

TECHNICAL ADVANCE

Floral induction in tissue culture: a system for the analysis of LEAFY-dependent gene regulation

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Summary

We have developed a versatile floral induction system that is based on ectopic overexpression of the transcription factor LEAFY (LFY) in callus. During shoot regeneration, flowers or floral organs are formed directly from root explants without prior formation of rosette leaves. Morphological and reporter gene analyses show that leaf-like structures are converted to floral organs in response to LFY activity. Thus, increased levels of LFY activity are sufficient to bypass normal vegetative development and to direct formation of flowers in tissue culture. We found that about half of the cultured cells respond to inducible LFY activity with a rapid upregulation of the known direct target gene of LFY, *APETALA1* (*AP1*). This dramatic increase in the number of LFY-responsive cells compared to whole plants suggested that the tissue culture system could greatly facilitate the analysis of LFY-dependent gene regulation by genomic approaches. To test this, we monitored the gene expression changes that occur in tissue culture after activation of LFY using a flower-specific cDNA microarray. Induction of known LFY target genes was readily detected in these experiments. In addition, several other genes were identified that had not been implicated in signaling downstream of LFY before. Thus, the floral induction system is suitable for the detection of low abundance transcripts whose expression is controlled in an LFY-dependent manner.

Keywords: flower development, floral induction, floral meristem identity, LEAFY, callus, gene expression analysis.

Introduction

The LEAFY (LFY) transcription factor is a central regulator of the switch from vegetative to reproductive development and is required for proper induction of the floral organ identity genes (Weigel and Meyerowitz, 1993; Weigel and Nilsson, 1995; Weigel *et al.*, 1992). Despite its pivotal role in *Arabidopsis* development, the molecular events downstream of LEAFY activation (e.g. signaling and regulatory steps, the identity of the genes regulated by LFY) are poorly understood. Among the few direct target genes of LFY that are currently known are *APETALA1* (*AP1*), *AGAMOUS* (*AG*), and *APETALA3* (*AP3*) (Busch *et al.*, 1999; Lamb *et al.*, 2002;

Parcy *et al.*, 1998; Wagner *et al.*, 1999). These genes, all encoding members of the MADS-box family of transcription factors, play distinct roles in flower development, which is reflected in their different spatial and temporal expression patterns. This, in turn, indicates that the mechanisms by which LFY regulates the three genes are different (Parcy *et al.*, 1998; see below). *AP1* functions as a meristem identity as well as a floral organ identity gene. It is initially expressed uniformly in young floral primordia, but the domain of expression becomes restricted to sepals and petals at later developmental stages because of repression

by AG (Gustafson-Brown *et al.*, 1994; Mandel *et al.*, 1992). The initial phase of *AP1* expression has been shown to be directly dependent on LFY, whereas the late phase of expression is only indirectly affected by it (Parcy *et al.*, 1998; Wagner *et al.*, 1999; Weigel and Meyerowitz, 1993). In a recent study, it was shown that the *AP1* paralog *CAULIFLOWER* (*CAL*) is also directly regulated by LFY (William *et al.*, 2004). The floral organ identity gene *AP3* is required, together with *PISTILLATA* (*PI*), for the development of petals and stamens. *AP3* expression is essentially restricted to developing petals and stamens (Jack *et al.*, 1992) by the activities of *LFY*, *AP1*, and *UNUSUAL FLORAL ORGANS* (*UFO*) (Ng and Yanofsky, 2001). LFY appears to act in both a direct and an indirect manner to regulate *AP3* expression, with the latter pathway depending at least in part on *AP1* (Lamb *et al.*, 2002; Parcy *et al.*, 1998). Lastly, *AG* is required for the development of stamens and carpels, and also plays a role in establishing the determinacy of the floral meristem. *AG* expression is restricted to the center of the developing flower and depends on the combined activities of LFY and the homeodomain protein *WUSCHEL* (Lenhard *et al.*, 2001; Lohmann *et al.*, 2001). LFY has been shown to bind *in vitro* to sequences in regulatory regions of *AP1*, *CAL*, *AP3*, and *AG*, but whereas deletion or mutation of the binding sites in *AG* largely eliminated the enhancer's activity, mutation of the putative LFY-binding sites in the *AP3* promoter did not obviously disrupt *AP3* expression (Busch *et al.*, 1999; Lamb *et al.*, 2002; Parcy *et al.*, 1998).

It is clear from these examples that the dissection of the regulatory cascade that exists downstream of LFY presents important challenges. First, in the plant, only a small subset of cells responds to endogenous LFY activity (Busch *et al.*, 1999; Wagner *et al.*, 1999; Weigel and Meyerowitz, 1993). Second, as summarized above, different tissues (or the same tissue at different developmental stages) respond differently to LFY, and LFY targets can be regulated by multiple pathways some of which do not depend on LFY itself.

A comparison of the gene expression profiles at the shoot apex of wild-type and *lfy-12* mutant plants upon transfer from short to long days (an inductive signal for flowering) led to the identification of a group of genes whose activation of expression is LFY dependent (Schmid *et al.*, 2003). This group included the LFY targets described above, the organ identity co-factor genes *SEPALLATA* (*SEP*)1–*SEP*3, and 10 other genes that had not been associated with LFY before. However, whether these novel genes are immediately downstream of LFY, and how they are integrated into a regulatory network, remains to be elucidated.

In another study, an LFY fusion protein with inducible activity was used to assess gene expression changes in whole seedlings (William *et al.*, 2004). This study led to the identification of 15 potential LFY target genes, 5 of which are directly regulated by LFY. However, the previously

known direct LFY target gene *AP1* was not detected, most likely because of the dilution effect caused by using a mixture of tissues in which only a small portion of cells are responding to ectopic LFY activity (Wagner *et al.*, 1999; William *et al.*, 2004).

No overlap was observed between the genes identified in the two studies, despite the fact that the same type of 'whole genome' microarray was used. This indicates that the list of genes that are controlled, directly or indirectly, by LFY is not yet comprehensive, and that new experimental approaches are needed to allow a more complete description of LFY-dependent gene regulation.

In this report, we describe a floral induction system that is based on ectopic expression of LFY in tissue culture. We demonstrate that during shoot regeneration, LFY activity is sufficient to induce flower formation without prior formation of vegetative organs. Furthermore, compared to whole plants, the portion of cells that are competent to respond to LFY is greatly increased. We have performed microarray experiments to demonstrate the suitability of the floral induction system for the identification of genes downstream of LFY and detected expression changes for several known LFY target genes as well as genes that had not been associated with LFY before. The advantages of the described approach compared to other experimental systems as well as possible limitations are discussed.

Results and discussion

Floral induction in tissue culture

A fusion between LFY and the hormone-binding domain of the rat glucocorticoid receptor (GR) results in a biologically functional form of LFY that can be activated post-translationally in plants by treatment with the synthetic steroid hormone dexamethasone (Wagner *et al.*, 1999). We investigated the effect of increased LFY activity during regeneration (callus formation) from root explants of 21-day-old seedlings. Living plant cells de-differentiate when removed from their tissue context and placed on callus-inducing medium (CIM). After subsequent incubation on shoot-inducing medium (SIM), newly formed meristems give rise to new plants that essentially recapitulate normal seedling development (Banno *et al.*, 2001; Cary *et al.*, 2001). The shoot meristems first generate rosette leaves and then primary inflorescences that bear the typical two types of lateral appendages: initially secondary inflorescence branches subtended by cauline leaves and later the reproductive structures, the flowers.

To assay the effect of LFY overexpression during plant regeneration in tissue culture, root explants of 35S:LFY-GR transgenic plants were treated with dexamethasone while on CIM and then transferred to SIM to induce shoot

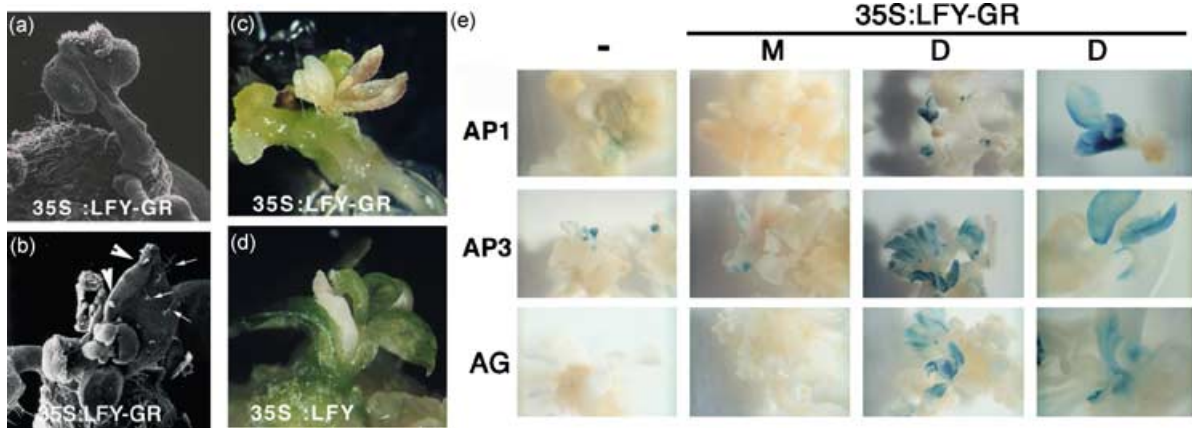


Figure 1. Overexpression of LFY in regenerating root explants. (a, b) Scanning electron microscope pictures of the organs that developed from dexamethasone-treated 35S:LFY-GR root explants after placement on SIM. (a) Carpel with enlarged valves. (b) Chimeric organ showing leaf- and carpel-like structures. Note ovules on the left half (arrowheads) and stellate trichomes on the right half of the central organ (arrows). (c, d) Flowers develop directly from the root explants of 35S:LFY-GR (c) or 35S:LFY (d) plants. (e) GUS staining of reporter lines for the floral organ identity genes *AP1*, *AP3*, and *AG*. The promoters driving GUS expression in the plants are indicated to the left of each row. Samples were untreated (-), treated with 0.1% ethanol (M) or with 5 μM dexamethasone in 0.1% ethanol (D). All pictures were taken at equal magnification (7×), except for the right-most column that shows representative samples at higher magnification (21×). Root explants from 35S:LFY-GR plants were treated with 5 μM dexamethasone for 7 days while on CIM prior to transfer to SIM. Assays were performed after 21 days on SIM.

regeneration. Instead of rosette leaves, flowers or floral organs formed directly from the root explants during regeneration in the majority of the explants (Figure 1a–c). This was not observed in mock-treated root explants derived from the same plants. Thus, after activation of the LFY-GR fusion protein, regenerating explants in tissue culture completely bypass vegetative development. An identical effect was observed when non-chimeric LFY was expressed in

callus under control of a 35S promoter (Figure 1d), indicating that the observed phenotype was not because of an altered activity of the LFY-GR fusion protein.

The immediate formation of flowers in the absence of leaves suggests that elevated levels of LFY activity in callus cause a conversion of leaves into floral organs. In support of this hypothesis, we occasionally observed chimeric organs (Figure 1b) that had both leaf traits (trichomes)

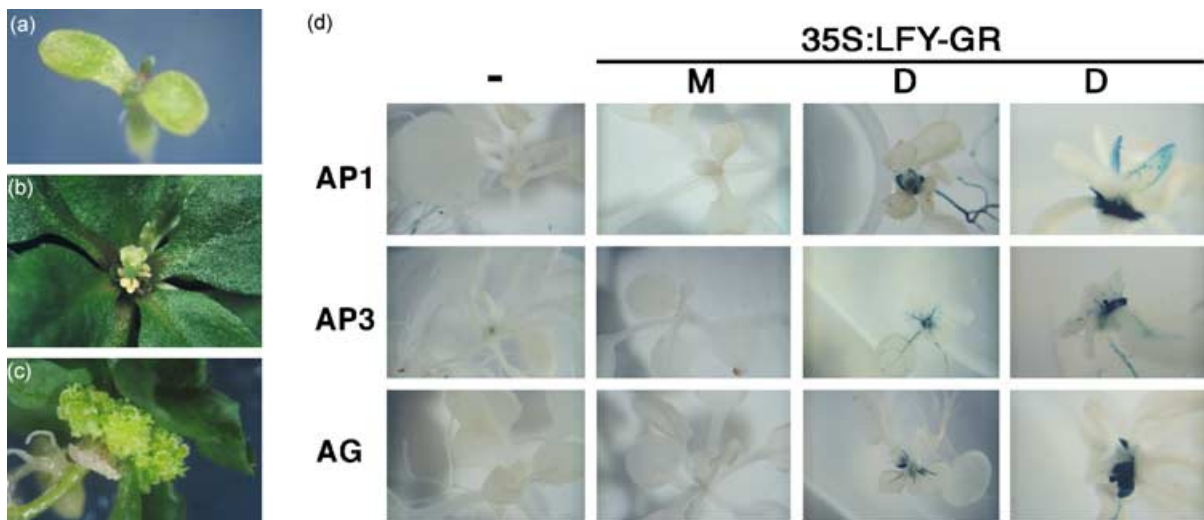


Figure 2. Overexpression of LFY-GR in seedlings. (a–c) 35S:LFY-GR seedlings treated with 5 μM dexamethasone from day 1 to day 7 after germination. Plants were photographed 10 days (a) or 21 days after the end of the treatment (b,c). (b) Top view onto rosette and (c) side view of the axil of a cotyledon. (d) GUS staining of reporter lines for the floral organ identity genes *AP1*, *AP3*, and *AG*. The promoters driving GUS expression in the seedlings are indicated to the left of each row. Samples were untreated (-), treated with 0.1% ethanol (M), or treated with dexamethasone in 0.1% ethanol (D). All pictures were taken at equal magnification (7×); except for the right-most column that shows representative samples at higher magnification (21×).

and carpel traits (ovules and stigmatic papillae). To further test this hypothesis, we investigated the expression of the floral organ identity genes in regenerating plants derived from 35S:LFY-GR root explants. The activity of the β -glucuronidase (GUS) reporter driven by the promoters of *AP1*, *AP3*, or *AG* (Gustafson-Brown *et al.*, 1994; Sieburth and Meyerowitz, 1997; Tilly *et al.*, 1998) was analyzed in 35S:LFY-GR-regenerating plants that were hormone- or mock-treated during callus induction. Only a low background of GUS staining was observed in the leaf-like organs regenerated from root explants of the GUS-expressing lines or from mock-treated plants carrying the GUS-reporter constructs as well as 35S:LFY-GR (Figure 1e). By contrast, strong GUS staining was observed for all reporter constructs in the structures regenerated from dexamethasone-treated root explants of 35S:LFY-GR plants (Figure 1e). Thus, increased levels of LFY activity, when present during regeneration in tissue culture, are sufficient to cause floral homeotic gene expression. Taken together with the chimeric leaf/carpel organs observed (Figure 1b), the data indicate that during regeneration on SIM, increased levels of LFY protein are sufficient to bypass vegetative development and to direct formation of the reproductive structures.

Recently, shoot regeneration has been studied extensively in *Arabidopsis* (Banno *et al.*, 2001; Cary *et al.*, 2001, 2002; Catterou *et al.*, 2002; Che *et al.*, 2002; Mordhorst *et al.*, 2002; Ozawa *et al.*, 1998). Shoot apical meristems are formed in tissue culture between 3 and 6 days after transfer to SIM, and their initiation appears to be regulated essentially as during embryo development (Cary *et al.*, 2002; Mordhorst *et al.*, 2002). When we treated root explants of 35S:LFY-GR plants with dexamethasone, floral structures were formed after transfer of the treated explants to SIM (see above). Thus, increased LFY-GR activity or increased levels of another downstream factor likely persist until formation of the new shoot meristems to change meristem identity.

Previous results have shown that overexpression of LFY in seedlings results in precocious flower formation, yet by itself is not sufficient to cause plants to bypass vegetative development (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999; Weigel and Nilsson, 1995). The 35S:LFY overexpression phenotype results in an accelerated transition to the reproductive phase: formation of fewer rosette leaves than in the wild type, and transformation of most inflorescences with subtending bracts into flowers. Given that activation of 35S:LFY-GR in callus resulted in flower formation without prior initiation of rosette leaves, we next tested whether the enhanced response to LFY activity can also be observed in seedlings. For this, we treated 35S:LFY-GR seedlings with dexamethasone in a fashion comparable to that used in the callus system and monitored the consequence of this treatment on the phenotype as well as on homeotic gene

expression (Figure 2). Most 35S:LFY-GR seedlings arrested growth after forming two small primary leaves (Figure 2a) when treated with dexamethasone from day 1 to day 7 after germination. A subset of the arrested plants recovered from the growth arrest and formed a series of rosette leaves before terminating in a single flower (Figure 2b). All inflorescence primordia (primary and axillary) in these plants, even the inflorescence primordia in the axils of the cotyledons, were converted to flowers or to floral structures (carpellid organs, Figure 2c). Thus, as described previously for overexpression of LFY, overexpression of LFY-GR results in conversion of inflorescence branches with subtending cauline leaves into flowers, but does not result in flower formation in lieu of rosette leaf formation.

We next tested the expression of *AP1*, *AP3*, and *AG* in 7-day-old dexamethasone- and mock-treated seedlings using the above-mentioned GUS reporter lines. No significant GUS staining was observed in seedlings of the untreated GUS reporter lines or in the mock-treated 35S:LFY-GR GUS lines (Figure 2d), consistent with published data, demonstrating that the reporter constructs are flower specific (Gustafson-Brown *et al.*, 1994; Sieburth and Meyerowitz, 1997; Tilly *et al.*, 1998). In contrast, strong GUS staining was observed in hormone-treated 35S:LFY-GR seedlings, primarily in the axils of the leaves and cotyledons. This is where the axillary inflorescences are located, which are converted to flowers or floral structures in response to LFY-GR overexpression (Figure 2c). Thus, in seedlings, overexpression of LFY-GR causes transformation of inflorescence branches with cauline leaves into flowers or floral structures because of activation of floral homeotic gene expression in the inflorescence primordia. The phenotype of dexamethasone-treated 35S:LFY-GR seedlings is thus similar to that observed for overexpression of non-chimeric LFY (Weigel and Nilsson, 1995).

These findings indicate that LFY is sufficient to induce flower formation in lieu of the vegetative program only during plant regeneration in the callus system. Previous studies have shown that overexpression of LFY can cause the formation of flowers immediately after germination in seedlings when the level of the flowering time regulator FT is also increased (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999). Therefore, the increased response to LFY overexpression in callus may be because of the presence of an activator of the floral program in this tissue (such as FT) or because of the absence of a repressor (such as AGAMOUS-LIKE24 (AGL24); Yu *et al.*, 2004).

LFY regulates AP1 expression in tissue culture

In order to determine whether this floral induction system can be used for the analysis of LFY-dependent gene regulation, we next tested the effect of short activation of LFY-GR on the expression of the known LFY target gene *AP1*. GUS

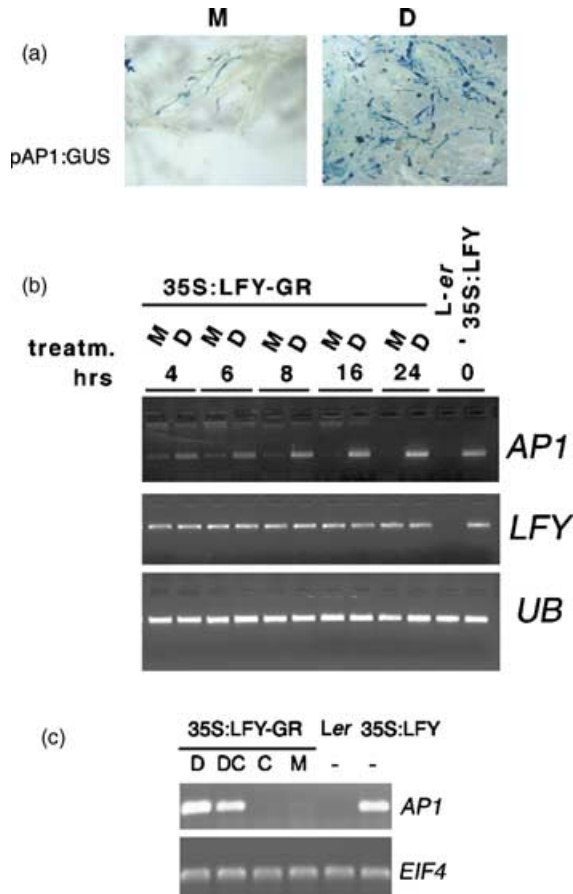


Figure 3. Effect of activation of 35S:LFY-GR in root explants on CIM. (a) GUS staining performed on 35S:LFY-GR root explants containing an *AP1* reporter construct treated with 0.1% ethanol (M) or with 5 μM dexamethasone in 0.1% ethanol (D) for 8 h. (b) RT-PCR analysis of root explants treated for varying durations (4–24 h) with D or M solution. Gene-specific primers for *AP1*, *LFY*, and *UBIQUITIN* (*UB*) were used. The genotypes of the explants are indicated. *LFY* is over-expressed to approximately the same level in root explants from 35S:LFY-GR and 35S:LFY plants. (c) RT-PCR analysis with gene-specific primers for *AP1* using root explants treated for 8 h with D and M solutions as described in (a), as well as with D solution containing 10 μM cycloheximide (DC) and M solution containing 10 μM cycloheximide (C). Primers specific for the ubiquitously expressed eukaryotic elongation factor 4 (*EIF4*) gene were used to test for equal template amount. The respective plant genotypes are indicated. Root explants generated from 3-week-old seedlings were placed on CIM for 1 week. On day 7, the explants were used for the experiments.

activity of the reporter line described above was monitored in root explants on CIM after an 8-h-long treatment with dexamethasone or mock solution. Untreated (not shown) or mock-treated root explants exhibit a low background of staining (Figure 3a; left panel). A strong induction of GUS activity was observed in the dexamethasone-treated tissue (Figure 3a; right panel), suggesting that a single treatment of root explants on CIM is sufficient to cause the rapid upregulation of a known LFY target gene.

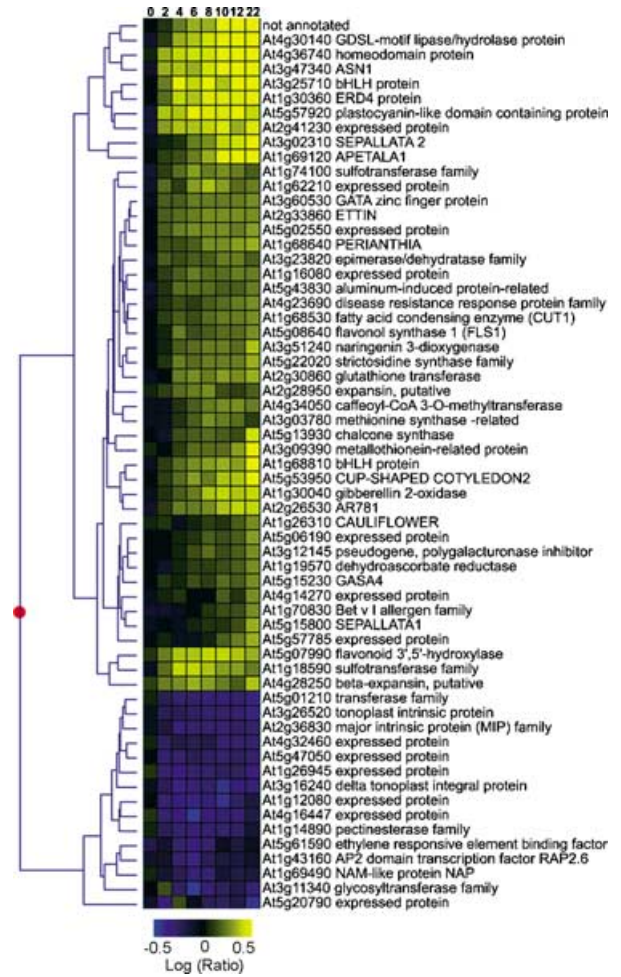


Figure 4. Cluster diagram of genes whose expression changed significantly in response to LFY-GR activation. Root explants of 35S:LFY-GR plants were treated for 0, 2, 4, 6, 8, 10, 12, or 22 h with dexamethasone or were mock treated. Four biologically independent sets of samples were hybridized to a flower-specific cDNA array, and the data were analyzed as described in Experimental procedures. A total of 61 genes were identified as significantly changed in the microarray experiments in at least one of the time points (P -value < 0.001 and fold change > 1.75). Genes that were upregulated in dexamethasone-treated samples are shown in yellow and downregulated genes in blue. The intensities of the colors increase with increasing expression differences as indicated on the bottom. The diagram was generated using \log_{10} transformed expression ratios.

Furthermore, the number of cells that are able to respond to LFY-GR activation by *AP1* induction is very high. An estimated 50% of the cells in the root explants respond to LFY activation by upregulation of *AP1*. By contrast, less than 1% of the cells in a seedling (only the cells in the flower primordia *anlagen*) respond to LFY by upregulating *AP1* expression (Wagner *et al.*, 1999). Thus, the tissue culture system allows us to overcome one of the major obstacles encountered in using entire multicellular eukaryotic

organisms for genome-wide expression analyses, by greatly increasing the ratio of cells that are responsive to LFY to the total number of cells.

Genome-wide microarray-based analyses require quantitative differences in the mRNA levels of a gene of interest between the experimental and control samples. Therefore, we determined whether we could detect quantitative upregulation of an LFY target such as *AP1* using the tissue culture system. Toward this end, we used semiquantitative RT-PCR to analyze *AP1* expression levels after different treatment durations either with dexamethasone-containing or mock solution. Treatment of 35S:LFY-GR root explants with the steroid hormone for all durations (from 4 h up to 24 h) led to successively increasing *AP1* mRNA levels, while mock-treated explants exhibited only background levels of *AP1* expression (Figure 3b). Thus, expression of the known LFY target gene *AP1* increases rapidly and quantitatively in response to 35S:LFY-GR activation in root explants. In addition, the upregulation of *AP1* expression is not caused by altered LFY activity because of fusion to the GR hormone-binding domain as 35S:LFY root explants show a similar increase in *AP1* expression when compared to wild-type controls (Figure 3b). The observed upregulation of *AP1* in untreated 35S:LFY explants further suggests that the observed effect in the 35S:LFY-GR lines is not caused non-specifically by the dexamethasone treatment.

Moreover, the upregulation of *AP1* expression is independent of protein synthesis in the callus system: root explants treated for 8 h with dexamethasone alone or simultaneously with dexamethasone and the translation inhibitor cycloheximide had elevated levels of *AP1* transcript (Figure 3c). Cycloheximide efficiently inhibits protein synthesis in a variety of plant tissues (Sablowski and Meyerowitz, 1998; Wagner *et al.*, 1999). In contrast, root explants that were treated with cycloheximide alone or were mock-treated showed only background expression of *AP1* (Figure 3c). Taken together, these data indicate that in the tissue culture system described here many aspects of *AP1* induction by LFY are conserved. Furthermore, the large portion of cells responding to LFY (see above) suggests that the tissue culture system is well suited for the analysis of events downstream of LFY by genome-wide expression analyses.

Microarray analysis

To further characterize LFY-dependent gene regulation in the tissue culture system, we performed microarray experiments using a cDNA array whose composition is strongly enriched for flower/inflorescence-specific transcripts (Wellmer *et al.*, 2004). This microarray contains 10 816 elements representing approximately 5000–6000 genes. Root explants of 35S:LFY-GR plants were treated with dexamethasone-containing or mock solution, and

tissue was collected after 0, 2, 4, 6, 8, 10, 12, and 22 h. RNA was isolated from these samples and dye-labeled RNA fragments derived from dexamethasone, and mock-treated samples were co-hybridized to the cDNA array. We performed hybridizations with four biological replicates to allow a robust statistical analysis (see Experimental procedures for details). A total of 61 genes with significant changes in gene expression in at least one of the time points were identified. Most of these genes were upregulated in response to dexamethasone treatment, whereas only a few elements were significantly downregulated (Figure 4).

The hybridization results were confirmed for selected genes by semiquantitative RT-PCR (Figure 5) using independently prepared samples. In addition, all of the genes tested, except for *At1g30140*, showed elevated transcript levels in untreated 35S:LFY root explants relative to the wild type (Figure 5), indicating that they respond to LFY and not to dexamethasone itself. To verify this finding, we determined whether any of the identified genes were significantly changed in dexamethasone-treated compared to mock-treated wild-type callus. RNA was isolated from two biological replicates after 4, 12, and 22 h, and microarray experiments were performed. None of the identified genes (Figure 4) showed significant changes in these control experiments (data not shown).

Of the known direct target genes of LFY with well-defined roles during early flower development, induction of *AP1* and *CAL*, but not of *AP3* or *AG*, was detected (Figure 6a,c). This result is in agreement with the previous finding that the meristem identity activity of LFY and the role of LFY in induction of the floral homeotic genes are separable (Lee *et al.*, 1997; Wagner *et al.*, 1999).

Of the three *SEP* genes, we detected significant expression changes for *SEP1* and *SEP2* (Figure 6a) but not for *SEP3* (Figure 6c). Induction of *SEP1* and *SEP2* was confirmed by RT-PCR using gene-specific primers (Figure 5). For *SEP3*, by contrast, we were not able to detect significant expression changes by RT-PCR (not shown). As all three *SEP* genes are activated in an LFY-dependent manner (Schmid *et al.*, 2003), these results suggest that the mode of regulation for the *SEP* genes is different. This idea is supported by the fact that *SEP1* and *SEP2* on the one hand, and *SEP3* on the other hand, have different expression patterns. Whereas *SEP1* and *SEP2* are expressed in all four whorls of young floral meristems, *SEP3* expression is excluded from the first whorl (Flanagan and Ma, 1994; Savidge *et al.*, 1995). Thus, the lack of *SEP3* induction in our experiments could be explained by the fact that its expression requires a co-activator that is not present in the callus system. Alternatively, a whorl-specific repressor of *SEP3* might be constitutively expressed in the tissue culture system that prevents its LFY-dependent induction.

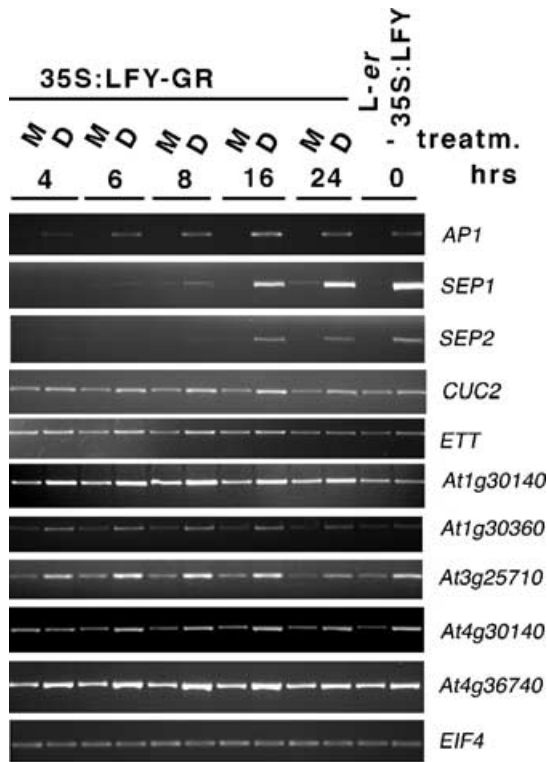


Figure 5. Verification of microarray results. Several genes that were identified to be upregulated in response to LFY-GR activation were tested by semiquantitative RT-PCR using gene-specific primers. Root explants of 35S:LFY-GR were treated with either 5 μ M dexamethasone in 0.1% ethanol (D) or with 0.1% ethanol (M) for 4, 6, 8, 16, or 24 h. As a control, root explants from untreated 35S:LFY and wild type (*Landsberg erecta* (*Ler*)) plants were included. Equal amounts of total RNA from each time-point and genotype were used. Primers specific for the ubiquitously expressed *EIF4* gene were used to test for equal template presence in all reactions. Treatments and genotypes used are indicated. Genes tested are indicated on the right.

Of the recently identified genes that show LFY-dependent expression (Schmid *et al.*, 2003; William *et al.*, 2004), only two genes were represented by the known elements of the flower-specific microarray used in this study. Of these two genes, one (*At3g47340*) was upregulated in our experiments in agreement with the previous results, while no significant induction was observed for *At4g21590*.

Two known floral regulators that were weakly induced in the experiment are *PERIANTHIA* (*PAN*) and *ETTIN* (*ETT*) (Figure 6b). Both genes are expressed in floral meristems when LFY becomes active; however, their expression appears unchanged in *lfy* mutants (Chuang *et al.*, 1999; Sessions *et al.*, 1997). This suggests that LFY is not required for their induction but may be involved in controlling their expression levels.

Among the 61 genes identified here that respond to LFY-GR activity, 14 (23%) encode known or predicted transcriptional regulators (for Gene Ontology predictions, see Supplementary Material). This percentage is significantly higher (*P*-values < 0.01; Fisher's exact test) than that of transcription factors in the *Arabidopsis* genome (approximately 5.9%; Riechmann *et al.*, 2000) or the estimated percentage of transcription factors represented on the flower-specific cDNA array (approximately 7.5%), suggesting that LFY, in agreement with its role as a master regulator, controls especially the expression of other regulatory proteins that execute the developmental programs required for flower development.

An enrichment of proteins involved in transcription or in signal transduction was also observed among the 15 genes identified in the study by William *et al.* (2004). In this study, LFY-GR was activated in seedlings and expression changes were monitored with 'whole genome' microarrays. While this approach led to the identification of novel LFY response genes, expression of previously known LFY target genes, such as *AP1*, was below the limit of detection, in all

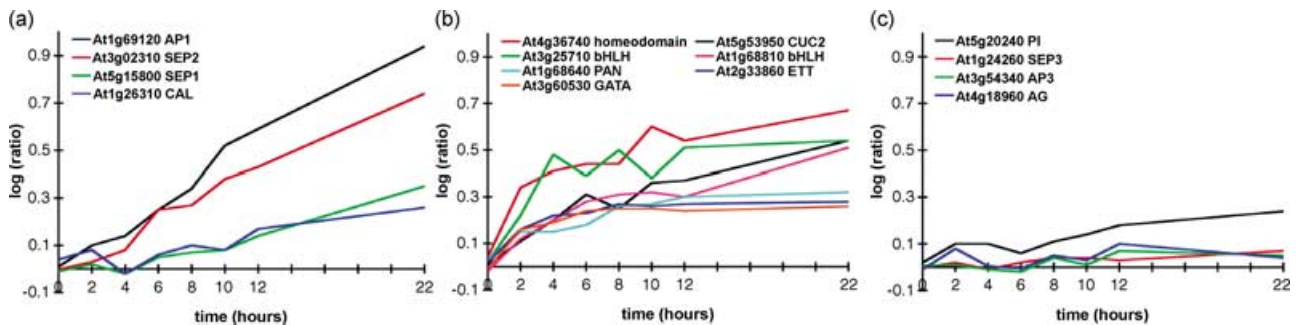


Figure 6. Expression changes for selected transcription factors after LFY-GR activation. (a) Genes previously implicated in floral meristem or organ identity that were judged to show significant expression changes in the microarray experiments. (b) Additional transcription factor genes whose expression changed significantly. (c) Genes previously implicated in floral organ identity that did not show significant expression changes. Gene identifiers and gene symbols or abbreviated gene names are indicated. The diagram was generated using \log_{10} transformed expression ratios. The data were analyzed as described for Figure 4.

probability because of the dilution effect caused by using a mixture of tissues in which only a small portion of cells are responding to LFY activity. In contrast, the use of the floral induction system readily allowed us to detect gene expression changes for these low-abundance transcripts. Thus, the main advantages of the tissue culture system described here are the strong enrichment of cells that respond to LFY activity and the ease with which large amounts of LFY-responsive tissue can be collected for detailed time course experiments.

The formation of flowers and the identification of known LFY target genes after activation of LFY-GR shows that the genes required downstream of LFY are correctly controlled in the tissue culture system and can be detected by microarray analysis. However, it is possible that some of the newly identified genes may respond to ectopic LFY activity only in callus, but do not respond to endogenous LFY in young floral meristems. This could be because of a different cellular competence of the cultured cells compared to meristematic cells and/or because of the overexpression of the factor. Thus, LFY-dependent regulation of the identified genes should be verified in whole plants, for example by comparing their expression patterns in wild-type plants and *lfy* mutants by *in situ* hybridizations.

An additional limitation of the system is that expression changes for genes that are repressed by endogenous LFY activity might not be detected because they are not expressed in callus. For example, repression of *AGL24* is an important step for the promotion of flower development (Yu *et al.*, 2004). However, while probes for *AGL24* were present on the microarray used in this study, we were not able to detect downregulation of this gene, suggesting that it may not be expressed in tissue culture.

In summary, we have described a floral induction system that reproduces many aspects of the transition to flowering observed in *Arabidopsis* and that can be used to study gene regulation downstream of LFY. As many important regulatory genes are expressed in a spatially and temporally restricted fashion, the tissue culture approach described here might be useful beyond the analysis of LFY function.

Experimental procedures

Plant growth and tissue culture

Arabidopsis seedlings were surface sterilized with 50% bleach, 0.1% Triton X-100 for 10 min, washed with sterile water (five times), and plated on 0.5× MS medium. Seedlings were grown in continuous light (100 μmol m⁻² sec⁻¹) for 3 weeks. Plates were inverted, and roots were collected for growth on CIM. Root fragments were cut to 1 cm length prior to placement on CIM plates. Growth on CIM was allowed for 7 days. After 7 days, root explants were transferred to SIM. To regenerate shoots or flowers, plants were transferred to fresh SIM plates every 3 weeks. Outgrowths were usually observed by week 4 on SIM.

Dexamethasone treatment of roots explants/seedlings

For 7-day treatments (Figures 1 and 2), root explants were submerged in 5 μM dexamethasone while on CIM. The dexamethasone solution was substituted with a fresh dexamethasone solution daily. Seedlings were submerged in 5 μM dexamethasone, 0.1% ethanol for 7 days on 0.5× MS plates as described above for root explants. On the last day of the treatment, root explants or seedlings were washed with sterile water and transferred to fresh SIM or 0.5× MS plates, respectively.

Shorter dexamethasone treatments were initiated such that the treatment was finished at the same time (5 PM) on day 7 on CIM. At this stage, explants were frozen in liquid nitrogen for RT-PCR analyses or for microarray analysis. For GUS assays, tissue was fixed in 90% ice-cold acetone. GUS staining was performed as described previously by Sieburth and Meyerowitz (1997). Cycloheximide was used in a final concentration of 10 μM in 0.1% ethanol.

RNA isolation, reverse transcription, and PCR

Total RNA used for microarray experiments was isolated using the RNeasy RNA Isolation Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA used for RT-PCR was isolated using TRI reagent (Molecular Research Center Inc., Cincinnati, OH, USA). Up to 100 μg of RNA for each sample was further purified using RNeasy columns (Qiagen) and eluted in a final volume of 50 μl. Two micrograms of RNA was reverse transcribed in a 20-μl reaction using the Thermoscript kit (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions. Up to 0.8 μl of the cDNA was used in a 25-μl PCR with Platinum TAQ polymerase (Invitrogen). PCR (94°C, 3 min; followed by cycles of 94°C, 30 sec; 56°C, 30 sec; 72°C, 1 min; followed by 72°C, 4 min) was performed for 24–32 cycles, depending on target gene expression levels. Optimal cycle number was determined empirically to be in the linear range for each target gene. Primer sequences are listed in Supplementary Material.

Probe labeling and microarray hybridization protocols

Probe labeling and hybridization protocols were described previously by Wellmer *et al.* (2004). In brief, first- and second-strand cDNA was synthesized from 3 μg of total RNA using a poly(A) primer with a T7 promoter sequence. *In vitro* transcription was performed using the Megascript T7 kit (Ambion, Austin, TX, USA) and aminoallyl-UTP. Dyes were covalently linked to the amplified RNA, and the labeled RNA was fragmented. The fragments were then hybridized to the array. For replicate experiments, the dyes used for labeling RNA derived from dexamethasone or mock-treated samples were switched to avoid dye-related artifacts.

Data acquisition and statistical analysis

Analysis of microarray data was performed as follows. Scanning of slides and quantitation of signal intensities was performed as described by Wellmer *et al.* (2004), using an Axon GenePix 4000 A scanner and the associated GENEPIX PRO 4.1 software (Axon Instruments, Union City, CA, USA). The GenePix.gpr files were processed in the Rosetta Resolver Gene Expression Data Analysis System (Rosetta Biosoftware, Kirkland, WA, USA). Processing in this system consists of error correction and calculation of a *P*-value of differential expression using the intensity error estimation from the .gpr file. Error correction consists of a simplified version of the algorithm described by Schadt *et al.* (2001), in which a piece-wise

linear function replaces smoothing splines to fit and correct intensity non-linearity. Calculation of *P*-values consists of a statistic that combines additive and multiplicative error components in both channels of a two-colored experiment. The resultant ratio profiles (that is, two-channel, error-processed microarray scans) were combined into ratio experiments in the Resolver system as described by Stoughton and Dai (2002). Within the resolver system, we performed the analysis at the so-called sequence level, i.e. when there are multiple datapoints for the same gene in the same hybridization (as a result of reporter or probe sequences from that gene appearing more than once on the microarray; see Wellmer *et al.* (2004)), such data are combined. When combining data, it is assumed that the ratio measurement with the lowest overall error is closest to the true value for that sequence, and weights are constructed such that the feature or reporter with the lowest error is given the greatest weight (Stoughton and Dai, 2002).

Upon request, all novel materials described in this publication will be made available in a timely manner for non-commercial research purposes.

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Supplementary Material

The following material is available from <http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2127/TPJ2127.sm.htm>

Table S1 Results of clustering. Genes included in the cluster diagram (Figure 4)

Table S2 Gene ontologies. Gene ontology annotations of the identified genes. Annotations were obtained with the Gene Ontology annotation search tool at TAIR (<http://www.arabidopsis.org/tools/bulk/go/>)

Table S3 Primers. Primers used for RT-PCR. Sequences of primers are given

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Microarray data have been deposited at the Gene Expression Omnibus data repository at NCBI (<http://www.ncbi.nlm.nih.gov/geo/>) under the Accession number GSE1411.