Molecular basis for the specification of floral organs by APETALA3 and PISTILLATA

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How different organs are formed from small sets of undifferentiated precursor cells is a key question in developmental biology. To understand the molecular mechanisms underlying organ specification in plants, we studied the function of the homeotic selector genes APETALA3 (AP3) and PISTILLATA (PI), which control the formation of petals and stamens during Arabidopsis flower development. To this end, we characterized the activities of the transcription factors that AP3 and PI encode throughout flower development by using perturbation assays as well as transcript profiling and genomewide localization studies, in combination with a floral induction system that allows a stage-specific analysis of flower development by genomic technologies. We discovered considerable spatial and temporal differences in the requirement for AP3/PI activity during flower formation and show that they control different sets of genes at distinct phases of flower development. The genomewide identification of target genes revealed that AP3/PI act as bifunctional transcription factors: they activate genes involved in the control of numerous developmental processes required for organogenesis and repress key regulators of carpel formation. Our results imply considerable changes in the composition and topology of the gene network controlled by AP3/PI during the course of flower development. We discuss our results in light of a model for the mechanism underlying sex-determination in seed plants, in which AP3/PI orthologues might act as a switch between the activation of male and the repression of female development.

To gain detailed insights into the processes controlled by floral organ identity factors during morphogenesis, we analyzed the activities of the B function regulators APETALA3 (AP3) and PISTILLATA (PI) from Arabidopsis throughout flower development. AP3 and PI are closely related MADS domain proteins that are thought to act as obligate heterodimers (12–14). Only few direct target genes of AP3/PI have been described, which include the A function gene APETALA1 (API) (15), NAP (NAC-like, activated by AP3/PI) (16), two GATA transcription factors (17), and three related BANQUO genes (18), which also encode transcriptional regulators. To unravel the gene network underlying B function on a global scale, we used gene perturbation assays, transcript profiling, and genomewide localization studies, in combination with a floral induction system, which allows the analysis of regulatory processes during early flower development by genomic technologies. We supplemented these molecular data with a morphological analysis of the spatial and temporal requirement for AP3/PI activity during flower development. Taken together, our results provide a molecular framework for the control of organ specification by B function.

Results

Genes and Processes Controlled by AP3/PI. To unravel the transcriptional program underlying B function, we identified genes whose expression depends on AP3/PI activity during early flower development. To this end, we used a floral induction system, which allows the collection of a large number of flowers that are at approximately the same developmental stage (19). Morphological and molecular analyses have shown that flowers produced by this system can serve as a model for the early phase of flower development (19). To perturb B function activity, we introduced the null mutant alleles ap3-3 and pi-1, respectively, into the floral induction system. Activation of flower development in these genetic backgrounds led to the formation of flowers with petal-to-sepal and stamen-to-carpel transformations (Fig. 1 A–E), as expected for B function mutants. We collected mutant flowers at different stages of early flower development (stages according to ref. 20) and compared their gene expression profiles, by whole-genome microarray analysis, with those of corresponding flowers in which B function was not affected. As expected, the number of differentially expressed genes identified in these experiments increased

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Flowers are typically composed of four organ types, which are disposed in four floral whorls. From the outside of the flower to the center, they are sepals, petals, stamens, and carpels (the subunits of the gynoecium). The developmental fate of these different types of organs is specified by a small number of floral organ identity genes. The pivotal role of these genes was uncovered through the analysis of mutants that form flowers with homeotic transformations, i.e., the replacement of one type of organ with another (1–4). Based on the morphological defects of the individual mutants and their genetic interactions, it was proposed that the floral organ identity genes act in a combinatorial manner and have distinct functions during flower development, with the so-called A function genes being required for the formation of sepals and petals, B function genes for petal and stamen development, and C function genes for the formation of stamens and carpels. This well-established ABC model of floral organ identity specification (5) has provided, since its introduction more than 20 y ago, an invaluable framework for the analysis of the genetic mechanisms underlying the formation and evolution of flowers.

Molecular characterization of the floral organ identity genes in different species revealed that they encode transcription factors and belong, with few exceptions, to the family of MADS domain proteins (1–3). The floral organ identity factors were shown to form higher-order complexes together with flower-specific cofactors, which are also MADS domain transcription factors (6, 7). Further insights into the molecular functions of these regulators have come from the recent identification of some of their target genes and interacting proteins (7–11). Despite this progress, our understanding of the developmental mechanisms that mediate the specification and formation of floral organs is still vastly incomplete.
with progressing flower development due to accumulative effects of B function perturbation (SI Appendix, Fig. S1). A search for overrepresented Gene Ontology (GO) terms in the 2,100 differentially expressed genes (Dataset SI) revealed numerous enriched functional categories (Fig. 1F and SI Appendix, Fig. S2). These included several implicated in organogenesis and in particular, in petal and stamen development, in agreement with the known role of the B function genes during flower formation. Additional GO terms that were enriched among the differentially expressed genes included, for example, genes involved in pattern formation, cellular differentiation, and in the response to the phytohormone gibberelin, which has important functions during floral organ development (21). Genes involved in the regulation of transcription were also highly enriched in the dataset. In fact, a gene family enrichment analysis showed that transcription factor families with known roles in flower development were strongly overrepresented among the differentially expressed genes (Fig. 1G). We further found regulators of carpel development to be enriched in the dataset. Notably, a test for coordinated directionality of expression changes within the enriched GO terms revealed that these genes were predominantly up-regulated in very young (i.e., stage ∼3–4) flowers of ap3 and pi mutants (Fig. 1H and SI Appendix, Fig. S2). Because this response occurred shortly after the onset of AP3/PI expression at stage 3 (13, 14), it suggested that these carpel developmental genes are normally repressed by the B function regulators during early flower formation. In agreement with this conjecture is the observation that the gene CRABS CLAW (CRC), which responded strongly in the microarray experiments, is precociously expressed in young floral buds of B function mutants, with its expression expanding from the fourth into the third floral whorl (22) (SI Appendix, Fig. S3).

functions of AP3/PI in Early- and Late-Stage Flowers. Expression of floral organ identity genes commences during early floral stages and continues for most of floral organ development (3). This prolonged expression implies that these genes are not only involved in the specification of organ primordia but also in the control of organ maturation during later developmental stages (10, 23). To gain insights into the molecular basis of these different activities, we identified genes whose expression depends on the B function regulators at distinct stages of flower development. To this end, we conducted conditional gene perturbation experiments by introducing the temperature-sensitive AP3 mutant allele ap3-3 (4) into the floral induction system. We found that inactivation of AP3 through a shift from permissive to nonpermissive temperatures led to a strong transcriptional response in young floral buds (stage ∼3–4), whereas, in slightly older buds (stage ∼5–6), only minor effects on gene expression occurred (Fig. 1F and SI Appendix, Fig. S2). In whole inflorescences of ap3-3 mutant plants, representing flowers predominantly of intermediate and late developmental stages (24), we again detected widespread transcriptional changes after a perturbation of AP3 activity. A GO analysis of the resulting datasets revealed considerable differences between gene groups identified as enriched at early and late stages of development (Fig. 1F and SI Appendix, Fig. S2). Furthermore, genes responding early showed, as expected, a significant overlap (P < 0.0001, Pearson χ² test) with genes identified as differentially expressed in the null mutant experiments, whereas genes identified at late stages did not. The latter genes included many with known roles in microsporogenesis (i.e., pollen formation; Fig. 1F and SI Appendix, Fig. S2), a process that commences at intermediate stages of flower development and is completed shortly before anthesis. These results together provide molecular evidence for an involvement of AP3/PI in the control of different processes during petal and stamen organogenesis and indicate that they regulate largely unique sets of genes at distinct phases of flower development.

Spatial and Temporal Requirement for B Function. We next investigated whether the strong reduction in the number of differentially expressed genes detected in the ap3-1 microarray experiments around stage 6 was indicative of a temporarily reduced role of the B function regulators in flower development. To this end, we generated functional artificial microRNAs (amiRNAs) (25) against AP3 and PI (Fig. 2 and SI Appendix, Fig. S4 and Table S1). We then expressed these amiRNAs from an ethanol
and function at different developmental stages of microarray data, we measured mRNA levels in these stages. SPL activity led to changes in the expression of genes that are known or suspected to be transcriptionally regulated by AP3. By using the inducible amiRNA lines, we assessed the requirement of B function regulators after induction of AP3-amirNA expression in the floral induction system. Plants were treated with ethanol vapor 3 d after induction of flower formation, or were mock-treated, for different time periods (as indicated). Recovery of AP3 mRNA accumulation after a pulsed induction of AP3-amirNA expression in the floral induction system. Plants were exposed to ethanol vapor for 6 h (green bar) 2 d after the induction of flower development. Following an 18-h recovery period, tissue was collected at different time points (as indicated), and AP3 mRNA levels relative to those in mock-treated flowers were determined. Expression of selected genes (SI Appendix, Table S3) includes full gene names) in flowers of different stages (s) after an amiRNA-mediated perturbation of AP3 function at different developmental stages (Fig. 2) led to the identification of genes whose expression depends on AP3/PI activity. To distinguish between direct and indirect target genes, we identified the binding sites of AP3 and PI on a genomewide scale. To this end, we generated constructs that mediate the expression of AP3 and PI, respectively, fused to GFP under the regulatory regions of the endogenous genes (SI Appendix, Fig. S6). When expressed in ap3 and pi plants, these fusion proteins led to a full rescue of the mutant phenotypes. Subsequently, we crossed these lines into the floral induction system and collected floral buds at stage 5. With this material, we conducted ChIP assays with GFP-specific antibodies followed by next-generation sequencing (ChIP-Seq).

**Genomewide Localization of AP3/PI Binding Sites.** The microarray experiments described here led to the identification of genes whose expression depends on AP3/PI activity. To distinguish between direct and indirect target genes, we identified the binding sites of AP3 and PI on a genomewide scale. To this end, we generated constructs that mediate the expression of AP3 and PI, respectively, fused to GFP under the regulatory regions of the endogenous genes (SI Appendix, Fig. S6). When expressed in ap3 and pi plants, these fusion proteins led to a full rescue of the mutant phenotypes. Subsequently, we crossed these lines into the floral induction system and collected floral buds at stage 5. With this material, we conducted ChIP assays with GFP-specific antibodies followed by next-generation sequencing (ChIP-Seq).
Analysis of the ChIP-Seq data revealed more than 1,500 high-confidence binding sites for AP3 and PI in the Arabidopsis genome (Fig. 4 and Dataset S2). To validate the binding data, we tested and confirmed selected binding sites in independent ChIP experiments followed by quantitative real-time PCR analysis (Fig. 4B). We also studied the effect of transfer DNA insertions within binding sites located in the vicinity of the floral regulators UNUSUAL FLORAL ORGANS (UFO) (28) and RABBIT EARS (RBE) (29), which respond transcriptionally to the perturbation of B function (Fig. 2F). We found that these insertions led to phenotypes resembling those of the corresponding loss-of-function mutants (SI Appendix, Fig. S7), implying functionality of the binding sites.

Further support for the validity of the ChIP-Seq data came from the analysis of the locations of binding sites in the Arabidopsis genome. We observed that many of these sites were located in close proximity to transcription start sites (Fig. 5A), in agreement with what has been found for other transcription factors in Arabidopsis (11, 30, 31). We also identified, through de novo predictions, overrepresented DNA sequence motifs in the regions bound by AP3/PI. We found CArG box-like sequences (consensus, 5′-CC(A/T)6GG-3′), the canonical binding sites of MADS domain proteins (12), to be highly enriched (Fig. 5B). In this analysis, we also identified several other sequence motifs as over-represented (SI Appendix, Fig. S8). Although we currently do not know whether they are functionally relevant in the context of AP3/PI-dependent gene regulation, we noted that G-boxes (consensus, 5′-CACGTG-3′), which serve as binding sites for basic leucine-zipper and basic helix-loop-helix transcription factors (32), are significantly overrepresented (P < 0.05) at binding sites in promoters of genes that are repressed by the B function regulators compared to those of activated genes (Fig. 5C).

The ChIP-Seq experiments described earlier revealed that AP3/PI associate with many sites in the Arabidopsis genome. In agreement with the idea that AP3/PI act as obligate heterodimers, we found that the genomewide binding patterns for AP3 and PI were very highly correlated (Fig. 5D and SI Appendix, Fig. S8) and, in many cases, indistinguishable (Fig. 4I). We next compared the binding data for AP3/PI to those recently published for several floral regulators, which included the MADS domain proteins AP1 and SEPALLATA3 (SEP3) (11, 30), as well as the structurally unrelated transcription factors LEAFY (LFY) (31, 33) and AP2 (8). We detected a high degree of correlation in the case of AP1, SEP3, and LFY, but not for AP2 (Fig. 5D and SI Appendix, Fig. S8). These results are in agreement with the finding that AP1 and SEP3 can interact with the B function regulators in the transcriptional complexes that control petal (AP1 and SEP3) and stamen (SEP3) development, respectively (7), and the observation that LFY binds to many genes also targeted by AP1 and SEP3 (31). Despite the considerable degree of overlap between the different datasets, we also detected clear binding pattern disparities (e.g., Fig. 4I). Whether they are the result of different experimental setups or reflect bona fide differences in the binding specificities of the transcription factors remains to be elucidated.

**Target Genes of the Bifunctional Transcription Factors AP3/PI.** A GO term analysis of the genes bound by AP3 and PI (defined as genes with at least one binding site in the region from 3 kb upstream to 1 kb downstream of the transcribed sequence) revealed as enriched many of the same terms as the analysis of the ap3/pi microarray data described earlier (Fig. 4A). These include, for example, genes involved in stamen development, pattern formation (i.e., “regionalization”) and in the regulation of transcription. In total, 469 of the 2,100 genes that were detected as differentially expressed in the null mutant microarray experiments (~22%; Dataset S1) are bound by AP3/PI, implying that they are under direct control of the B function regulators. This list of high-confidence target genes contains many known floral regulators, including genes involved in processes such as organ boundary formation and in the response to different hormones (SI Appendix, Table S2). In agreement with the results from our microarray experiments, which suggested that regulators of carpel formation are under transcriptional control of AP3/PI during early flower development, we found binding sites in the putative promoters of many of the corresponding genes (Fig. 4A and SI Appendix, Fig. S3). An analysis of the directionality of gene expression changes of the high-confidence targets genes revealed a similar number of genes that were up- and down-regulated upon B function perturbation (Fig. 6B). Thus, AP3/PI
appear to act as bifunctional transcription factors in the control of flower development.

**Discussion**

The genomewide identification of AP3/PI binding sites and target genes revealed that these transcription factors control the expression of more than 460 genes, which can be assigned to different functional categories involved in a multitude of cellular and developmental processes commonly associated with organogenesis. This implies that AP3/PI directly participate in the regulation of many of the pathways and genes that are required for petal and stamen formation. Our results also show that AP3/PI control different processes during early and late flower development and indicate considerable differences in the spatial and temporal requirement of B function. Through conditional gene perturbation experiments we found that, in *Arabidopsis*, the fate of stamens is specified much earlier than that of petal primordia, which appear to remain uncommitted until intermediate stages of flower development. This finding is in agreement with what has been reported previously for flowers of *Antirrhinum majus* (snapdragon) (27), a species that is distantly related to *Arabidopsis*. Unexpectedly, morphological and molecular data from our experiments support the idea of a temporary reduction in the requirement for B function after stamen primordia have been specified. Thus, the control of stamen formation by B function appears to be discrete, whereas that of petal development is more continuous.

It has been suggested that AP3/PI regulate the expression of only a few transcription factor-coding genes and thus act relatively directly in the control of petal and stamen morphogenesis (34). However, we detected differential expression for a large number of such genes in young *ap3* and *pi* mutant flowers (~13% of differentially expressed genes; ~26% of high-confidence targets). In contrast, in more mature flowers, which are represented here through the analysis of gene expression in *ap3-l* inflorescences, the number of differentially expressed genes that code for transcription factors was much reduced compared with early stages and similar to their genomewide distribution of ~6% (35). These results imply that, during early developmental stages, AP3 and PI mediate floral organ formation to a considerable extent by controlling the expression of other transcriptional regulators, whereas, during late stages, AP3/PI may act more directly in the control of organogenesis or, alternatively, may function through only a few downstream factors. Together with our discovery that AP3/PI regulate different sets of genes at distinct phases of flower formation, these findings suggest that the composition and the topology of the gene network underlying AP3/PI function changes considerably over developmental time.

It has been proposed that a key event in the evolution of seed plants was the establishment of partially nonoverlapping expression domains of B and C function genes that originated from an ancient duplication event. Hence, sex determination could be mediated by a simple switch-like mechanism in which the presence

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**Fig. 5.** Genomewide binding data for AP3/PI. (A) Distribution of binding sites around transcription start sites. Plot showing the number and relative distribution of ChIP-Seq peaks in the proximity (from 3,000 bp upstream to 1,000 bp downstream) of transcribed regions of potential target genes. Binding site positions within transcribed regions (shaded box) were normalized relative to their length. Distribution of CArG (B) and G-boxes (C) within 400-bp windows around PI binding sites in the promoters of up-regulated (blue line) or down-regulated (red line) genes, or of all genes in the dataset (green line). (D) Comparison of binding data for selected floral regulators. Heat maps show binding data from different ChIP-Seq experiments for genomic regions surrounding 1,852 high-confidence PI binding sites (data for MADS domain proteins are shown in blue, and data for LFY and AP2 in red). Normalized Poisson enrichment scores are depicted in a color-code scale. All data were sorted (from top to bottom) according to descending PI peak height.

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**Fig. 6.** Correlation between GO terms enriched in the microarray and ChIP-Seq datasets. (A) Plot showing the correlation between GO terms identified in null mutant microarray (combined data from *ap3-3* and *pi-1, 5-d time point*) and ChIP-Seq (PI) experiments. Selected terms are labeled (Dataset S4 provides a full list). For these three highly significant terms, rounded coordinates (xy) are listed in the upper right corner. (B) Proportion of direct targets among genes identified as up- or down-regulated in the *ap3-3* and *pi-1* experiments and in the *ap3-1* experiment.
or absence of B function results in the formation of male or female structures, respectively (56). Our results show that, in Arabidopsis, the B function regulators AP3/PI are bifunctional transcription factors that activate or repress downstream targets, thus acting as a context-dependent transcriptional switch. In some cases, this appears to happen in concert with C function, for example, in the activation of the key regulator of microsporogenesis, SPL (Figs. 2I and 4I). On the contrary, we found that AP3/PI suppress genes, such as those involved in carpel development (e.g., CRC), which are otherwise activated by C function (9), implying opposing regulatory roles. Based on these results, we propose that the molecular mechanism for sex-determination in seed plants depends on the interplay of synergistic and antagonistic interactions between B and C function regulators.

Materials and Methods

Previously published Arabidopsis strains used in this study included the following: 35S:AP1-GR ap1-1 cal-1 (19), ap3-1 (4), ap3-3 (14), and pi-1 (4). Plants were grown on a soil:vermiculite:perlite (3:1:1) mixture at 20 °C under constant illumination with cool white fluorescent light unless indicated otherwise. Cloning strategies and methods used for PCR-based genotyping of mutant alleles and transgenes are described in SI Appendix. Plant transformations, quantitative RT-PCR assays, and in situ hybridizations were carried out as outlined in SI Appendix. Transcript profiling experiments were done by using custom microarrays (Agilent Technologies) and a previously described protocol (11). ChiP-seq analysis was performed, with minor modifications, as described previously (11). Analysis of microarray and ChiP-seq data were done using custom R scripts and functions provided by the Bioconductor project. Detailed experimental and data analysis procedures are provided in SI Appendix.

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