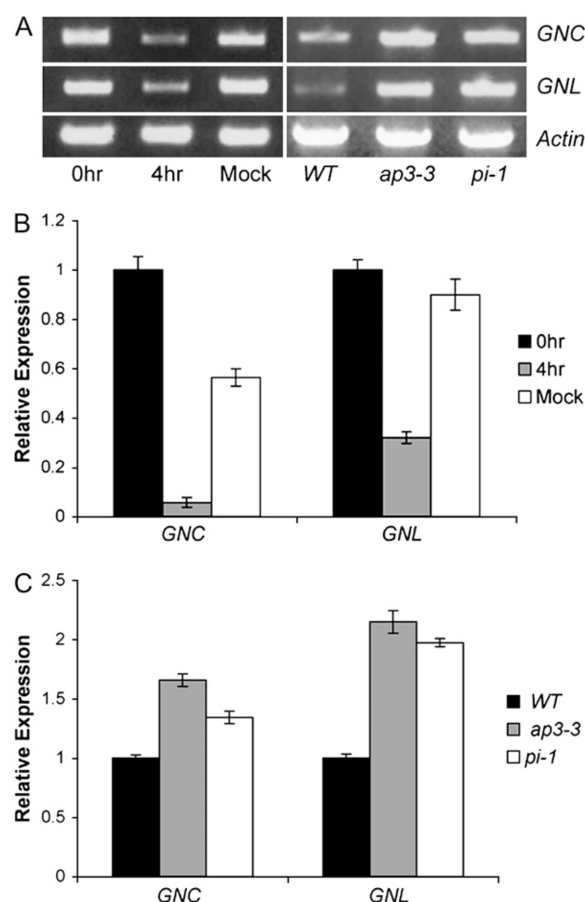

Figure 1. *GNC* and *GNL* are targets of AP3/PI. A, Expression levels of *GNC* and *GNL* by RT-PCR in 0-h and 4-h dex- and mock-treated flowers and in the wild type (*WT*), and *ap3-3* and *pi-1* mutant flowers. B, Quantified expression levels of *GNC* and *GNL* by RT-PCR in 0-h and 4-h dex- and mock-treated flowers. Average expression levels from three replicates were normalized to actin with wild type scaled to 1. C. Quantified expression levels of *GNC* and *GNL* by RT-PCR in the wild type (*WT*), and *ap3-3* and *pi-1* mutant flowers. Average expression levels from three replicates were normalized to actin with wild type scaled to 1.

GNC and GNL Have Roles in the Leaves and Flowers

GNC and *GNL* are both expressed most strongly in the cauline and rosette leaves of wild-type plants and to a lesser extent in the flowers, siliques, and stems (Fig. 3A). To further characterize these expression patterns in the flower, we carried out in situ hybridizations. These data indicated that in young stage 3 flowers, *GNC* expression is detected throughout the floral bud (Fig. 3B). By stage 6, *GNC* expression is largely restricted to the inner whorls of the flower, specifically the petals, stamens, and carpels (Fig. 3C). In older flowers, from stage 8 onward, *GNC* expression is detectable in the petals, stamen filaments, and carpels with weaker expression in the anthers of the stamens (Fig. 3, D and E). Thus, AP3/PI may repress strong *GNC* expression in the anthers at later stages. The expression pattern of *GNL* overlaps with that of *GNC*, suggesting that the two genes may share redundant functions. *GNL* expression is first detected in at stage 3 throughout the entire floral bud (Fig. 3F). By stage 6, strongest expression is restricted to the inner three whorls (Fig. 3G). As the flower matures, *GNL* expression in the stamens decreases compared to the expression in the petals and carpels, suggesting that AP3/PI may repress *GNL* in the stamens (Fig. 3, H and I).

To explicitly test whether AP3/PI repress *GNC* and *GNL* expression in a spatially limited manner, we examined the expression domains of these genes in

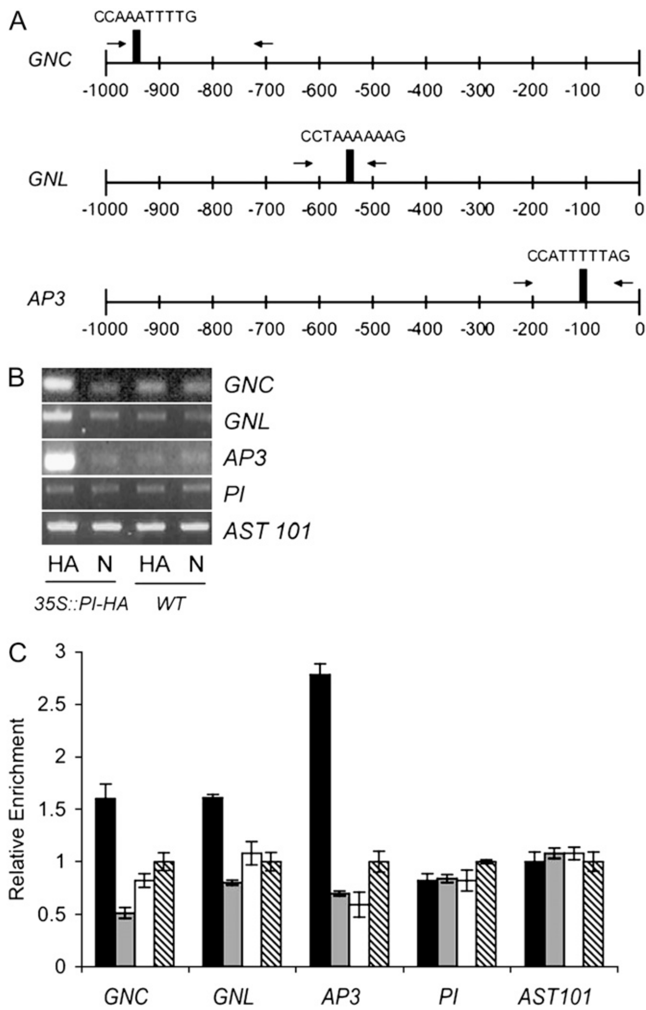

Figure 2. *GNC* and *GNL* are directly regulated by AP3/PI. A, CArG-like boxes in the *GNC*, *GNL* and *AP3* promoters. Black boxes indicate the position of the CArG-like box and the arrows indicate the position of the primers used for ChIP PCR. B, ChIP PCR using primers flanking the CArG-like boxes indicates that there is significant enrichment in these regions of the *GNC*, *GNL* and *AP3* promoters. *35S::PI-HA* and wild-type (*WT*) nuclear extracts were immunoprecipitated with α-HA antibody (HA) or normal serum (N). C, Quantified enrichment levels in the *GNC*, *GNL*, *AP3*, *PI*, and *AST101* promoters. Black bars represent *35S::PI-HA* samples immunoprecipitated with α-HA antibody. Gray bars represent *35S::PI-HA* samples immunoprecipitated with normal serum. White bars represent wild-type samples immunoprecipitated with α-HA antibody. Cross-hatched bars represent wild-type samples immunoprecipitated with normal serum. Average enrichment levels from three replicates were normalized to wild-type samples immunoprecipitated with normal serum scaled to 1.

ap3-3 and *pi-1* mutant flowers. The *ap3-3* and *pi-1* mutants display similar homeotic phenotypes, in which petals are transformed into sepalloid organs and stamens are transformed into carpeloid organs. Consistent with a role in modulating the overall levels of expression of *GNC* and *GNL*, expression of each gene was detectable in the inner three whorls, including third-whorl tissues, of *ap3-3* and *pi-1* flowers (Fig. 3, J–M).

Analyses of T-DNA insertional mutations in both *GNC* and *GNL* indicated that they have partially redundant roles in chlorophyll biosynthesis. T-DNA insertional mutations for each gene were obtained from the SALK collection (Alonso et al., 2003); both *gnc* (SALK 001778) and *gnl* (SALK 21362C) homozygous mutations resulted in undetectable levels of transcripts, suggesting both mutations are complete loss-of-function alleles (Fig. 4, A and C). The rosette leaves of homozygous *gnc* mutant plants were a paler green compared to wild type (Fig. 4B), as reported in Bi et al. (2005). Homozygous *gnl* mutants also displayed a similar phenotype, with paler rosette and cauline leaves (Fig. 4B). We extracted chlorophyll from mutant and wild-type rosette leaves to determine if mutants had decreased chlorophyll levels. In agreement with Bi et al. (2005), we found that chlorophyll levels were decreased significantly in *gnc* mutant rosette leaves (Fig. 4D). We also detected a decrease in chlorophyll levels in other parts of the plants including the cauline leaves and the siliques (Fig. 4D). Chlorophyll extrac-

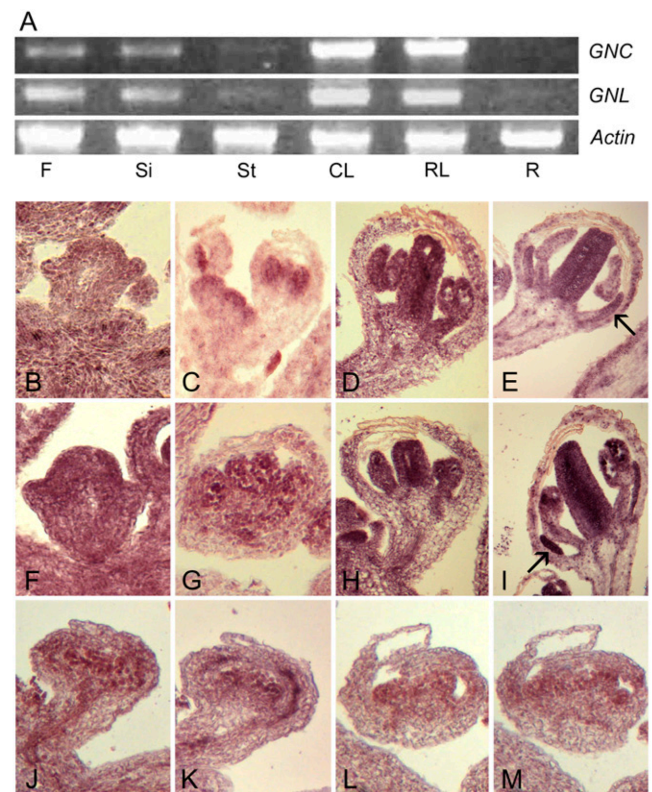

Figure 3. Expression patterns of *GNC* and *GNL*. Expression of *GNC* and *GNL* in flowers (F), siliques (Si), stems (St), cauline leaves (CL), rosette leaves (RL), and roots (R) by RT-PCR. Expression of *GNC* in stage 4 (B), stage 4 to 6 (C), stage 8 (D), and stage 10 (E) wild-type flowers by in situ hybridization. Expression of *GNL* in stage 4 (F), stage 6 (G), stage 8 (H), and stage 10 (I) wild-type flowers. Arrows indicate strong petal expression at later stages of development. Expression of *GNC* (J) and *GNL* (K) in serial sections of *ap3-3* mutant flower buds. Expression of GNC (L) and *GNL* (M) in serial sections of *pi-1* mutant flower buds.

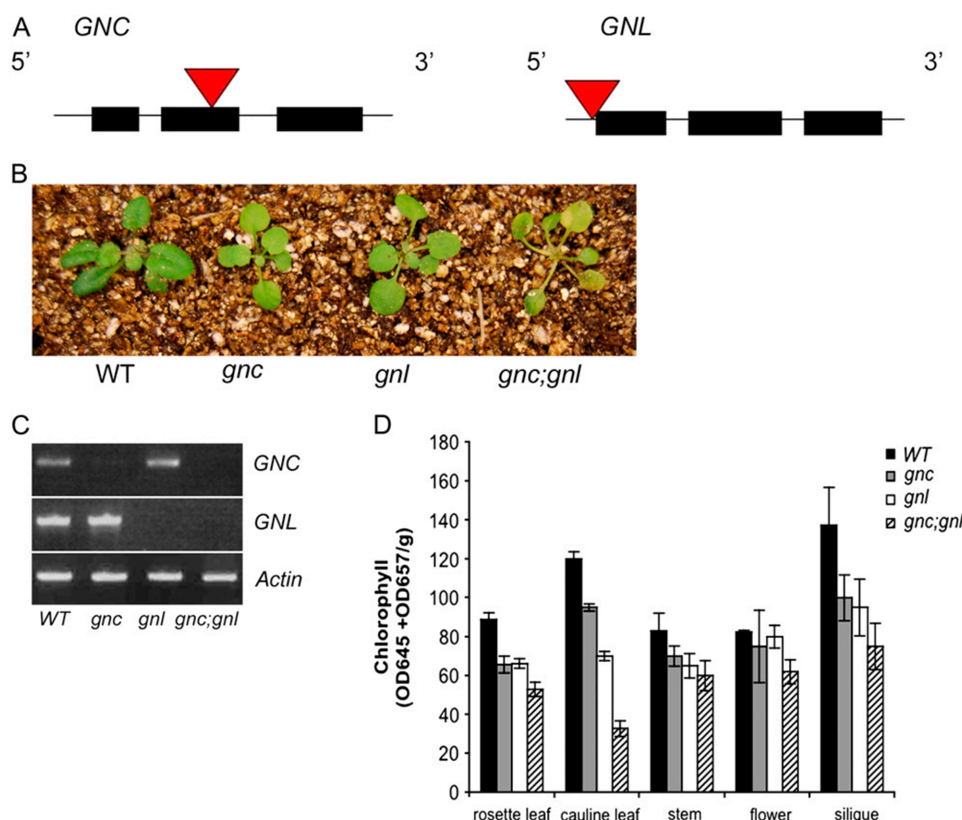

Figure 4. Mutational analysis of *GNC* and *GNL*. A, Gene structure of *GNC* and *GNL* indicating the position of T-DNA insertions. B, *gnc*, *gnl*, and *gnc;gnl* mutants have paler leaves than the wild type. C, *GNC* and *GNL* expression is abolished in single and double mutants. D, Chlorophyll levels are decreased in *gnc*, *gnl*, and *gnc;gnl* mutants versus the wild type.

tions indicated that *gnl* mutants also have decreased chlorophyll levels (Fig. 4D). Leaves of double *gnc;gnl* mutants are even paler green than the single mutants and have lower chlorophyll levels in all plant tissues assayed (Fig. 4, B and D), suggesting that *GNC* and *GNL* have similar and partially overlapping roles in promoting chlorophyll biosynthesis throughout the plant.

Regulation of Sugar-Sensing Genes

Based on transcriptional profiling, it has been proposed that GNC regulates genes involved in carbon metabolism and sugar sensitivity (Bi et al., 2005). Specifically, two hexose transporter genes, *AtPLT6* and *AtSTP13* were down-regulated in the leaves of *gnc* mutants compared to wild type (Bi et al., 2005). On the other hand, *HXK1*, a hexokinase, was up-regulated 1.5-fold in *gnc* mutants, while *HXK2* expression was unchanged (Bi et al., 2005). Although no chlorophyll biosynthesis genes have been identified as GNC targets, sugar production and sensitivity are linked to photosynthesis and plant growth. Photosynthesis is down-regulated in the presence of high sugar levels, whereas sugar is essential for the growth of the plant (Jang et al., 1997). Thus, down-regulation of genes involved in carbon metabolism and sugar sensitivity may contribute to the pale leaf phenotype seen in *gnc* mutants. To determine if GNL shares redundant regulatory roles with GNC, we tested if expression of *HXK1*, *HXK2*, *AtSTP13*, and *AtPLT6* was altered in the leaves and flowers of *gnl* mutants. We found that in the leaves and flowers, *HXK1* levels were significantly up-regulated in *gnc* and *gnl* single and double mutants, while *HXK2* levels were unchanged (Fig. 5, A–C; Supplemental Fig. S1, A and D). *AtPLT6* expression, on the other hand, decreased in the leaves and flowers of both the *gnc* and *gnl* single and double mutants (Fig. 5, A–C). Overall, GNC and GNL may have redundant roles in regulating *HXK1* and *AtPLT6* expression to regulate sugar sensitivity.

Surprisingly, the expression pattern of *AtSTP13* in *gnc* and *gnl* mutants varied in the leaves compared to the flowers. In the leaves, *AtSTP13* expression decreased significantly in the leaves of *gnc* and *gnl* single and double mutants (Fig. 5, A and B). However, *AtSTP13* expression increased in the flowers of *gnc* and *gnl* single and double mutants, suggesting that GNC and GNL differentially regulate *AtSTP13* in different parts of the plant (Fig. 5, A and C; Supplemental Fig. S1, B and E). Based on *AtSTP13* promoter::reporter gene fusions that show expression in multiple senescing tissues as well as specifically in the vasculature of young petals, *AtSTP13* has been suggested to have a role in regulating petal development (Norholm et al., 2006). In situ hybridizations indicate that *AtSTP13* is highly expressed in the anthers at later stages of flower development (Supplemental Fig. S1, A and D). Together, these results suggest that the differential expression of *AtSTP13* in petals and stamens may reflect flower-specific AP3/PI dependent regulation.

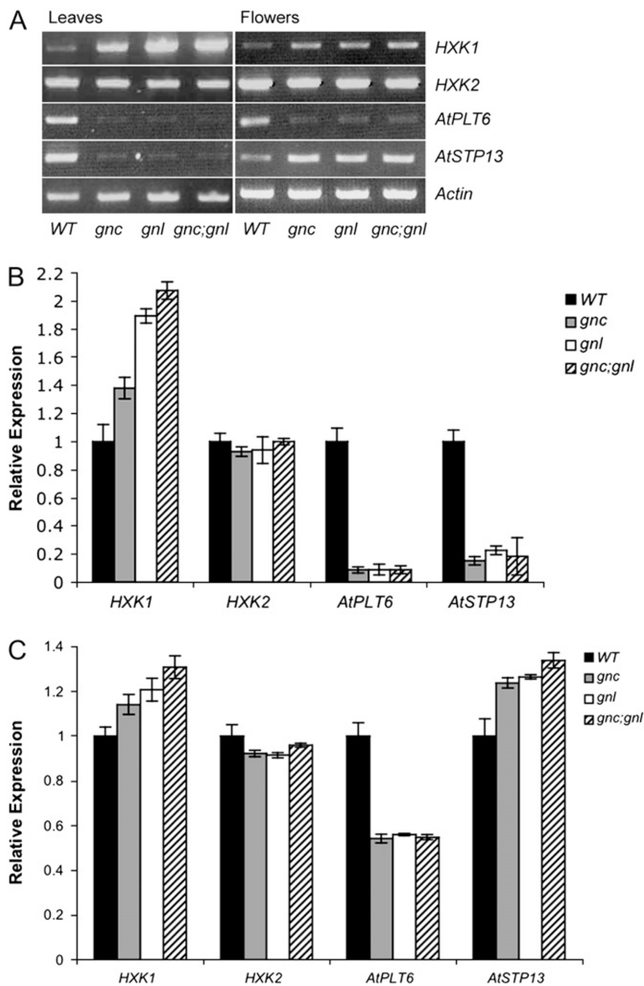

Figure 5. *GNC* and *GNL* regulate the expression of sugar-sensing genes. A, RT-PCR expression of genes involved in sugar sensitivity in the wild type and in mutant plants. B, Quantified expression levels of sugar-sensing genes in the leaves by RT-PCR. Average expression levels from three replicates were normalized to actin with the wild type scaled to 1. C, Quantified expression levels of sugar-sensing genes in the flowers by RT-PCR. Average expression levels from three replicates were normalized to actin with the wild type scaled to 1.

To further define the AP3/PI pathway, we tested if *HXK1*, *HXK2*, *AtSTP13*, and *AtPLT6* expression was altered in either *ap3-3* and *pi-1* or *35S::AP3* and *35S::PI* mutant flowers as compared to wild type. Our data indicate that in the flower, AP3/PI negatively regulate *GNC* and *GNL* and that GNC and GNL negatively regulate *HXK1* and *AtSTP13* in flowers and positively regulate *AtPLT6* expression. Thus, we would expect *HXK1* and *AtSTP13* expression to decrease while *AtPLT6* expression should increase in *ap3-3* and *pi-1* mutant flowers. *HXK2* expression should remain unchanged because it is not regulated by GNC or GNL. In agreement with our predictions, we found that *HXK1* and *AtSTP13* expression decreased significantly in *ap3-3* and *pi-1* mutants while *HXK2* expression remained unchanged (Fig. 6, A and C). However, we found *AtPLT6* expression also decreased significantly in the mutants (Fig. 6, A and C). In *35S::AP3* and *35S::PI* plants, *HXK1* and *AtSTP13* expression increased as predicted, while *HXK2* expression remained unchanged (Fig. 6, A and B). *AtPLT6* expression, however, also increased in *35S::AP3* and *35S::PI* plants (Fig. 6, A and B). Although the expression pattern of *AtPLT6* differs from our predictions, these results could indicate that there exists a more complicated regulatory pathway controlling the expression of this sugar-transporter gene, such as input from regulatory factors independent of AP3/PI. Thus, it appears that AP3/PI have roles in regulating genes involved in

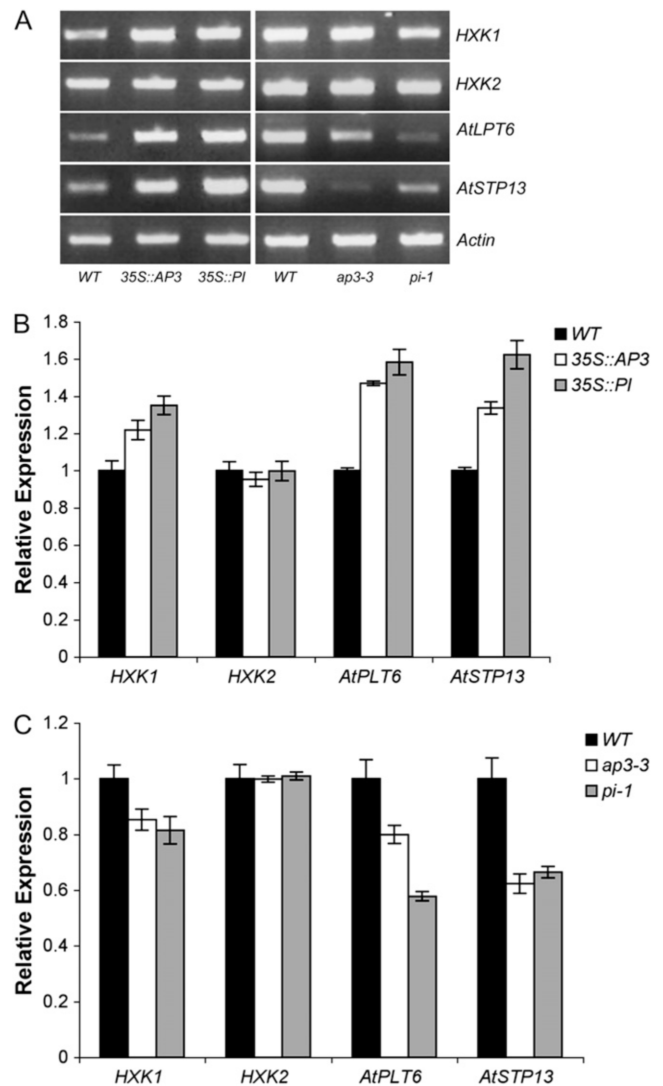

Figure 6. AP3 and PI regulate the expression of sugar-sensing genes. A, Expression levels of *HXK1*, *HXK2*, *AIPLT6*, and *AISTP13* by RT-PCR in *35S::AP3* and *35S::PI* and *ap3-3* and *pi-1* mutant flowers. B, Quantified expression levels in *35S::AP3* and *35S::PI* flowers by RT-PCR. Average expression levels from three replicates were normalized to actin with the wild type scaled to 1. C, Quantified expression levels in *ap3-3* and *pi-1* mutant flowers by RT-PCR. Average expression levels from three replicates were normalized to actin with the wild type scaled to 1.

sugar metabolism and sensing through the direct regulation of *GNC* and *GNL*, but that other unidentified regulatory factors are also likely to be involved.

GNC and GNL Regulate Genes Previously Identified as AP3/PI Targets

Because our data indicate that *GNC* and *GNL* are regulated by AP3/PI in flowers, genes downstream of GNC and GNL should also be part of the AP3/PI pathway. Using microarray analyses, Bi et al. (2005) identified 46 genes that were significantly repressed in *gnc* mutants and thus positively regulated by GNC. One of these genes, *At2g29350*, a putative tropinone reductase, was also identified in our microarray screen as a gene that was up-regulated after AP3 induction and thus positively regulated by AP3/PI (Supplemental Tables S3 and S4). Three genes identified as targets of GNC, *At4g30270* (endo-xyloglucan transferase), *At4g35770* (senescence-associated protein), and *At2g15890* (unknown protein; Bi et al., 2005), were also identified as genes that were positively regulated by AP3/PI (Zik and Irish, 2003). Based on expression analyses, *At4g30270* and *At2g15890* were suggested to act early in petal and stamen development (Zik and Irish, 2003). *At4g35770* was suggested to be expressed in both petals and stamens (Zik and Irish, 2003) and in situ hybridizations indicate that this gene is not only expressed in these tissues, but also in the sepals at high levels at later stages of flower development (Supplemental Fig. S1C). Additionally, *At1g57990*, encoding a purine transporter-like protein, was identified by Wellmer et al. (2004) as a petal-specific gene.

To determine the regulatory hierarchies controlling the expression of these five genes, we assayed their expression in various mutant combinations. First we tested if the expression of these five genes is altered in the leaves and flowers of *gnc* and *gnl* single and double mutants (Fig. 7, A–C). Using RT-PCR, we found that the expression of *At2g29350* in the leaves and flowers decreased significantly in both *gnc* and *gnl* mutants and was nearly undetectable in *gnc;gnl* double mutants. *At4g35770* expression decreased significantly in the leaves and flowers of *gnc* mutants and was nearly abolished in *gnl* single and *gnc;gnl* double mutants (Fig. 7, A–C; Supplemental Fig. S1, C and F). *At1g57990* expression decreased significantly in the leaves and flowers of *gnc* and *gnc;gnl* double mutants but to a lesser extent in the leaves and flowers of *gnl* single mutants. *At4g30270* expression was significantly decreased in the leaves and flowers of *gnc* and *gnl* single and double mutants. In the leaves, *At2g15890* expression decreased significantly, while in the flowers *At1g15890* expression increased in the *gnc*, *gnl*, and *gnc;gnl* double mutants. Thus, GNC and GNL share partially redundant roles in regulating *At2g29350*, *At4g35770*, *At4g30270*, *At1g57990*, and *At2g15890* expression in the leaves and flowers. Furthermore, GNC and GNL differentially regulate *At2g15890* in the leaves versus the flowers.

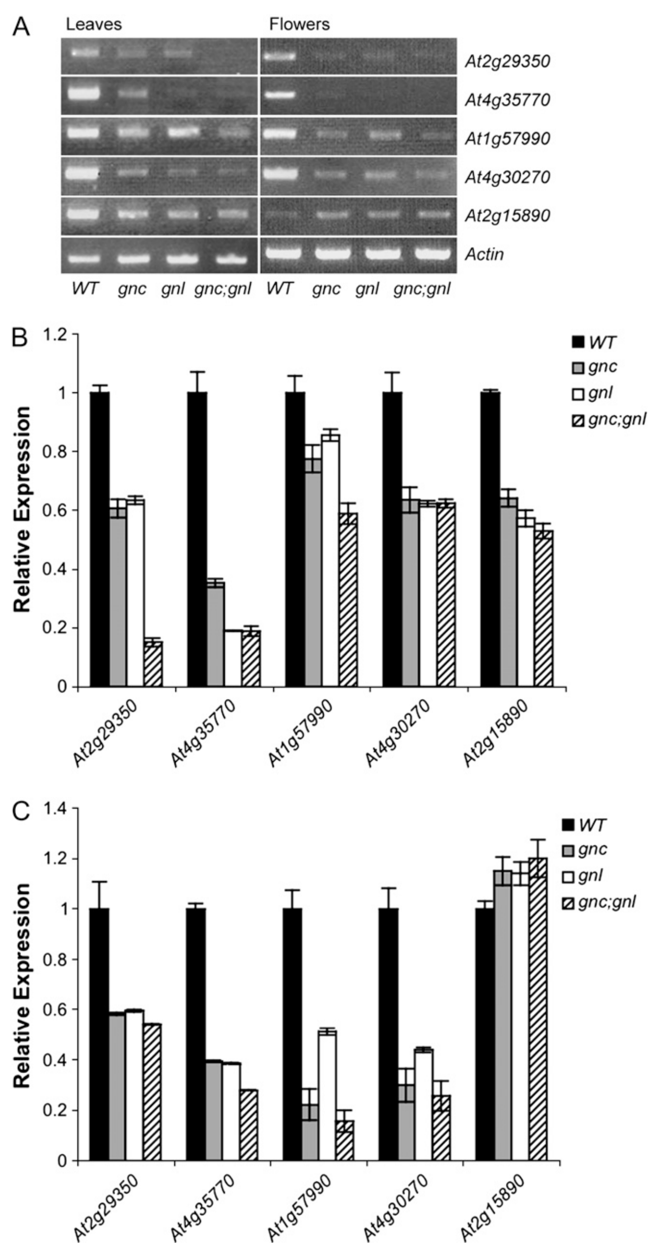

Figure 7. *GNC* and *GNL* regulate putative AP3/PI target genes. A, Expression of target genes by RT-PCR in *gnc* and *gnl* single and double mutants. B, Quantified expression levels of target genes in the leaves by RT-PCR. Average expression levels from three replicates were normalized to actin with the wild type scaled to 1. C, Quantified expression levels of target genes in the flowers by RT-PCR. Average expression levels from three replicates were normalized to actin with the wild type scaled to 1.

Next we wanted to confirm that *At2g29350*, *At4g35770*, *At1g57990*, *At4g30270*, and *At2g15890* expression is regulated by AP3/PI. Microarray data suggests that in the flower, *At2g29350*, *At4g35770*, *At4g30270*, and *At2g15890* expression is positively regulated by AP3/PI (Zik and Irish, 2003; data not shown). Thus, expression of these genes should decrease in *ap3-3* and *pi-1* mutants compared to wild type. We

found that expression of all four genes did decrease significantly in these mutants (Fig. 8, A and C). On the other hand, if these genes are positively regulated by AP3/PI we would expect their expression to increase in *35S::AP3* and *35S::PI* transgenic plants compared to wild type. We found that the expression of all five genes did increase in the transgenic flowers, further supporting the idea that these genes are positively regulated by AP3/PI (Fig. 8, A and B).

The RT-PCR data described above support the idea that these five genes are regulated by AP3/PI as well

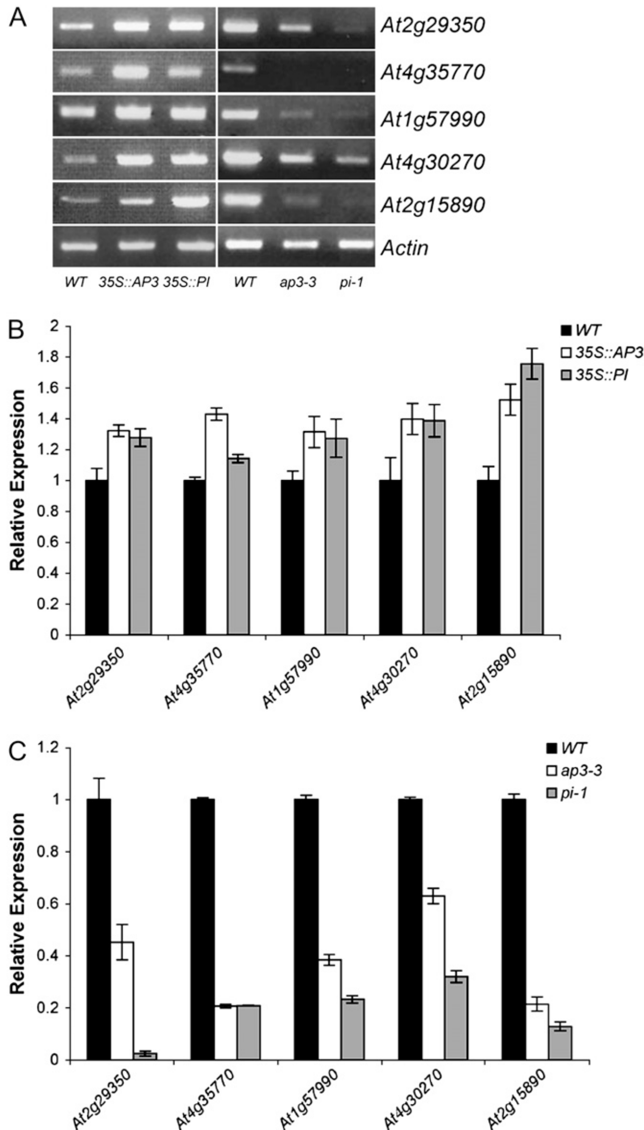

Figure 8. AP3 and PI regulate targets of GNC and GNL. A, Expression of target genes in *35S::AP3* and *35S::PI* and *ap3-3* and *pi-1* mutant flowers. B, Quantified expression levels of target genes in *35S::AP3* and *35S::PI* flowers by RT-PCR. Average expression levels from three replicates were normalized to actin with the wild type scaled to 1. C, Quantified expression levels of target genes in *ap3-3* and *pi-1* mutant flowers by RT-PCR. Average expression levels from three replicates were normalized to actin with the wild type scaled to 1.

as by GNC and GNL. However, we found that in the flower AP3/PI negatively regulate *GNC* and *GNL*, which in turn positively regulate *At2g29350*, *At4g35770*, *At1g57990*, and *At4g30270*, and negatively regulate *At2g15890* expression. In this simple scenario, AP3/PI should negatively regulate *At2g29350*, *At4g35770*, *At1g57990*, and *At4g30270*, and positively regulate *At2g15890* expression. As expected, we found that *At2g15890* expression was positively regulated by AP3/PI (Fig. 8). However, we also found that *At2g29350*, *At4g35770*, *At1g57990*, and *At4g30270* expression was positively regulated by AP3/PI. This inconsistency was also found for *AtPLT6* expression as described earlier. Thus, the AP3/PI regulatory pathway controlling *At2g29350*, *At4g35770*, *At1g57990*, and *At4g30270* expression must involve input from additional unidentified factors or be independent of GNC and GNL.

DISCUSSION

A Gene Regulatory Network Controlled by AP3/PI

Through a microarray-based screen for downstream targets of AP3/PI, we identified *GNC* as a gene that is negatively regulated by AP3/PI. We also found that AP3/PI negatively regulate the paralog of *GNC*, *GNL*, suggesting that AP3/PI may have important roles in down-regulating many genes to ensure the proper development of petals and stamens. We have also shown by ChIP that AP3/PI can bind to CArG-like boxes present in the *GNC* and *GNL* promoters, suggesting that AP3/PI act to directly regulate the transcription of these targets. AP3/PI could be acting as transcriptional repressors through recruiting specific corepressors to transcriptional complexes at these promoters, or through affecting histone modifications of the promoter regions. The MADS domain proteins AP1 and SEPALLATA3 have been shown to act as components of transcriptional repression complexes through interactions with specific corepressor proteins (Sridhar et al., 2006) while AGL15, another MADS domain protein, has recently been shown to act as a transcriptional repressor through recruiting histone deacetylase to target gene promoters (Hill et al., 2008). Based on these observations, it seems likely that AP3/PI are interacting with as yet unidentified cofactors to mediate their negative regulatory effects; moreover, the mechanism of repression at each target promoter may be distinct.

Microarray-based expression analyses have been informative in identifying targets of AP3/PI (Zik and Irish, 2003; Wellmer et al., 2004; this work) as well as targets of GNC (Bi et al., 2005). Genes recovered as targets of GNC are potentially targets of AP3/PI. In particular, any genes expressed in petals and/or stamens are likely to be positively regulated by AP3/PI, albeit likely in an indirect manner. Eight genes, in particular, were shown to be regulated by both GNC

and AP3/PI. Our microarray data confirmed positive regulation by AP3/PI for one of these eight genes, *At2g29350* (putative tropinone reductase), based on up-regulation after AP3 induction. We also found that *AtSTP13* expression was up-regulated after dex induction but expression also increased after mock treatment, suggesting that at 4 h *AtSTP13* expression levels are likely changing in response to the treatment and not in response to AP3 induction. The expression profiles of the six other genes *AtPLT6*, *HXK1*, and *At4g35770* (senescence-associated protein), *At1g57990* (purine transporter-like protein), *At4g30270* (endoxyloglucan transferase), and *At2g15890* (unknown protein) were not found to change significantly or consistently across our replicates after AP3 induction. Although our microarray screen does not confirm seven of these eight genes as targets of AP3/PI, this is likely due to the design of our experiment. In identifying genes that are up- or down-regulated 4 h after AP3 induction, we aimed to identify genes regulated directly by AP3/PI. At 4 h after AP3 induction, the expression levels of these seven genes may not yet be affected by AP3/PI function or expression changes may not yet have reached levels detectable by microarray analysis. However, by examining the expression profiles of genes recovered in our microarray screen as well as the screens mentioned previously, we were able to confirm that these eight genes are coordinately regulated by AP3/PI, GNC, and GNL.

Surprisingly, the characterization of the expression patterns of these genes in various mutant and transgenic backgrounds revealed that their regulation was more complex than would be expected. Three genes, *HXK1*, *AtSTP13*, and *At2g15890*, are negatively regulated by GNC and GNL, and positively regulated by AP3 and PI; these observations can be explained by a simple linear pathway of negative regulation (Fig. 9). However, it is clear that *AtSTP13* and *At2g15890* are differentially regulated in vegetative tissues because their expression is dependent on GNC and GNL activity in leaves. The other five genes, *AtPLT6*, *At1g57990*, *At2g29350*, *At4g30270*, and *At4g35770*, are positively regulated by GNC and GNL in flowers as well as leaves. However, their expression in flowers is also positively regulated by AP3/PI. This points to the possibility that additional factors play a role, either through modulating GNC and GNL activity, or independently thereof, in regulating the expression of these genes in floral tissues.

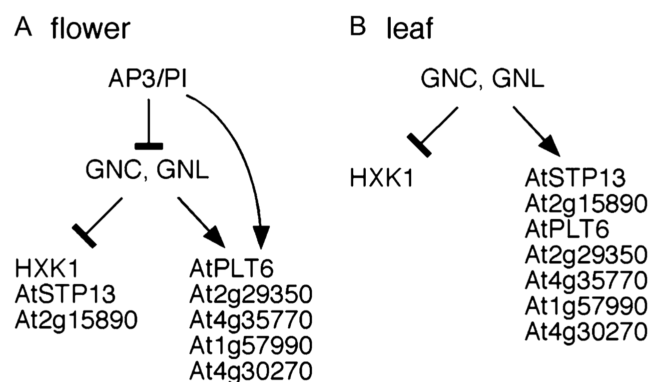

Figure 9. The regulatory cascade controlled by *AP3*, *PI*, *GNC*, and *GNL*. A, Summary of regulatory interactions occurring in the flower. Arrows indicate positive and bars indicate negative regulatory interactions. Genes for which functions have not yet been demonstrated are indicated by their locus names; these include At2g15890 (annotated as unknown protein), At2g29350 (putative tropinone reductase), At4g35770 (senescence associated protein), At1g57990 (purine transporter-like protein), and At4g30270 (endoxyloglucan transferase). These genes are likely indirect targets of AP3/PI. B, Summary of regulatory interactions in the leaf. Note that AtSTP13 and At2g15890 are regulated in a different manner as compared to the flower.

AP3/PI Regulate Pathways Required for Nitrogen Metabolism, Sugar Sensing, and Photosynthesis

We have shown that *GNC* and *GNL* expression levels are negatively regulated by AP3/PI in flowers, likely through direct transcriptional repression. GNC and GNL are the only two out of 30 GATA transcription factors that were found to be nitrate inducible (Wang et al., 2003; Price et al., 2004; Scheible et al., 2004; Bi et al., 2005). Nitrogen and carbon metabolism are closely linked and it has been shown that carbon metabolites regulate genes that are involved in nitrogen acquisition and metabolism (Coruzzi and Bush, 2001). Furthermore, our observation that GNC and GNL both negatively regulate *HXK1* and *AtSTP13* expression directly implicates these GATA transcription factors in modulating sugar sensing. HXK1 has been found to act as both a metabolic enzyme in the hexose assimilation pathway, as well as a component of a transcriptional complex involved in negatively regulating photosynthetic gene expression (Cho et al., 2006). This unexpected dual role for HXK1 thus directly links both sugar metabolism and sugar-dependent transcriptional responses. In turn, these observations together suggest that a cascade of negative regulatory interactions culminate in the HXK1-dependent negative regulation of photosynthetic gene expression in floral tissues. AtSTP13 has also been shown in vitro to have hexose transporter activity (Norholm et al., 2006; Buttner, 2007). Thus, the down-regulation of chlorophyll levels in *gnc* and *gnl* mutants likely reflects the lack of appropriate regulation of carbon and nitrogen metabolism and consequent photosynthetic responses.

The negative regulation of *GNC* and *GNL* by AP3/PI implies that chlorophyll levels are regulated in part by these floral homeotic MADS-box gene products. Our in situ data indicates that GNC and GNL are expressed strongly in the petals, stamen filaments, and the carpels, and to a lesser extent in the anthers of the stamens. *GNC* and *GNL* expression appears to be regulated by AP3/PI because *ap3* and *pi* mutants show higher levels of expression of *GNC* and *GNL*. This is consistent with a model whereby AP3/PI, presumably in conjunction with tissue-specific factors, regulates expression of these

GATA transcription factors in particular floral tissues, including the stamens.

AP3/PI Control Genes Involved in Cell Wall Remodeling

AP3/PI, GNC, and GNL all positively regulate the expression of *At4g30270*, which encodes a putative endoxyloglucan transferase, an enzyme that modifies xyloglucan, which is a major structural component of the plant cell wall (Campbell and Braam, 1999; Zik and Irish, 2003). *At4g30270* is a member of a tandemly duplicated region of three endoxyloglucan transferase genes in Arabidopsis (Rose et al., 2002) and has been variously named *AtXTH24* (Rose et al., 2002) or *meri5* (Medford et al., 1991) although no specific physiological function has yet been ascribed to this gene. *At4g30270* is expressed in multiple tissues, but notably at high levels in petals and sepals (Becnel et al., 2006) suggesting that this gene may have relatively specific roles in remodeling cell walls during specific aspects of floral organ growth and/or differentiation. The combined positive regulatory effects of AP3/PI, GNC, and GNL together may act to ensure the appropriate up-regulation of *At4g30270* during petal cell division or expansion.

Additionally, AP3/PI may have roles in regulating senescence because senescence is delayed in *ap3-3* and *pi-1* mutants compared to wild type (Zik and Irish, 2003). This may be mediated by the GNC and GNL dependent up-regulation of *AtSTP13*, which is expressed in cells undergoing programmed cell death (Buttner, 2007; Supplemental Fig. S1). We also recovered *At4g35770* that encodes a senescence-associated protein, which is positively expressed in response to AP3/PI and to GNC and GNL.

Together, these results indicate that AP3/PI can act to regulate transcriptional cascades that in turn are necessary for integrating energy requirements with developmental and environmental signals to promote appropriate floral organ type differentiation.

MATERIALS AND METHODS

Plant Material and Growth Condition

Arabidopsis (*Arabidopsis thaliana*) plants were grown on 12:3:1 mix of vermiculite:soil:sand at 22°C under long-day conditions (16-h-light/8-h-dark cycle). The mutant lines (*ap3*-3 and pi-*1*) and transgenic lines (*ap3*-3; *35S*::*PI*; *35S*::*AP3-GR* [*AP3-GR*]; *35S*::*AP3*; and *35S*::*PI-HA*) are in the Landsberg *erecta* background. The SALK T-DNA insertion lines (SALK 001778 and SALK 21362C) are in the Columbia background. The *AP3-GR* line was a gift from Robert W.M. Sablowski (John Innes Centre, Norwich, UK; Sablowski and Meyerowitz, 1998). The *35S*::*PI-HA* line was a gift from Naomi Nakayama (Yale University, New Haven, CT; Sundstrom et al., 2006).

Microarray Analysis

Floral buds from *35S*::*AP3-GR*, *35S*::*PI*, *ap3-3* plants were treated with dex (0.015% silwet, 0.1% ethanol, and 5 μM dex) or mock (0.015% silwet and 0.1% ethanol), collected at 0 and 4 h, and snap frozen in liquid nitrogen. Total RNA was extracted using Trizol (GibcoBRL) according to the manufacturer's instructions and purified using the Qiagen Rneasy kit (QIAGEN). Labeled complementary RNA was hybridized to the Affymetrix ATH1 GeneChip arrays (Affymetrix). Hybridization signals were detected using the Agilent GeneArray scanner and quantified by the Microarray suite software (MAS 5.0; Affymetrix). For comparisons, the overall intensity of each probe set on the array was scaled to a target intensity value of 500.

RT-PCR Analysis

Total RNA was extracted from plant tissue using Trizol (GibcoBRL) according to the manufacturer's instructions. Complementary DNA was synthesized using Superscript III Rnase reverse transcriptase (Invitrogen) according to the manufacturer's instructions. RT-PCR quantification was done using the ImageJ software (rsb.info.nih.gov/ij/) and RT-PCR products were normalized to an actin control. The gene-specific primers used to analyze expression are listed in Supplemental Table SVI.

ChIP

Nuclear extracts were prepared using MC, M1, M2, and M3 buffers as described in Ito et al. (1997). Following sonication, purified chromatin was immunoprecipitated using either a commercially available monoclonal anti-HA antibody or normal mouse serum (Santa Cruz Technology). Fractions corresponding to bound and unbound DNA samples were used as templates for ChIP PCR using primers flanking the CArG-like boxes identified in the promoter regions of each gene using the RSA tools software (rsat.ulb.ac.be/rsat/). Enrichment quantification and analysis was done using the ImageJ software (rsb.info.nih.gov/ij/). The primers used for ChIP PCR are listed in Supplemental Table S5.

In Situ Hybridization

In situ probes were generated by PCR amplification of complementary DNA using gene-specific primers containing T7 RNA polymerase-binding sites. T7 RNA polymerase (New England Biolabs) was used to transcribe digoxygenin-labeled UTP (Roche) probes. Tissue was fixed in 4% paraformaldehyde (Sigma) and embedded in Paraplast X-tra (Monoject Scientific). Sections (8 μm) were fixed to Probe-on-Plus slides at 42°C (Fisher Scientific). Procedures for in situ prehybridization, hybridization, and detection were performed as described previously (Carr and Irish, 1997). The primers used to make in situ probes are listed in Supplemental Table S4.

SALK Line Analysis

Homozygous SALK lines were identified by PCR genotyping for the presence of the T-DNA insertion. RNA was extracted from homozygous plants using the Trizol reagent (GibcoBRL) according to the manufacturer's instructions. RT-PCR analysis, as described above, was used to check for abolishment of the transcript.

The primers used to verify SALK 001778 (GNC) and SALK 21362C (GNL) lines are listed in Supplemental Table S5.

Chlorophyll Extraction and Measurement

Tissue was snap frozen in liquid nitrogen and then chlorophyll was extracted using 80% acetone as described in Lichtenthaler, 1987. Absorbance was measured at 645 and 657 nm and chlorophyll content was calculated using: $(20.2 \times A_{645} + 8.02 \times A_{657})$/g fresh weight.

Microarray data from this article have been deposited with the NCBI Gene Expression Omnibus data repository (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE9702.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Expression of selected downstream targets of *GNC* and *GNL*.

Supplemental Table S1. Genes with expression profiles that changed after dex treatment.

Supplemental Table S2. Genes with expression profiles that changed after mock treatment.

Supplemental Table S3. Genes with expression profiles that changed only after dex treatment.

Supplemental Table S4. One-hundred putative targets of AP3/PI.

Supplemental Table S5. Confirmed targets of AP3/PI.

Supplemental Table S6. Primer list.

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