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TECHNICAL ADVANCE

Gene network analysis of *Arabidopsis thaliana* flower development through dynamic gene perturbations

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SUMMARY

Understanding how flowers develop from undifferentiated stem cells has occupied developmental biologists for decades. Key to unraveling this process is a detailed knowledge of the global regulatory hierarchies that control developmental transitions, cell differentiation and organ growth. These hierarchies may be deduced from gene perturbation experiments, which determine the effects on gene expression after specific disruption of a regulatory gene. Here, we tested experimental strategies for gene perturbation experiments during *Arabidopsis thaliana* flower development. We used artificial miRNAs (amiRNAs) to disrupt the functions of key floral regulators, and expressed them under the control of various inducible promoter systems that are widely used in the plant research community. To be able to perform genome-wide experiments with stage-specific resolution using the various inducible promoter systems for gene perturbation experiments, we also generated a series of floral induction systems that allow collection of hundreds of synchronized floral buds from a single plant. Based on our results, we propose strategies for performing dynamic gene perturbation experiments in flowers, and outline how they may be combined with versions of the floral induction system to dissect the gene regulatory network underlying flower development.

Keywords: flower development, *Arabidopsis thaliana*, gene regulatory network, gene perturbation, inducible promoter systems, artificial miRNAs, technical advance.

INTRODUCTION

Flower development is an excellent model system for studying plant organogenesis. Our understanding of the molecular processes that govern the formation of flowers has improved significantly in recent decades; however, large gaps remain (Wellmer *et al.*, 2014). In particular, our knowledge of the activities of many floral regulators and the topology of the gene regulatory networks (GRNs) that they control is incomplete. Genomic approaches have revealed that the GRNs underlying the formation of flowers are elaborate and complex, particularly at early stages of development (Gomez-Mena *et al.*, 2005; Wellmer *et al.*, 2006; Kaufmann *et al.*, 2010; Wuest *et al.*, 2012; O'Maoileidigh *et al.*, 2013; Pajoro *et al.*, 2014), during which many transcriptional regulators are involved. To unravel and understand these networks, researchers have relied heavily on gene perturbation experiments followed by reverse

transcription coupled to quantitative real-time PCR, microarray analysis, and, more recently, deep sequencing of mRNA populations (RNA-Seq). Generally, these perturbation experiments rely on comparisons between mutant and wild-type (or reference) plants regarding the relative abundance of mRNA species derived from whole inflorescences that contain a mixture of floral stages. Additionally, the mutant plants used in these studies were often derived from 'static' mutations [i.e. transfer DNA insertions, point mutations, constitutive expression of artificial microRNAs (amiRNAs) or RNA interference (RNAi) constructs]. There are several drawbacks when applying this strategy in Arabidopsis: (i) the differences in size between old and young flowers lead to a dilution of transcripts from early floral stages in mRNA preparations from whole inflorescences, (ii) the morphological differences between mutant and reference plants may be dramatic, resulting in detection of gene expression changes that are not due to the primary perturbation, and (iii) static perturbation experiments often overlook stage-specific functions of the gene of interest as the mutation is present from the onset of development. Together or individually, these shortcomings may distort results and lead to inappropriate conclusions about regulatory interactions. Furthermore, if genome-wide stage-specific experiments are performed, collection of specific floral stages is hampered by the fact that flowers are initiated sequentially in Arabidopsis, so that only one flower of a specific developmental stage is found in each plant at any one time.

To circumvent these issues and to allow stage-specific analysis of the GRN underlying the formation of flowers, dynamic perturbations of gene activities may be combined with methods to isolate floral tissues at specific stages of flower development. Dynamic gene perturbations may facilitate direct comparison of molecular profiles without introducing tissue-source bias. Moreover, these perturbations may be induced in order to investigate stage-specific functions of regulators of interest. Fluorescence-activated cell sorting (FACS) and laser capture microdissection (LCM) have been successfully used to isolate specific plant tissues for gene expression profiling (Birnbaum *et al.*, 2003; Wuest *et al.*, 2010; Liu *et al.*, 2011; Mantegazza *et al.*, 2014). However, as both FACS and LCM require expensive instrumentation and involve time-consuming procedures, their general applicability is limited (Wang *et al.*, 2012). Furthermore, use of these techniques for genome-wide localization studies through chromatin immunoprecipitation, as well as for proteomic or metabolomic approaches, is limited as large quantities of tissue are required for these types of analyses (Smaczniak *et al.*, 2012; Graciet *et al.*, 2014). To facilitate the isolation of homogenous stage-specific floral tissue, a flower induction system may be used that allows collection of hundreds of synchronized floral buds from a single plant (Wellmer *et al.*, 2006). This system is based on specific activation of a fusion protein between the key floral regulator APETALA1 (AP1) and the hormone-binding domain of the rat glucocorticoid receptor (GR) in an *ap1 cauliflower* (*cal*) double mutant background, which accumulates large numbers of inflorescence-like meristems (Bowman *et al.*, 1993). The floral induction system has been widely used for analysis of Arabidopsis flower development in combination with a broad range of experimental approaches (Ito *et al.*, 2007; Jiao *et al.*, 2008; Das *et al.*, 2009; Sun *et al.*, 2009, 2014; Jiao and Meyerowitz, 2010; Kaufmann *et al.*, 2010; Smaczniak *et al.*, 2012; Wu *et al.*, 2012; Wuest *et al.*, 2012; O'Maoileidigh *et al.*, 2013; Pajoro *et al.*, 2014).

In this study, we investigated various experimental strategies for dynamic gene perturbations to allow stage-specific analysis of the GRNs underlying flower development. To this end, we used transgenic lines that express amiRNAs that target key floral regulators under the control of various inducible promoter systems, and compared the widely used dexamethasone-dependent OPpro/GR-LhG4 system, the ethanol-dependent AlcApro/35S:AlcR system, and the 17β-estradiol-dependent LexApro/XVE promoter system, which have been previously used to facilitate enhancement, re-introduction or depletion of gene activities (Lloyd *et al.*, 1994; Zuo and Chua, 2000; Roslan *et al.*, 2001; Deveaux *et al.*, 2003; Craft *et al.*, 2005; Sun *et al.*, 2009). We also describe the generation of a series of improved floral induction systems, and assess their potential for analyzing flower development when combined with amiRNA-mediated gene knockdowns.

RESULTS

Dynamic perturbation of floral regulators via amiRNA-mediated knockdowns

To facilitate gene perturbations in plants, amiRNAs may be designed to target individual genes without apparent off-target effects (Schwab *et al.*, 2006). We previously identified amiRNAs that perturb the activity of the floral homeotic genes *AGAMOUS* (*AG*) and *APETALA3* (*AP3*) (Wuest *et al.*, 2012; O'Maoileidigh *et al.*, 2013), and placed them under the control of the AlcApro/35Spro:AlcR ethanol-inducible promoter system (Caddick *et al.*, 1998; Roslan *et al.*, 2001; Deveaux *et al.*, 2003) (constructs 35Spro>>AG-amiRNA^{Alc} and 35Spro>>AP3-amiRNA^{Alc}, respectively) (Figure 1 and Figure S1a). Analysis of the kinetics of the knockdown showed that mRNA levels of both genes reached a minimum approximately 24 h after onset of a 6 h treatment with ethanol vapor, and that they subsequently remained low for an additional 24 h before they recovered over a period of several days (Wuest *et al.*, 2012; O'Maoileidigh *et al.*, 2013). We further found that the reduction of *AG* and *AP3* mRNA levels resulted in floral

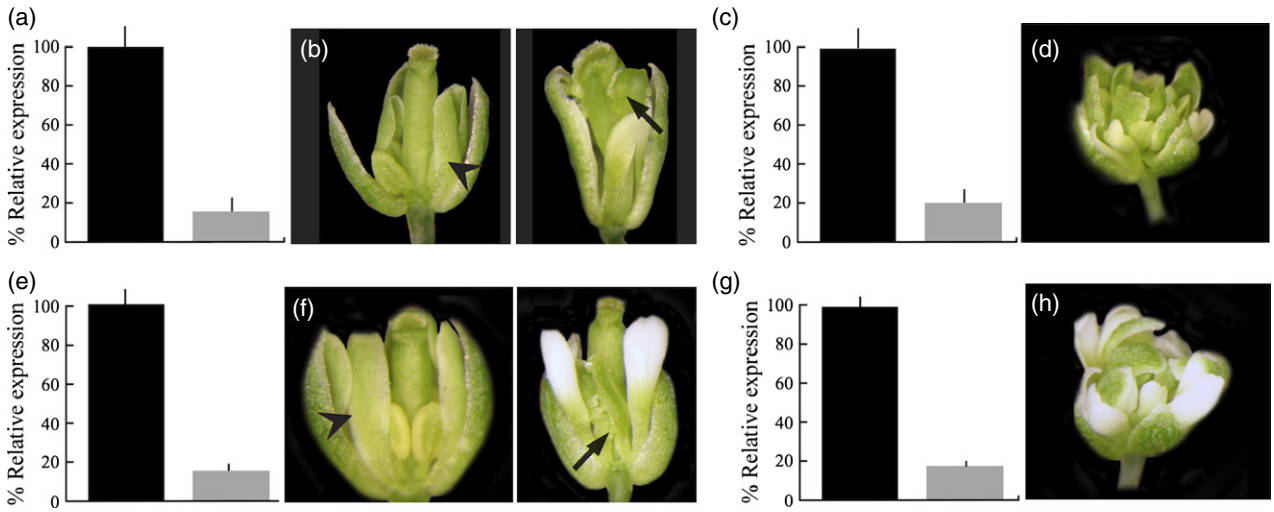

Figure 1. amiRNA-mediated gene perturbations using the AlcApro/AlcR and OPpro/GR-LhG4 promoter systems.
(a–d) Response of 35Spro>>AP3-amiRNA^{Alc} plants (a,b) and 35Spro>>AG-amiRNA^{Alc} plants (c,d) to ethanol treatment.
(a,c) Results of quantitative real-time PCR assays showing (a) *AP3* and (c) *AG* mRNA levels in stage 1–10 flowers of mock-treated plants (black bars) and ethanol-treated plants (gray bars), 24 h after the start of a 6 h ethanol vapor treatment.
(b) A single 24 h treatment with ethanol vapor resulted in conversion of petals to sepals (left panel, arrowhead) and of stamens to carpels (right panel, arrow) in 35Spro>>AP3-amiRNA^{Alc} lines. The images were taken 10 and 15 days, respectively, after the ethanol treatment.
(d) Five 6 h pulses of ethanol vapor (with 66 h recovery periods between treatments) resulted in homeotic conversion of stamens to petals and carpels to sepals in addition to a loss of floral meristem determinacy in 35Spro>>AG-amiRNA^{Alc} lines. The image was obtained 19 days after the first ethanol treatment.
(e–h) Response of 35Spro>>AP3-amiRNA$^{GR\text{-}LhG4}$ plants (e,f) and 35Spro>>AG-amiRNA$^{GR\text{-}LhG4}$ plants (g,h) to dexamethasone treatment.
(e,g) Results of quantitative real-time PCR assays showing (e) *AP3* and (g) *AG* mRNA levels in stage 1–10 flowers of mock-treated plants (black bars) and dexamethasone-treated plants (gray bars), 24 h after treating inflorescences with a solution containing 10 μM dexamethasone.
(f) A single dexamethasone treatment resulted in conversion of petals to sepals (left panel, arrowhead) and of stamens to carpels (right panel, arrow). The images were taken 9 and 16 days after the dexamethasone treatment.
(h) Three dexamethasone treatments resulted in conversion of stamens to petals and carpels to sepals in addition to a loss of floral meristem determinacy. The image was obtained 18 days after the first dexamethasone treatment.
Values in (a), (c), (e) and (g) are means and SEM of four biological replicates. A sepal was removed from the flowers shown in (b), (d) and (f).

phenotypes resembling those of strong *ag* and *ap3* mutant alleles, respectively (Figure 1b,d and Table 1).

To assess whether similar results may be achieved using the OPpro/GR-LhG4 dexamethasone-inducible promoter system (Craft *et al.*, 2005), we transformed wild-type plants with constructs containing either OPpro:AP3-amiRNA/35Spro:GR-LhG4 or OPpro:AG-amiRNA/35Spro:GR-LhG4 (O'Maoileidigh *et al.*, 2013) (termed 35Spro>>AG-amiRNA$^{GR\text{-}LhG4}$ and 35Spro>>AP3-amiRNA$^{GR\text{-}LhG4}$ hereafter) (Figure S1b). Treatment of the inflorescences of the resulting transgenic plants with a dexamethasone-containing solution led to a dramatic reduction of *AP3* and *AG* mRNA levels (Figure 1e,g), and formation of flowers that resembled those of the corresponding mutants (Figure 1f,h). We further found that the kinetics of the amiRNA-mediated knockdown in plants carrying OPpro/GR-LhG4 were similar to those for the AlcApro/35Spro:AlcR promoter system; however, the time until recovery of mRNA accumulation commenced was considerably extended with the OPpro/GR-LhG4 system (Figure S2).

A closer examination of the transgenic plants obtained showed that the proportion of independent OPpro/GR-LhG4-based lines that exhibited mutant phenotypes was higher than for lines in which amiRNA expression was under the control of the AlcApro/AlcR promoter system (Table 1), suggesting that the dexamethasone-responsive promoter system may be better suited for inducible gene perturbations during flower development.

We also tested the ability of the LexApro/XVE β-estradiol-inducible promoter system (Zuo and Chua, 2000) to drive amiRNA expression in flowers by placing the *AP3* amiRNA and *AG* amiRNA sequences downstream of the LexA promoter, and transforming wild-type plants with constructs containing LexApro:AP3-amiRNA/$G_{10\text{-}90}$pro:XVE and LexApro:AG-amiRNA/$G_{10\text{-}90}$pro:XVE, respectively (Figure S1c). We did not observe any *ap3* or *ag*-like phenotypes after the inflorescences of primary transformants or their progeny were treated with solutions containing up to 50 μM β-estradiol (Table 1).

Global effects of induced amiRNA-mediated gene perturbation on gene expression

The results of the experiments described above indicate that both the ethanol-dependent AlcApro/AlcR promoter system and the dexamethasone-dependent OPpro/GR-LhG4 promoter system may be used for amiRNA-mediated knockdown of floral regulatory gene activity. To test whether the amiRNA lines are also suitable for studying

Table 1 Artificial miRNAs directed against *AG* and *AP3* driven by various inducible promoter systems

Line/ induction system	Number of independent lines	Phenotypes			
		Strong	Intermediate	Weak	None
35Spro>>AG-amiRNA					
OPpro/ GR-LhG4	21	10	6	3	2
AlcApro/ AlcR	23	1	2	9	11
LexApro/ XVE	15	0	0	0	15
35Spro>>AP3-amiRNA					
OPpro/ GR-LhG4	16	7	2	5	2
AlcApro/ AlcR	22	6	2	2	12
LexApro/ XVE	6	0	0	0	6

Plants were treated with either ethanol vapour for 24 h, or once with a 10 μM dexamethasone-containing solution or once with a 10 μM 17β-estradiol-containing solution. Weak *ag* mutant phenotypes included male sterility due to defects in stamen elongation and pollen production, as well as carpel shape defects and partial loss of floral meristem termination. Intermediate *ag* mutant phenotypes included partial homeotic transformations of stamens and carpels and strong loss of floral meristem determinacy. Strong *ag* mutant phenotypes refer to full homeotic organ transformations as observed in *ag-1* null mutants. Weak *ap3* mutant phenotypes included male sterility, due to defects in stamen elongation and pollen production, and greenish petals. Intermediate *ap3* mutant phenotypes included the presence of carpelloid stamens and conversion of petals to sepals. Strong *ap3* mutant phenotypes refer to full homeotic organ transformations as observed in *ap3-3* null mutants. One potentially 'leaky' line showing mild carpel defects was identified for the 35Spro>>AG-amiRNA^{AlcR} construct. This line was excluded from further analysis.

the global effects of gene perturbations on gene expression, we collected stage 1–10 flowers of 35Spro>>AG-amiRNA$^{GR\text{-}LhG4}$ and 35Spro>>AP3-amiRNA$^{GR\text{-}LhG4}$ plants that had been treated for 24 h with either a dexamethasone-containing solution or a mock solution. In parallel, we collected stage 1–10 flowers of 35Spro>>AG-amiRNA^{AlcR} and 35Spro>>AP3-amiRNA^{AlcR} plants that had been mock-treated or treated with ethanol vapor for the same duration. Using whole-genome microarray analysis, we then compared the gene expression profiles of the inducer-treated samples with those of their mock-treated counterparts. The differentially expressed genes (DEGs) identified in these experiments included a significant number of target genes of the AP3 and AG transcription factors identified at early floral stages (Data S1) (Wuest *et al.*, 2012; O'Maoileidigh *et al.*, 2013), indicating that the degree of gene knockdown was sufficient to affect the expression of genes known to act downstream of the floral homeotic regulators. Furthermore, a gene ontology (GO) analysis led to identification of enriched GO terms related to known functions of the floral homeotic transcription factors (e.g. 'floral whorl development', 'carpel development' or 'transcription factor activity'). Unexpectedly, these terms were identified mainly in the experiments performed with the OPpro/GR-LhG4 promoter system (Figure 2b, Figure S3b and Data S2). We further found that GO terms associated with biotic and abiotic stress responses were enriched in the microarray datasets (Figure 2b, Figure S3b and Data S2), with the number of DEGs assigned to these terms being considerably larger in the datasets from the AlcApro/AlcR-containing lines (Data S2). The differential expression of these stress-related genes accounts at least in part for the much larger number of DEGs identified in the experiments performed using the AlcApro/AlcR promoter system compared with those performed using the OPpro/GR-LhG4 promoter system (Figure 2a and Figure S3a). Based on these results, we hypothesized that at least some of the DEGs identified in the microarray experiments may not have responded transcriptionally due to perturbation of floral homeotic gene function, and that, in the case of the experiments performed with the AlcApro/AlcR promoter system, misexpression of a large number of stress-related genes may have concealed the enrichment of GO terms associated with AG or AP3 functions.

To understand why genes related to biotic and abiotic stress response pathways were differentially expressed in the microarray experiment, we first tested the effects that the inducers ethanol and dexamethasone alone have on gene expression. To this end, we selected genes that are associated with stress responses (Data S2) and that exhibited expression changes after activation of amiRNA expression using the AlcApro/AlcR and OPpro/GR-LhG4 promoter systems. We then used quantitative real-time PCR to measure the expression of these selected DEGs in flowers and seedlings of wild-type plants after treatment with ethanol vapor or a dexamethasone-containing solution. In case of ethanol treatment, we found that expression of several of the selected DEGs was slightly altered compared to mock-treated plants (Figure 3), but these changes were overall much smaller than those observed in the microarray experiments. In contrast, dexamethasone treatment did not significantly affect expression of any of the genes tested (Figure S4). Next, we determined whether activation of the promoter systems and/or the induction of amiRNA expression influences the transcription of genes independently of the effect of a specific amiRNA on its intended target gene. To this end, we activated *AG* amiRNA expression from both the AlcApro/AlcR and OPpro/GR-LhG4 promoter systems in seedlings, in which *AG* is not expressed. In case of 35Spro>>AG-amiRNA^{AlcR} seedlings, we found that the selected DEGs were strongly differentially expressed after ethanol treatment (Figure 3b), while in the 35Spro>>AG-amiRNA$^{GR\text{-}LhG4}$ line, the transcriptional response of these genes after exposure to dexamethasone was more variable

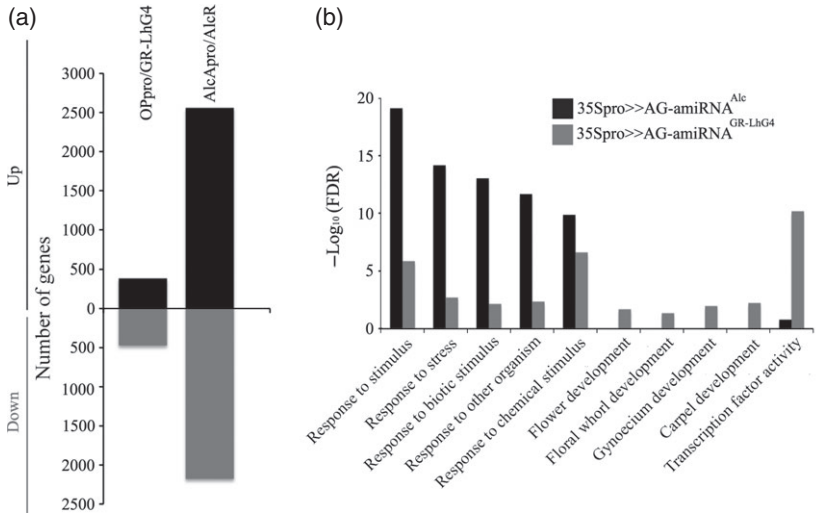

Figure 2. Global analysis of genes responding to induction of *AG* amiRNA expression from two promoter systems.
(a) Number of differentially expressed genes identified by microarray analysis as up-regulated (black bars) and down-regulated (gray bars) in 35Spro>>AG-amiRNA$^{GR\text{-}LhG4}$ and 35Spro>>AG-amiRNA^{Alc} plants (as indicated) after induction of amiRNA expression.
(b) Selected GO categories enriched among the differentially expressed genes identified in the microarray experiments with the 35Spro>>AG-amiRNA^{Alc} (black bars) and 35Spro>>AG-amiRNA$^{GR\text{-}LhG4}$ (gray bars) lines. Note the absence of enrichment for GO terms relating to known AG functions in the experiments using the AlcApro/AlcR promoter system. Negative decadal logarithms of FDR values are shown. Five stress-related GO terms are shown on the left, and five terms known to be associated with AG function are shown on the right.

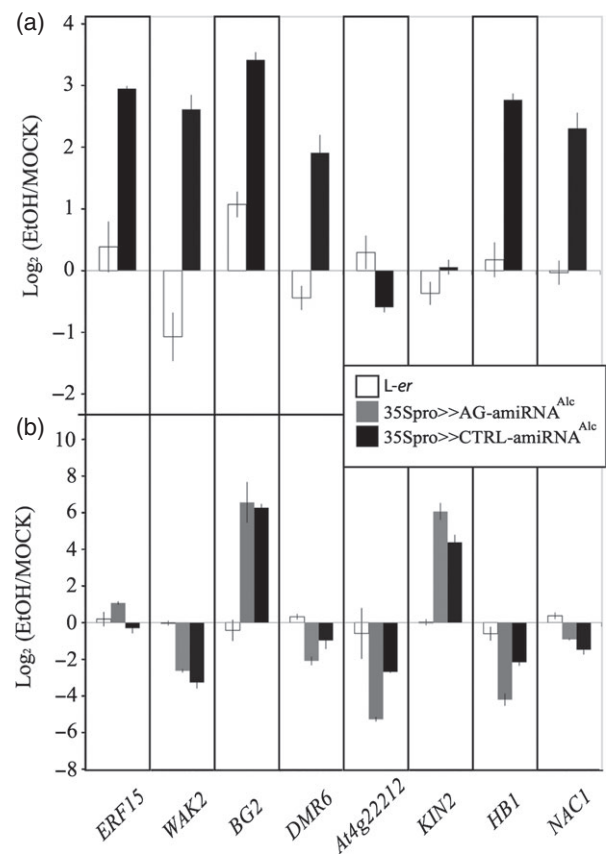

Figure 3. Transcriptional response of selected genes after induction of amiRNA expression using the AlcApro/AlcR promoter system.
(a) Expression of selected genes (as indicated) in flowers of non-transgenic wild-type plants (white bars) and AP1pro:AP1-GR$_{FIS}$ 35Spro>>CTRL-amiRNA^{Alc} plants (black bars) treated with ethanol (EtOH) vapor compared with mock-treated control plants of the same genotype.
(b) Expression of selected genes in seedlings of wild-type plants (white bars), 35Spro>>AG-amiRNA^{Alc} plants (gray bars), and AP1pro:AP1-GR$_{FIS}$ 35Spro>>CTRL-amiRNA^{Alc} plants (black bars) treated with ethanol (EtOH) vapor compared with mock-treated control plants of the same genotype.
Log_2-transformed expression ratios derived from quantitative real-time PCR assays are shown. Values are means ± SEM of three biological replicates.

and overall much reduced compared with 35Spro>>AG-amiRNA^{Alc} seedlings (Figure S4b). The results of these experiments imply that the differential expression detected for stress-related genes in the microarray experiments, especially with the AlcApro/AlcR promoter system, was not caused by an amiRNA-mediated perturbation of floral homeotic gene activity. To investigate this possibility further, we designed a control amiRNA (termed CTRL amiRNA) that contains all sequence features of a normal amiRNA but is predicted to not target any Arabidopsis transcripts. We then expressed this amiRNA under the control of the AlcApro/AlcR promoter system (termed 35Spro>>CTRL-amiRNA^{Alc}), and identified lines that express *AlcR* and, after ethanol treatment, amiRNA precursors at levels similar to those in 35Spro>>AG-amiRNA^{Alc} plants to allow direct comparison of the effects on gene expression (Figure S5). In agreement with the possibility that many stress-response genes exhibited differential expression in the microarray experiments independently of amiRNA-mediated target gene perturbation, we found that, in these 35Spro>>CTRL-amiRNA^{Alc} lines, the selected DEGs responded strongly to ethanol treatment in both flowers and seedlings (Figure 3a,b). Thus, for gene perturbation experiments using amiRNAs in combination with the AlcApro/AlcR promoter system in particular, rigorous experimental controls must be implemented to distinguish genes that respond due to knockdown of target gene activity from genes that exhibit non-specific effects (see Discussion).

Floral induction systems for stage-specific gene perturbation experiments

To be able to perform gene perturbation assays at specific stages of flower development, we sought to combine use of the inducible amiRNA lines described above with a floral induction system that allows the collection of hundreds of synchronized floral buds from a single plant (Wellmer

et al., 2006). This floral induction system (FIS) was initially based on expression of the AP1–GR fusion protein from the constitutive CaMV 35S promoter in an *ap1-1 cal-1* double mutant background (termed 35Spro:AP1-GR_{FIS} hereafter) (Wellmer *et al.*, 2006). In order to avoid effects on gene expression stemming from over-expression of AP1–GR in a domain much larger than that for the endogenous *AP1* gene (Mantegazza *et al.*, 2014), we generated transgenic lines that drive expression of AP1–GR from the *AP1* regulatory region in *ap1-1 cal-1* plants (termed AP1pro:AP1-GR_{FIS} hereafter) (Figure S6b) (O'Maoileidigh *et al.*, 2013). To confirm that introduction of the transgene resulted in a spatio-temporal expression of *AP1–GR* similar to that of endogenous *AP1*, we performed *in situ* hybridizations using a *GR* antisense probe (Figure 4h,i). In agreement with the known expression pattern of *AP1* (Mandel *et al.*, 1992), we found

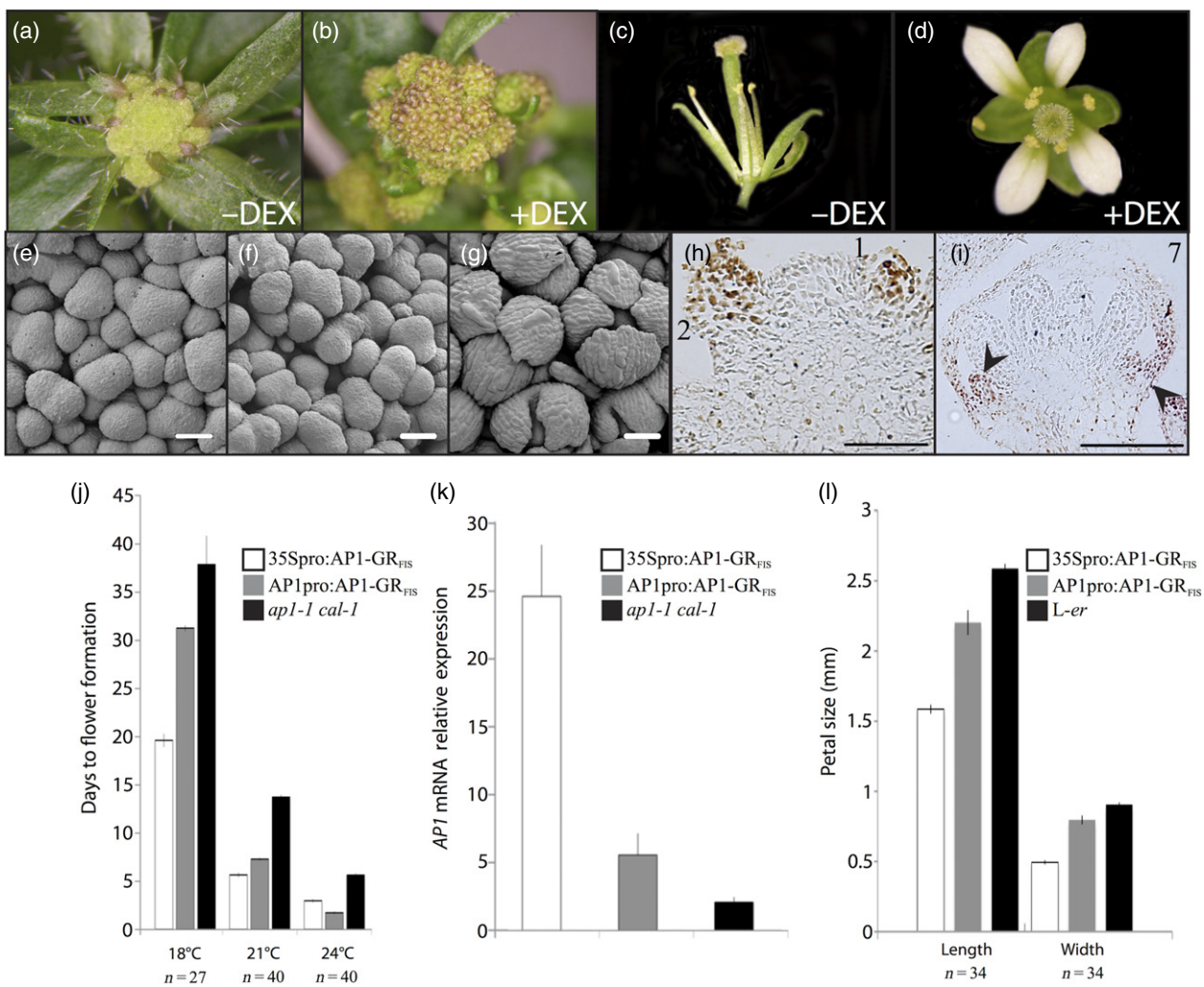

Figure 4. Characterization of the AP1pro:AP1-GR_{FIS} floral induction system.
(a,b) Inflorescence-like meristems of untreated AP1pro:AP1-GR_{FIS} plants (a) and AP1pro:AP1-GR_{FIS} plants (b) 5 days after treatment with a solution containing dexamethasone (DEX).
(c) Flower formed by an untreated AP1pro:AP1-GR_{FIS} plant.
(d) Flower of an AP1pro:AP1-GR_{FIS} plant approximately 14 days after treatment with a dexamethasone-containing solution.
(e–g) Scanning electron micrographs of inflorescence-like meristems of an *ap1-1 cal-1* double mutant (e), an untreated AP1pro:AP1-GR_{FIS} plant (f) and an AP1pro:AP1-GR_{FIS} plant (g) 5 days after treatment with a dexamethasone-containing solution. Scale bars = 100 μm.
(h,i) Expression of *AP1–GR* in flowers. Results of *in situ* hybridization with a GR antisense probe are shown. At stages 1-2 (h), *AP1–GR* expression is found throughout floral primordia, while in stage 7 flowers (i), its expression is restricted to the outer two whorls (arrowheads). Scale bars = 50 μm (h) and 100 μm (i).
(j) Timing of flower formation after bolting in the absence of dexamethasone treatment in 35Spro:AP1-GR_{FIS} plants (white bars), AP1pro:AP1-GR_{FIS} plants (gray bars) and *ap1-1 cal-1* plants (black bars) at three growth temperatures (as indicated).
(k) Results of quantitative real-time PCR assays to assess *AP1* mRNA levels in untreated inflorescence-like meristems of 35Spro:AP1-GR_{FIS} plants (white bar), AP1pro:AP1-GR_{FIS} plants (gray bar) and *ap1-1 cal-1* plants (black bar).
(l) Petal dimensions of flowers of 35Spro:AP1-GR_{FIS} plants (white bars) and AP1pro:AP1-GR_{FIS} plants (gray bars) treated once with a dexamethasone-containing solution, and of untreated wild-type (L*er*) plants (black bars).
Values in (j) and (l) are means and SEM of the indicated number of samples. Values in (k) are means and SEM of three biological replicates.

that *AP1–GR* mRNA accumulated throughout young floral meristems (Figure 4h) and in sepals and petals at later stages of flower development (Figure 4i).

We next tested the response of AP1pro:AP1-GR_{FIS} plants to treatments with a dexamethasone-containing solution. Similar to the 35Spro:AP1-GR_{FIS} line, a single treatment of AP1pro:AP1-GR_{FIS} inflorescence-like meristems led to synchronous formation of flower buds (Figure 4b,g) and restored sepal and petal development (Figure 4d), while mock-treated plants did not respond phenotypically (Figure 4a,c,e,f). Flowers of AP1pro:AP1-GR_{FIS} plants more closely resembled wild-type flowers than those formed by 35Spro:AP1-GR_{FIS} plants. This was especially apparent for the petals of AP1pro:AP1-GR_{FIS} flowers, which were significantly longer and wider than petals of 35Spro:AP1-GR_{FIS} flowers (Figure 4l and Figure S6c). A similar but more pronounced effect on petal development was observed when we crossed the strong *AG* allele *ag-1* (Bowman *et al.*, 1989), which produces flowers with reiterations of petals and sepals in place of stamens and carpels, into both the 35Spro:AP1-GR_{FIS} and AP1pro:AP1-GR_{FIS} backgrounds. While the petals of flowers in dexamethasone-treated 35Spro:AP1-GR_{FIS} *ag-1* plants were narrow and greenish (Figure S7a), the petals of similarly treated AP1pro:AP1-GR_{FIS} *ag-1* plants were broader and white, resembling wild-type petals (Figure S7b).

Non-treated *ap1-1 cal-1* double mutant plants produce abnormal flowers (lacking perianth organs) after a long delay compared with the wild-type (Bowman *et al.*, 1993). In comparison, untreated 35Spro:AP1-GR_{FIS} plants transition much earlier (Figure 4j), probably because small amounts of AP1–GR fusion protein are present in the nucleus even in the absence of dexamethasone (Kaufmann *et al.*, 2010). Thus, a relatively narrow window of time is available during which synchronous flowering may be achieved and experiments may be performed. When we assessed untreated AP1pro:AP1-GR_{FIS} plants, we found that the time to flowering was significantly prolonged compared with 35Spro:AP1-GR_{FIS} plants (Figure 4j), possibly as a consequence of the lower *AP1–GR* expression levels in the former line (Figure 4k). This effect is temperature-dependent, and is mainly observed in plants grown at 18°C (Figure 4j). At more elevated temperatures, this effect was diminished (21°C) and eventually disappeared (24°C). Therefore, as long as plants are grown at moderate temperatures (i.e. approximately 18°C), the time window during which AP1pro:AP1-GR_{FIS} may be used for experiments is extended compared to 35Spro:AP1-GR_{FIS} plants. Because *ap1-1 cal-1* double mutants accumulate inflorescence-like meristems over time (Bowman *et al.*, 1993), this delayed flowering response has the additional benefit that experiments may be performed with older plants from which more tissue for experimental analysis may be obtained.

The 35Spro:AP1-GR_{FIS} and AP1pro:AP1-GR_{FIS} lines constitutively express the *BAR* gene, which confers resistance to the herbicide glufosinate (Block *et al.*, 1987). To anticipate problems with linkage during genetic analyses, we isolated a second AP1pro:AP1-GR_{FIS} line (Figure S7g–i) with resistance to glufosinate, which contains the AP1pro:AP1-GR transgene on a different chromosome to the line discussed above (T-DNA positions and genotyping assays for both lines are described in Experimental procedures). Hence, depending on the location of the mutant allele or T-DNA insertion to be introduced into the floral induction system, one or the other line may be used for crosses to avoid genetic linkage. Furthermore, to improve the versatility of these floral induction systems, we generated alternative versions that are resistant to the antibiotic kanamycin (Figure S7d–f,j–l). If needed, these lines may be super-transformed, for example with plant transformation vectors conferring glufosinate resistance, thus facilitating the selection of secondary transformants.

To generate a dexamethasone-independent floral induction system that may be used in conjunction with the OPpro/GR-LhG4 promoter system for amiRNA delivery (or alternatively for conditional expression of a gene of interest), we translationally fused the last exon of *AP1* (as part of a genomic fragment containing the *AP1* locus) to the coding sequence of the mouse androgen receptor ligand-binding domain (AR) (Figure S6d), and introduced the transgene into *ap1-1 cal-1* plants (termed AP1pro:AP1-AR_{FIS} hereafter). Similar to the dexamethasone-dependent floral induction systems described above, treatment of AP1pro:AP1-AR_{FIS} inflorescence-like meristems with a solution containing 2 μM dihydrogentestosterone led to synchronous flower formation (Figure 5b) and restoration of sepal and petal formation (Figure 5d). In contrast, mock-treated AP1pro:AP1-AR_{FIS} plants (Figure 5a) (or plants treated with solutions containing 1 or 10 μM dexamethasone) did not flower synchronously, and the flowers produced (after a long delay) resembled those of *ap1-1 cal-1* double mutants (Figure 5g). The time to flowering of untreated AP1pro:AP1-AR_{FIS} plants was similar to that of *ap1-1 cal-1* mutants (Figure 5i), and did not change when growth temperatures were increased, suggesting that the aforementioned temperature sensitivity of the AP1pro:AP1-GR_{FIS} line may depend on the GR portion of the fusion protein. We next tested the level of *AP1–AR* mRNA in the inflorescence-like meristems of untreated AP1pro:AP1-AR_{FIS} plants, and found that it was similar to that of *AP1* mRNA in *ap1-1 cal-1* double mutants (Figure 5j).

To test whether the AlcApro/AlcR inducible promoter system may be used to generate a floral induction system, we placed the *AP1* coding sequence downstream of the AlcA promoter (Figure S6c) and transformed *ap1-1 cal-1* plants with a construct containing AlcApro:AP1/35Spro:AlcR (termed AlcApro>>$AP1_{FIS}$ hereafter). While mock-trea-

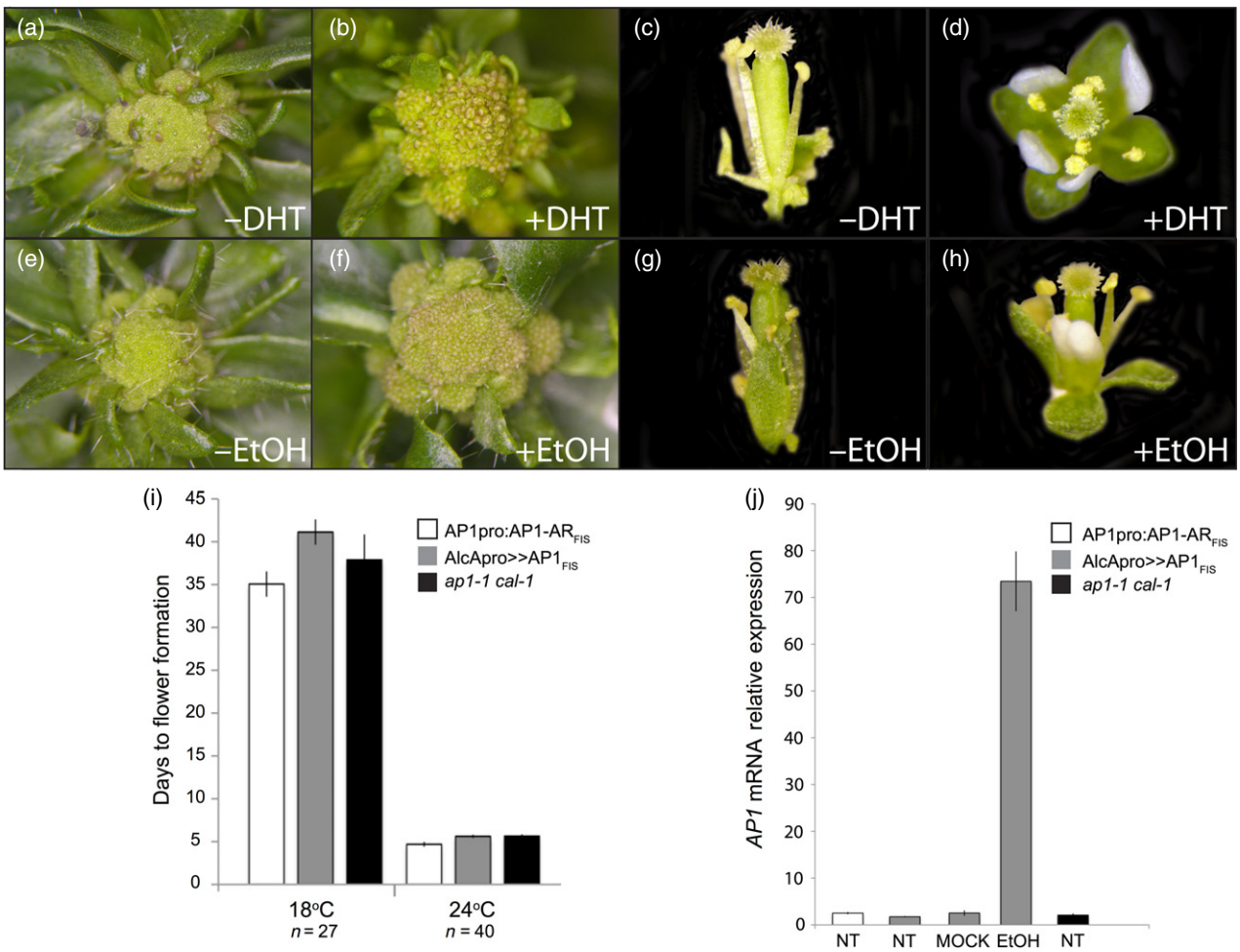

Figure 5. The AP1pro:AP1-AR$_{FIS}$ and AlcApro>>AP1$_{FIS}$ floral induction systems.
(a–d) Response of the AP1pro:AP1-AR$_{FIS}$ floral induction system to treatment with dihydrogentestosterone (DHT).
(a,b) Inflorescence-like meristems of AP1pro:AP1-AR$_{FIS}$ plants 5 days after treatment with a mock solution (a) or a DHT-containing solution (b).
(c,d) Mature flowers of untreated (c) and DHT-treated (d) AP1pro:AP1-AR$_{FIS}$ plants.
(e–h) Response of the AlcApro>>AP1$_{FIS}$ floral induction system to ethanol (EtOH) vapor treatment.
(e,f) Inflorescence-like meristems of AlcApro>>AP1$_{FIS}$ plants 10 days after mock treatment (e) or daily 3 h treatments with ethanol vapor (f).
(g,h) Mature flowers of untreated (g) and ethanol-treated (h) AlcApro>>AP1$_{FIS}$ plants.
(i) Time to flower formation of AP1pro:AP1-AR$_{FIS}$ plants (white bars), AlcApro>>AP1$_{FIS}$ plants (gray bars) and *ap1-1 cal-1* plants (black bars) at two temperatures (as indicated).
(j) Results of quantitative real-time PCR assays to assess *AP1* mRNA levels in inflorescence-like meristems of AP1pro:AP1-AR$_{FIS}$ plants (white bar), AlcApro>>AP1$_{FIS}$ plants (gray bars) and *ap1-1 cal-1* plants (black bar). AP1pro:AP1-AR$_{FIS}$ and *ap1-1 cal-1* inflorescences were untreated, while AlcApro>>AP1$_{FIS}$ plants were either untreated (NT), mock-treated (MOCK) or treated with ethanol vapor (EtOH) for 3 h daily for 5 days prior to harvesting the tissue.
Values in (i) are means and SEM of the indicated number of samples. Values in (j) are means and SEM of three biological replicates.

ted AlcApro>>AP1$_{FIS}$ plants did not respond phenotypically (Figure 5e), and eventually produced flowers resembling those of the *ap1-1 cal-1* mutant (Figure 5g), treatments with ethanol vapor led to synchronous flower development (Figure 5f), but only in up to approximately 15% of plants tested. Furthermore, the production of mature flowers was delayed by several days compared with the other floral induction systems described above, and they normally contained only one or two petals and partially restored sepals (Figure 5h). The incomplete response of the AlcApro>>AP1$_{FIS}$ line does not appear to be a consequence of a precocious AP1-independent transition to flower development, as we found that the time to flowering was similar in untreated AlcApro>>AP1$_{FIS}$ plants and *ap1-1 cal-1* double mutants (Figure 5i). Likewise, the limited response within the treatment population cannot be explained by insufficient *AP1* expression because we found that *AP1* mRNA accumulated to high levels after exposure to ethanol vapor (Figure 5j).

Finally, we generated transgenic lines for expression of *AP1* under the control of the LexApro/XVE β-estradiol-inducible promoter system (Zuo and Chua, 2000) in

inflorescences of the *ap1-1 cal-1* double mutant (Figure S6e). Although we were able to confirm induction of *AP1* expression in these lines when seedlings were treated in a liquid culture containing β-estradiol (Figure S8), neither primary transformants nor their offspring responded phenotypically when inflorescences were treated with solutions containing up to 10 μM β-estradiol.

Inducible gene perturbation in a floral induction system

We had previously combined the ethanol-inducible amiRNA lines for *AG* and *AP3* with the dexamethasone-dependent floral induction systems AP1pro:AP1-GR_{FIS} and 35Spro:AP1-GR_{FIS} to perturb gene activities at distinct developmental stages, and identified stage-specific functions of the floral organ identity genes (Wuest *et al.*, 2012; O'Maoileidigh *et al.*, 2013). As the results described above indicated that the OPpro/GR-LhG4 promoter system is more efficient for amiRNA-mediated gene knockdown and causes significantly fewer non-specific gene expression changes than the AlcApro/AlcR promoter system, we tested whether the 35Spro>>AG-amiRNA$^{GR\text{-}LhG4}$ and 35Spro>>AP3-amiRNA$^{GR\text{-}LhG4}$ lines are compatible with the dexamethasone-independent AP1pro:AP1-AR_{FIS} floral induction system. Because it has been reported that the dihydrogentestosterone used to activate the AP1–AR fusion protein may also stimulate activity of the glucocorticoid receptor (Viru and Korge, 1979), whose hormone-binding domain is part of the chimeric GR-LhG4 transcription factor, we first tested whether the OPpro/GR-LhG4 promoter system is activated by dihydrogentestosterone treatment. To this end, we isolated AP1pro:AP1-AR_{FIS} 35S>>AG-amiRNA$^{GR\text{-}LhG4}$ plants and monitored expression of the amiRNA precursor after treatment with various concentrations of dihydrogentestosterone that are sufficient to induce synchronous flowering in the AP1pro:AP1-AR_{FIS} floral induction system. We found that dihydrogentestosterone treatment of this line led to only a weak induction of amiRNA expression (up to approximately sixfold at the highest dihydrogentestosterone concentration tested) when compared to dexamethasone treatment (approximately 475-fold) (Figure 6a). We also treated 35Spro>>AG-amiRNA$^{GR\text{-}LhG4}$ and 35S>>AP3-amiRNA$^{GR\text{-}LhG4}$ plants with up to 10 μM dihydrogentestosterone, and did not observe any *ag*- or *ap3*-like mutant phenotypes. Thus, the dihydrogentestosterone treatments did not activate the OPpro/GR-LhG4 promoter system to an extent necessary to induce amiRNA-mediated gene perturbation.

Next, we tested whether *AG* and *AP3* mRNA levels were significantly reduced in AP1pro:AP1-AR_{FIS} 35S>>AG-amiRNA$^{GR\text{-}LhG4}$ and AP1pro:AP1-AR_{FIS} 35S>>AP3-amiRNA$^{GR\text{-}LhG4}$ plants. To this end, we first induced synchronous flowering with dihydrogentestosterone, and, after several days, treated the inflorescence-like meristems of these plants with either a mock solution or a dexamethasone-containing solution to induce amiRNA expression. We then harvested the tissue after 24 h, and used quantitative real-time PCR to detect a severe reduction in the levels of the amiRNA target genes (Figure 6b,c), suggesting that dexamethasone-inducible amiRNA lines are fully functional in the background of the AP1pro:AP1-AR_{FIS} floral induction system, and may be used for perturbation of gene activities during flower development.

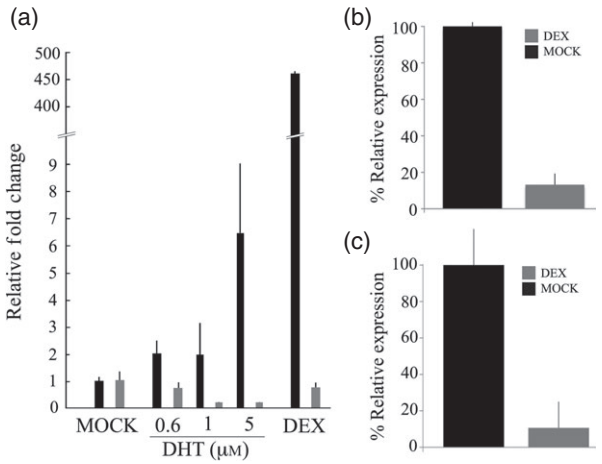

Figure 6. Dexamethasone-dependent induction of amiRNA expression in dexamethasone-independent floral induction systems.
(a) Results of quantitative real-time PCR assays monitoring amiRNA precursor levels (black bars) in inflorescence-like meristems of AP1pro:AP1-AR_{FIS} 35Spro>>AG-amiRNA$^{GR\text{-}LhG4}$ plants after mock treatment, treatment with solutions containing various concentrations of dihydrogentestosterone (DHT) (as indicated), or treatment with a solution containing 10 μM dexamethasone (DEX). Tissue was collected 24 h after the treatments. Gray bars represent amiRNA precursor levels in control samples in which reverse transcriptase was omitted from the cDNA synthesis reaction.
(b) Results of quantitative real-time PCR assays monitoring *AG* mRNA levels in flowers of AP1pro:AP1-AR_{FIS} 35Spro>>AG-amiRNA$^{GR\text{-}LhG4}$ plants that were treated once with a solution containing 2 μM DHT and then either mock-treated (black bar) or dexamethasone-treated (gray bar) after 3 days. Samples were collected 4 days after the DHT treatment.
(c) Results of quantitative real-time PCR assays monitoring *AP3* mRNA levels in flowers of AlcApro>>$AP1_{FIS}$ 35Spro>>AP3-amiRNA$^{GR\text{-}LhG4}$ plants that were treated daily, for a period of 10 days, with ethanol vapor for 3 h to direct flower formation. Inflorescences were then either mock-treated (black bar) or dexamethasone-treated (gray bar) 9 days after the onset of the ethanol treatments, and tissue was collected 24 h later. Values are means and SEM of at least three biological replicates.

DISCUSSION

In this study, we assessed experimental strategies for analysis of the GRN underlying flower development through gene perturbations. Gene perturbation assays are a standard approach for GRN analysis (Materna and Oliveri, 2008), but the methods used to alter gene functions vary widely depending on the organism under study. In plants, a number of approaches are commonly used to modify gene activities, and include, among others, constitutive or induced over-expression of genes, as well as gene knockdowns via RNA interference and amiRNAs. Because

amiRNAs may be designed so that they do not produce any off-target effects (Schwab *et al.*, 2006), they appear particularly well suited for GRN analyses in plants, especially for those strategies that involve use of genomic technologies to measure expression changes on a global scale after disruption of gene function. While the design of amiRNAs against specific target genes has been largely automated through development of sophisticated software programs, individual amiRNAs must be tested for their effects on target genes before they may be used in gene perturbation experiments. To this end, high-throughput approaches are now available (e.g. Hauser *et al.*, 2013), facilitating identification of functional amiRNAs and the systematic analysis of GRN components.

For analysis of GRNs involved in development, it is of particular importance to obtain data from distinct stages to understand how the structure and architecture of a GRN changes throughout ontogeny. To be able to specifically disrupt gene activities during flower development, we tested several inducible promoter systems that are widely used in plant research in combination with functional amiRNAs against the floral organ identity genes *AP3* and *AG* (Wuest *et al.*, 2012; O'Maoileidigh *et al.*, 2013). We found that the dexamethasone-dependent OPpro/GR-LhG4 promoter system and the ethanol-dependent AlcApro/AlcR promoter system work well for amiRNA-mediated knockdown of target genes. In contrast, we failed to isolate lines expressing the amiRNAs under the control of the LexApro/XVE β-estradiol-inducible promoter system that showed floral homeotic phenotypes. Taken together with the lack of floral induction in *ap1 cal* double mutant plants expressing *AP1* under the control of LexApro/XVE, these results suggest that the OPpro/GR-LhG4 and AlcApro/AlcR promoter systems may be better suited for the inducible expression of genes during flower development, and, in particular, for amiRNA-dependent perturbation of floral regulators. However, it is important to note that the LexApro/XVE system has been successfully used in several studies, including some on meristem development and flowering time control (e.g. Borghi *et al.*, 2010; Shen *et al.*, 2011).

When we tested the effects on global gene expression resulting from amiRNA-mediated knockdown of *AP3* and *AG* using the OPpro/GR-LhG4 and AlcApro/AlcR promoter systems, we noted considerable differences between the differentially expressed genes identified in these experiments. While some of the disparities between the datasets may be explained by slight differences in knockdown efficiencies and kinetics in the lines used, the response of stress-related genes, especially in the amiRNA lines using the AlcApro/AlcR promoter system, suggested that some of the transcriptional responses detected after induction of amiRNA expression may be independent of the effects on the amiRNA target genes. The results of additional experiments, in particular those with a control amiRNA (CTRL-amiRNA) that was designed to not target any transcripts in Arabidopsis, confirmed this possibility, and implied that these effects are caused by a combination of treatment of plants with the inducing substance, ethanol, and the high level of promoter system activity necessary for efficient knockdown of the intended target genes. However, we currently do not know whether activation of the AlcR transcription factor influences the expression of these genes directly or indirectly. As the AlcApro/AlcR promoter system has been used widely in plant research (Yu *et al.*, 2010; Hachez *et al.*, 2011; Wuest *et al.*, 2012; O'Maoileidigh *et al.*, 2013; Rosa *et al.*, 2013; Fujikura *et al.*, 2014; Simonini and Kater, 2014), including genome-wide approaches (Leibfried *et al.*, 2005; Anastasiou *et al.*, 2007; Busch *et al.*, 2010; Skylar *et al.*, 2010), our data highlight the need for extensive experimental controls to allow the best possible interpretation of the results obtained, and to distinguish between transcriptional effects caused by a perturbation of gene activity and those induced non-specifically as a consequence of activation of the promoter system. For the latter, the line that we generated that drives expression of the CTRL amiRNA under the control of the AlcApro/AlcR promoter system may be used.

To perform stage-specific gene perturbation experiments during flower development, we sought to combine the inducible amiRNA lines with a floral induction system in order to collect sufficient numbers of synchronized floral buds for genome-wide analyses. To this end, we generated and tested a series of floral induction systems in combination with the inducible promoter systems. We found that expression of the activatable version of the AP1 transcription factor from its own promoter resulted in floral induction systems that showed more complete rescue of the floral phenotypes of *ap1 cal* double mutants compared with ectopic expression of *AP1–GR* from a 35S promoter. Furthermore, plants of these improved floral induction systems may be used for experimental analysis during an extended time window compared with the 35S promoter-based line, as they transition to flowering more slowly. Because our results implied that the dexamethasone-dependent OPpro/GR-LhG4 promoter system is well suited for gene perturbation experiments in flowers, we further developed a dexamethasone-independent AP1pro:AP1-AR_{FIS} floral induction system that may be used in conjunction with the OPpro/GR-LhG4 promoter system. We found that induction of flower development in this line by treatment of plants with the androgen receptor ligand dihydrogentestosterone only activates the OPpro/GR-LhG4 promoter system very weakly, and to levels that do not appear to interfere with a stage-specific amiRNA-mediated perturbation of target genes. We further showed that induction of flower development and the induction of amiRNA expression may be performed sequentially and independently in these lines.

In summary, the series of floral induction systems described here constitute a versatile tool for dissection of GRNs during flower development, and may readily be combined with a range of inducible promoter systems to allow stage-specific knockdown or knock-in studies of specific genes of interest. Because use of the floral induction system allows collection of a large amount of synchronized flower buds, it is particularly well suited for genome-wide approaches, such as gene expression profiling or chromatin immunoprecipitation coupled to next-generation sequencing, but may also be used for phenotypic analyses (Wellmer *et al.*, 2006; Kaufmann *et al.*, 2010; Smaczniak *et al.*, 2012; Wuest *et al.*, 2012; O'Maoileidigh *et al.*, 2013; Pajoro *et al.*, 2014).

EXPERIMENTAL PROCEDURES

Plant growth

Plants were grown on a soil/vermiculite/perlite (3:1:1) mixture at 20°C (unless otherwise indicated) under constant illumination with cool white fluorescent light (36W, GE (http://catalog.gelighting.com/)). Previously described *Arabidopsis thaliana* strains used in this study include *ap1-1 cal-1* (Bowman *et al.*, 1993), 35Spro:AP1-GR *ap1-1 cal-1* (Wellmer *et al.*, 2006), OPpro:AG-amiRNA/35Spro:GR-LhG4 (O'Maoileidigh *et al.*, 2013), AlcApro:AG-amiRNA/35Spro:AlcR (O'Maoileidigh *et al.*, 2013), AP1pro:AP1-GR *ap1-1 cal-1* (O'Maoileidigh *et al.*, 2013) and AlcApro:AP3-amiRNA/35Spro:AlcR (Wuest *et al.*, 2012). The following lines were generated in this study, as described below: OPpro:AP3-amiRNA/35Spro:GR-LhG4, AP1pro:AP1-GR *ap1-1 cal-1* AlcApro:CTRL-amiRNA/35Spro:AlcR, AP1pro:AP1-AR *ap1-1 cal-1*, AlcApro:AP1/35Spro:AlcR *ap1-1 cal-1*, and kanamycin-resistant versions of the 35Spro:AP1-GR *ap1-1 cal-1* and AP1pro:AP1-GR *ap1-1 cal-1* floral induction systems.

Construction of the AP1pro:AP1–AR floral induction system

The mouse androgen receptor ligand-binding domain (AR) was amplified using oligonucleotides DM-658/DM-659 (Table S1) from cDNA derived from mouse testes RNA. The resulting PCR product was digested using *Xho*I/*Hin*dIII, and the fragment was cloned into the pBJ36 plasmid (Eshed *et al.*, 2001) that had been digested with the same restriction enzymes, resulting in pBJ36-AR. To generate the pBJ36-AP1pro:AP1-AR construct, a genomic fragment containing the *AP1* locus was PCR-amplified from Col-0 genomic DNA using primers DM-177/DM-180. The resulting PCR fragment was digested using *Xho*I/*Xba*I, and subcloned into pBJ36-AR treated with the same restriction enzymes. The AP1pro:AP1-AR translational fusion was then inserted into the plant transformation vector pML-BART (Eshed *et al.*, 2001) using *Not*I restriction sites, resulting in the plasmid pML-BART-AP1pro:AP1-AR. Plant populations doubly homozygous for the *ap1-1* and *cal-1* mutant alleles were transformed using *Agrobacterium tumefaciens* containing the pML-BART-AP1pro:AP1-AR plasmid. A transgenic line that responded to dihydrogentestosterone treatment by producing synchronous flower buds, sepals and petals in the *ap1-1 cal-1* background was chosen for further experimentation. Plants of this transgenic line that are homozygous for the AP1pro:AP1-AR transgene were subsequently identified by segregation analysis.

Construction of the kanamycin-resistant floral induction systems

The 35Spro:AP1-GR and AP1pro:AP1-GR fragments were digested using *Not*I from previously described pBJ36 derivatives (Wellmer *et al.*, 2006; O'Maoileidigh *et al.*, 2013), and ligated into pART27 (Gleave, 1992) that had also been digested with *Not*I. *Agrobacterium* containing these vectors were used to transform *ap1-1 cal-1* plants. Kanamycin-resistant transgenic lines that responded to treatment with a dexamethasone-containing solution by producing synchronous flower buds, sepals and petals in the *ap1-1 cal-1* background were chosen for further experimentation. Plants homozygous for the 35Spro:AP1-GR and AP1pro:AP1-AR transgenes, respectively, were subsequently identified by segregation analysis.

Construction of the AlcApro:AP1/35Spro:AlcR floral induction system

The *AP1* coding region was amplified from cDNA generated from RNA from flowers of accession Landsberg *erecta* (L*er*) using primers AR-33/AR-34 (Table S1), which incorporate *Xho*I and *Spe*I sites, respectively. The PCR fragment was digested using these two enzymes, and was inserted into the pBJ36-AlcApro plasmid that had been digested with *Xho*I and *Xba*I, resulting in plasmid pBJ36-AlcApro:AP1. This latter plasmid was then digested with *Not*I, and the AlcApro:AP1 fragment was ligated into pML-BART-35Spro:AlcR, which had also been digested with *Not*I. Plant populations doubly homozygous for the *ap1-1* and *cal-1* mutant alleles were transformed with *Agrobacterium* containing this pML-BART derivative. A transgenic line in the *ap1-1 cal-1* background that responded to ethanol vapor treatment by producing synchronous flower buds with the four types of floral organs was chosen for further experimentation. Plants homozygous for the AlcApro:AP1/35Spro:AlcR transgene were subsequently identified by segregation analysis.

Expression of *AP1* from the LexApro/XVE inducible system

The *AP1* coding region was amplified from cDNA generated from RNA of L*er* flowers using primers AR-33/AR-34 (Table S1), which incorporate *Xho*I and *Spe*I restriction sites. The resulting PCR fragment was cloned into the pER8 plasmid (Zuo and Chua, 2000) digested with the same enzymes, yielding pER8-LexApro:AP1/$G_{10\text{-}90}$pro:XVE. Plant populations doubly homozygous for the *ap1-1* and *cal-1* mutant alleles were transformed with *Agrobacterium* containing this plasmid, and transgenic plants were selected on half-strength MS agar medium supplemented with hygromycin.

Construction of the OPpro:AP3-amiRNA/35Spro:GR-LhG4 transgenic line

The *AP3* amiRNA was excised from pBJ36-35Spro:AP3-amiRNA (Wuest *et al.*, 2012) using *Bam*HI and *Eco*RI, and ligated into pBJ36-6xOPpro plasmid that had been digested with the same enzymes, resulting in plasmid pBJ36-6xOPpro:AP3-amiRNA. This plasmid was then digested with *Not*I, and the 6xOPpro:AP3-amiRNA fragment was cloned into pML-BART-35Spro:GR-LhG4, which had also been digested with *Not*I, resulting in pML-BART-6xOPpro:AP3-amiRNA/35Spro:GR-LhG4. Wild-type L*er* plants were transformed with *Agrobacterium* containing this vector, and transgenic plants were identified via their glufosinate-resistant phenotypes. A transgenic line that responded to dexamethasone treatment by producing sepals and carpels in the second and third whorls,

respectively, was chosen for further experimentation. Plants homozygous for the transgene were subsequently identified by genotyping. DM-5 and DM-494 (Table S2) were used to check the presence of the T-DNA, while DM-5 and DM-6 were used to identify the corresponding genomic region that lacked the T-DNA.

Construction of the LexApro:amiRNA/$G_{10\text{-}90}$pro:XVE transgenic lines

amiRNAs directed against *AP3* and *AG* were excised from pBJ36-35Spro:AP3-amiRNA (Wuest *et al.*, 2012) and pBJ36-35Spro:AG-amiRNA (O'Maoileidigh *et al.*, 2013) using *Bam*HI and *Eco*RI digestion. The fragments encoding the amiRNAs were ligated into the pER8 plasmid that had been digested with the same enzymes. L*er* plants were transformed with *Agrobacterium* containing this vector, and transgenic plants were identified based on their resistance to hygromycin.

Construction of the 35Spro>>CTRL-amiRNA$^{AlcA/AlcR}$ line

The CTRL amiRNA has no sequence complementarity to any annotated Arabidopsis transcript, and hence is not predicted to target any transcript using the WMD3-microRNA design tool (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi). The miR319 sequence encoded in the plasmid pRS300 (Schwab *et al.*, 2006) was used as a template for PCR-based mutagenesis as described previously (Schwab *et al.*, 2006) (see Table S1 for oligonucleotide sequences). The resulting CTRL amiRNA was inserted into the shuttle vector pBJ36 (Eshed *et al.*, 2001), containing the AlcA promoter (Caddick *et al.*, 1998) using *Eco*RI/*Bam*HI restriction sites. The AlcApro:CTRL-amiRNA fragment was then inserted into the plant transformation vector pART27 35Spro:AlcR (Gleave, 1992; Caddick *et al.*, 1998) using *Not*I restriction sites. This vector was used to transform AP1pro:AP1-GR *ap1-1 cal-1* using *Agrobacterium*. Transformants were identified based on their kanamycin-resistant phenotype. Once a suitable line was identified based on the expression levels of *AlcR*, homozygous lines were isolated by segregation analysis.

Mapping of T-DNA insertions

Genomic DNA preparations were used for TAIL-PCR (Liu *et al.*, 1995). Selected DNA bands were excised from agarose gels and sequenced using Sanger technology. Appropriate primers were designed to detect the T-DNA insertion (Table S2).

Genotyping the AP1pro:AP1-GR floral induction systems

Two separate transgenic lines (lines 3 and 5) that contained T-DNA insertions on different chromosomes were identified for the AP1pro:AP1-GR floral induction system containing the transgene that confers resistance to glufosinate. Primers DM-456 and DM-494 (Table S2) were used to detect the T-DNA insertions in genomic DNA of line 3, while DM-456 and DM-457 (Table S2) were used to detect genomic segments that did not contain the T-DNA insertion in this line. Primers DM-400 and DM-494 (Table S2) were used to detect the T-DNA insertions in genomic DNA of line 5, while DM-400 and DM-401 (Table S2) were used to detect genomic segments that did not contain the T-DNA.

Dexamethasone treatments

For all experiments with the dexamethasone-inducible floral induction systems, we used approximately 4-week-old plants. Flower development was induced as described by Wellmer *et al.* (2006) using a solution containing 10 μM dexamethasone (Sigma-Aldrich, http://www.sigmaaldrich.com/technical-service-home/product-catalog.html), 0.1% v/v ethanol and 0.015% v/v Silwet L-77 (De Sangosse, http://www.desangosse.co.uk/). For experiments performed with seedlings, 7-day-old seedlings grown under long-day conditions (16 h light/8 h dark) at 21°C on half-strength MS agar plates were transferred to half-strength MS liquid medium supplemented with 1 μM dexamethasone, 0.1% ethanol and 0.015% Silwet L-77. Seedlings were incubated for 1 day at room temperature. For mock treatments, dexamethasone was omitted.

Ethanol treatments

For all experiments involving the ethanol-inducible promoter, we used approximately 4-week-old plants. Flower development was induced as described by O'Maoileidigh *et al.* (2013) and Wuest *et al.* (2012). Briefly, pots were transferred to trays that may be covered with plastic lids (18 cm × 32 cm × 50 cm). Two 50 ml tubes containing 10 ml of 100% ethanol each were placed near the plants before the lid was closed. For the mock treatments, ethanol was replaced with water. For experiments performed with seedlings, 7-day-old seedlings grown under long-day conditions at 21°C on half-strength MS agar plates were transferred to half-strength MS liquid medium supplemented with 1% ethanol. Seedlings were incubated for 1 day at room temperature. For mock treatments, ethanol was omitted.

Dihydrogentestosterone treatments

In lines expressing AP1 fused to the AR domain, flower development was induced at approximately 4 weeks by using a solution containing 2 μM 5α-androst-16-en-3-one (dihydrogentestosterone) (Sigma-Aldrich), 2% v/v ethanol and 0.015% v/v Silwet L-77. For mock treatments, dihydrogentestosterone was omitted.

Estradiol treatments

Expression of *AP1* from the LexApro/XVE inducible system was induced at approximately 4 weeks using a solution containing 10 μM 17β-estradiol (Sigma-Aldrich), 0.1% dimethylsulfoxide and 0.015% Silwet L-77. For experiments with seedlings, 7-day-old seedlings grown under long-day conditions at 21°C on half-strength MS agar plates were transferred to half-strength MS liquid medium supplemented with 10 μM 17β-estradiol, 0.1% dimethylsulfoxide and 0.015% Silwet L-77. Seedlings were incubated for 1 day at room temperature. For mock treatments, 17β-estradiol was omitted. In some experiments, seedlings were germinated directly on half-strength MS agar plates containing 10 μM 17β-estradiol and 0.1% dimethylsulfoxide.

Scanning electron microscopy

Scanning electron microscopy was performed as described previously (Bowman *et al.*, 1989) using a Hitachi 4700 scanning electron microscope (http://hitachi-hta.com/). Briefly, untreated inflorescence-like meristems of approximately 4-week-old *ap1-1 cal-1* plants (Bowman *et al.*, 1993) and AP1pro:AP1-GR_{FIS} plants (O'Maoileidigh *et al.*, 2013) were harvested, as were AP1pro:AP1-GR_{FIS} inflorescence-like meristems 5 days after dexamethasone treatment, and prepared as described in (Fiume *et al.*, 2010).

In situ hybridizations

Non-radioactive *in situ* hybridizations were performed as previously described (Long and Barton, 1998). Part of the *GR* ligand-binding domain cDNA was amplified by PCR using primers DM-183 and FW-100 (Table S1). The PCR product was ligated into pGEM-T Easy (Promega, https://worldwide.promega.com/), and the result-

ing vector was sequenced to determine the orientation of the insert. Plants used for *in situ* hybridizations were the first-generation progeny (F_1) of a cross between AP1pro:AP1-GR *ap1-1 cal-1* and wild-type plants of accession L*er*.

Timing of flower formation in the floral induction systems in the absence of *AP1* induction

Plants were grown as indicated above. The number of days to flower formation was calculated as the time from when a given plant had bolted by approximately 1 cm until flowers appeared on the inflorescence-like meristems in the absence of *AP1* induction treatment. To do this, inflorescence-like meristems were inspected by light microscopy at least every second day. At least two independent biological replicates were performed.

Petal measurements

Petals were removed from flowers at anthesis, and photographed with an accompanying scale. ImageJ software (Schneider *et al.*, 2012) was used to measure the maximum width and length of each petal.

RNA preparation

Total RNA was isolated from tissue samples using a Plant Total RNA kit (Sigma-Aldrich). Quality of selected RNA samples was evaluated using a Bioanalyzer 2100 and a RNA Nano 6000 kit (Agilent, http://www.agilent.ie/home). DNase I treatments were performed using an on-column DNase I digestion set (Sigma-Aldrich).

RT-PCR experiments

cDNA synthesis was performed using total RNA preparations, oligo(dT) primers and RevertAid H Minus M-MuLV reverse transcriptase (Fermentas, https://www.lifetechnologies.com/ie/en/home/brands/thermo-scientific.html). PCRs were performed using gene-specific primers (Table S3) and DreamTaq DNA polymerase (Fermentas). The PCR products were analyzed using 1.5% agarose gels supplemented with ethidium bromide.

Quantitative Reverse transcriptase-PCR experiments

cDNA synthesis was performed using total RNA preparations, oligo(dT) primers and RevertAid H Minus M-MuLV reverse transcriptase (Fermentas). The relative transcript abundance of selected genes was determined using the LightCycler 480 system and the LC480 SYBR Green I Master kit (Roche, https://lifescience.roche.com/shop/home) (see Table S4 for a list of genes and the oligonucleotides used). Measurements were taken for three or four biologically independent sets of samples. In addition, all PCR reactions were performed twice for each cDNA (technical duplicates). LightCycler melting curves were obtained for the reactions, revealing single-peak melting curves for all amplification products. The amplification data were analyzed using the second derivative maximum method, and resulting C_p values were converted into relative expression values using the comparative C_T method (Livak and Schmittgen, 2001). One reference gene ('REF1') (Czechowski *et al.*, 2005) was used to normalize the data. The C_p values were averaged for each sample. Either control 'no RT' experiments or on-column DNase I treatments (Sigma-Aldrich) were performed.

Microarray experiments

We collected floral buds from stages 1–10 (approximately) from 25 plants 24 h after treatment with a dexamethasone-containing solution, mock solution, ethanol vapor or water vapor for the transgenic lines described above. Four biological replicates were generated for each treatment. RNA was isolated from the treated flowers as described above, and was co-hybridized to custom-designed microarrays (Agilent) as previously described (Kaufmann *et al.*, 2010). Microarray datasets have been deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) database under accession number GSE68157. Data for 35S>>AG-amiRNA$^{GR\text{-}LhG4}$ were taken from O'Maoileidigh *et al.* (2013), and are available under accession number GSE45939.

Microarray data analysis

Testing for differential gene expression was performed using linear models (Smyth, 2004) as described in the *Limma* user guide (Silver *et al.*, 2009). Our data analysis mainly focused on functional groups of genes (e.g. GO terms, gene/protein families) that are over-represented within lists of potentially mis-expressed genes. Therefore, the multiple testing adjustments were performed at the level of enrichment tests rather than at the level of selection of differentially expressed genes. Our gene selection strategy was based on declaring a gene as differentially expressed at a P value < 0.01. GO term enrichment analyses were performed using a hypergeometric test, and adjustments for multiple testing were performed using the Benjamini–Hochberg–Yekutieli procedure implemented using AgriGO online software (Du *et al.*, 2010). GO terms with a false discovery rate (FDR) < 0.05 were considered enriched in the datasets.

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SUPPORTING INFORMATION

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

Figure S1. Schematic representation of constructs used to express amiRNAs in an inducible manner.

Figure S2. Kinetics of *AP3* knockdown in 35Spro>>AP3-amiRNA$^{GR\text{-}LhG4}$ plants.

Figure S3. Genome-wide analysis of genes responsive to induction of an artifical microRNA targeting *AP3* using two inducible promoter systems.

Figure S4. Transcriptional response of selected genes after activation of the OPpro/35Spro:GR-LhG4 promoter system.

Figure S5. Transcriptional response of selected genes in response to the expression of a functional amiRNA.

Figure S6. Schematic representation of constructs used to generate the various floral induction systems.

Figure S7. Characterization of glucorcorticoid-based floral induction systems.

Figure S8. Expression of *AP1* in an estradiol-dependent manner using the LexApro/XVE system.

Table S1. Primer sequences used for generating constructs.

Table S2. Primer sequences used for TAIL-PCR.

Table S3. Primer sequences used for RT-PCR.

Table S4. Primer sequences used for quantitative real-time PCR.

Data S1. Differential expression of genes in response to induction of *AP3* and *AG* amiRNA expression using the AlcApro/AlcR and OPpro/GR-LhG4 promoter systems.

Data S2. GO terms identified as significantly enriched among differentially expressed genes derived from the microarray experiments with *AP3* and *AG* amiRNAs expressed using the AlcApro/AlcR and OPpro/GR-LhG4 promoter systems.

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