A Mechanistic Link between *STM* and *CUC1* during Arabidopsis Development[1][C][W][OA]

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The *KNOXI* transcription factor *SHOOT MERISTEMLESS* (*STM*) is required to establish and maintain the Arabidopsis (*Arabidopsis thaliana*) apical meristem, yet little is known about its direct targets. Using different approaches we demonstrate that the induction of *STM* causes a significant up-regulation of the organ boundary gene *CUP SHAPED COTYLEDON1* (*CUC1*), which is specific and independent of other meristem regulators. We further show that the regulation of *CUC1* by STM is direct and identify putative binding sites in its promoter. Continuous expression of *STM* in Arabidopsis leaf primordia also causes the activation of *CUC2-3*, as well as microRNA *MIR164a*, which provides a negative feedback loop by posttranscriptionally regulating *CUC1* and *CUC2*. The results bring new insights into the mechanistic links between *KNOXI* and *CUC* transcription factors and contribute to the understanding of the regulatory network controlled by *STM*.

In contrast to animals, plants continue to produce new organs throughout their life cycle. All aboveground parts of plants are derived from a small number of stem cells located at the shoot apical meristem. Two homeodomain transcription factors play key roles in meristem formation and maintenance. *WUSCHEL* (*WUS*) is expressed at the core of the meristem and defines the stem cell niche in the overlaying cells (Tucker and Laux, 2007), while *SHOOT MERISTEMLESS* (*STM*) is expressed throughout the meristem and prevents the differentiation of the meristematic cells (for review, see Hake et al., 2004; Hamant and Pautot, 2010; Hay and Tsiantis, 2010).

STM belongs to class I of *KNOX* homeodomain transcription factors. In Arabidopsis (*Arabidopsis thaliana*), the *KNOXI* subclass comprises *STM*, *KNAT1* (also called *BREVIPEDICELLUS*), *KNAT2*, and *KNAT6*, which can have partially overlapping functions in the shoot meristem (Scofield and Murray, 2006). KNOXI proteins interact with BELL1-like homedomain transcription factors, and these interactions determine their target affinity and subcellular localization (for review, see Hake et al., 2004; Hay and Tsiantis, 2010).

At least part of the functions of *WUS* and *STM* are performed through the control of hormone homeostasis and signaling. WUS directly represses a group of type-A *ARABIDOPSIS RESPONSE REGULATORs* involved in a negative feedback loop during cytokinin response (Leibfried et al., 2005; Busch et al., 2010). *STM* expression induces *ISOPENTENYL TRANSFERASE7* (*IPT7*), which encodes a key enzyme involved in cytokinin biosynthesis (Jasinski et al., 2005; Yanai et al., 2005). Conversely, ectopic *IPT* expression or exogenous cytokinin can partially rescue weak *stm* mutants (Jasinski et al., 2005; Yanai et al., 2005). *STM* also represses gibberellin activity by reducing the levels of the biosynthetic enzyme *GA 20-oxidase1* and increasing the levels of the catabolic enzyme *GA 2-oxidase1*, thus providing an environment of high cytokinin and low gibberellin (Sakamoto et al., 2001; Hay et al., 2002; Chen et al., 2004; Jasinski et al., 2005; Yanai et al., 2005).

The boundaries of the meristem are defined by members of the NAC family of transcription factors (Aida and Tasaka, 2006). In Arabidopsis, this function is redundantly performed by *CUP SHAPED COTYLEDON1* (*CUC1*), *CUC2*, and *CUC3* (Aida et al., 1997, 1999; Vroemen et al., 2003; Hibara et al., 2006). *CUC1* and *CUC2* are also posttranscriptionally regulated by microRNA (miRNA) miR164, which is encoded by a small gene family comprising three members, *MIR164a-c* (Laufs et al., 2004; Mallory et al., 2004; Baker et al., 2005; Nikovics et al., 2006; Sieber et al., 2007; Raman et al., 2008).

In Arabidopsis, *CUC* genes are required for the activation of *STM* during embryogenesis (Long et al., 1996; Aida et al., 1999), and it has been proposed that

[1] This work was supported by Agencia Nacional de Promoción Científica y Tecnlógica (Argentina), Human Frontiers Science Program, and Howard Hughes Medical Institute (grants to J.F.P.).

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www.plantphysiol.org/cgi/doi/10.1104/pp.111.177709

STM can in turn activate *CUC* expression (Aida et al., 1999, 2002; Takada et al., 2001; Kwon et al., 2006). Mutations in *STM* or two *CUC* genes compromise the population of self-renewing stem cells and cause fusions of the cotyledons. *KNOX* and *CUC* genes are recruited again at later stages of Arabidopsis development and are required for carpel and ovule development (Ishida et al., 2000; Pautot et al., 2001; Scofield et al., 2007). In many species, *KNOXI* and *CUC* genes are expressed in the leaf primordia and act in concert to sculpt the organ shape and generate compound leaves (Bharathan et al., 2002; Blein et al., 2008; Berger et al., 2009).

CUC1 and *CUC2* share a common ancestor, but have diverged significantly within the Brassicaceae (Hasson et al., 2011). While both of them are required for organ separation, specialization has been acquired for certain functions, such as the control of the serrations of an Arabidopsis simple leaf, which is regulated by the balance between *CUC2* and *MIR164a* genes (Nikovics et al., 2006; Hasson et al., 2010). *STM* expression also diverges within the Brassicaceae. While it is confined to the meristem in Arabidopsis, closely related species express *STM* in the leaf primordia and have more complex organs (Piazza et al., 2010).

Although the biological roles of the versatile developmental regulator *STM* are well characterized, little is known about its direct targets. In an attempt to bring insights into the network regulated by *STM*, we performed microarray experiments shortly after the induction of the transcription factor. We found that STM activates *CUC1*, and demonstrated that it directly binds to its promoter. Additionally, the long-lasting expression of *STM* also promotes the expression of *CUC2-3*, and *MIR164a*, which provides a negative feedback loop to adjust the final CUC level. These results provide new mechanistic insights into the regulatory network comprised by *KNOXI* and *CUC* transcription factors and miRNA miR164.

RESULTS

Genome-Wide Response to *STM* Levels

To start to explore the network controlled by *STM*, we analyzed the transcriptome of plants harboring an ethanol-inducible version of the transcription factor, an approach already used to identify targets of *WUS* (Leibfried et al., 2005). The selected transgenic plants did not show any obvious phenotypes when grown under normal conditions. However, one single treatment with ethanol was sufficient to cause leaf lobing, as expected for the ectopic expression of STM (Fig. 1A). These morphological changes were obvious 1 week after the induction (Fig. 1A).

It is known that KNOXI transcription factors interact with other proteins that regulate their activity (for review, see Hake et al., 2004; Hay and Tsiantis, 2010). Therefore, we generated an activated version of STM by preparing transgenic plants where the transcription factor is fused to the transactivation domain from the herpes simplex virus VP16. This strategy has been previously used in plants to detect transcription factor activity independently of the presence of coactivators

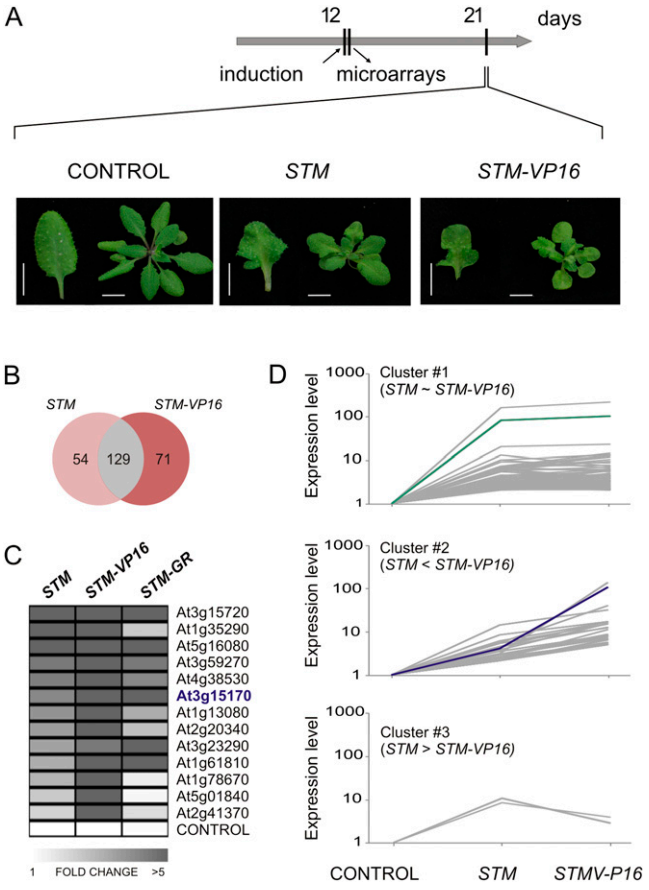

Figure 1. Genome-wide response to *STM* levels. A, Phenotype of *STM* and *STM-VP16* inducible lines 9 d after treatment with 0.6% ethanol. Samples for microarray experiments were collected 12 h after ethanol induction. The transgenic line used as control expresses *GUS* under the ethanol inducible promoter. Bars, 1 cm. B, Venn diagram showing the overlap of *STM* and *STM-VP16* up-regulated genes. C, Heat map representing relative expression levels in grayscale of 13 genes in three *STM* inducible systems: ethanol inducible *STM* and *STM-VP16*, and *STM-GR*. The genes were selected from those induced in STM and STM-VP16 (Fig. 1B). *CUC1* (At3g15170) is depicted in blue. The data shown are mean of two biological replicates ± SEM for microarray data (*STM* and *STM-VP16*) and three biological replicates ± SEM for RT-qPCR experiments in the case of *STM-GR* (see Supplemental Fig. S1 for details). As a control, we used the constitutively expressed gene *PROTEIN PHOSPHATASE2A*. D, The 129 genes induced by both *STM* and *STM-VP16* were classified in three clusters according to their relative expression levels in the Affimetrix microarrays. Cluster number 1 contains genes with similar levels in *STM* and *STM-VP16* transgenic plants (101 genes); cluster number 2 has genes with higher expression in *STM-VP16* than in STM (25 genes); cluster number 3 contains genes with higher expression in *STM* than in *STM-VP16* (three genes) using the criteria described in the text to select differentially expressed genes (logit-T 0.05; 2-fold change with GCRMA). *STM* is highlighted in green and *CUC1* in blue.

(e.g. Parcy et al., 1998). Treatment with ethanol of plants harboring an inducible *STM-VP16* transgene caused a higher degree of leaf lobing than that observed for *STM* alone (Fig. 1A).

We performed a transcriptome analysis on ATH1 microarrays, 12 h after the induction of *STM* and *STM-VP16*. For the profiling experiments we used the shoot apex that includes the meristem and developing leaves, as has been described previously (Leibfried et al., 2005). Genes that showed per-gene variance $P >$ 0.05 (logit-T; Lemon et al., 2003) and more than 2-fold change (GeneChip Robust Multiarray Averaging [GCRMA]; Irizarry et al., 2003) compared to control plants were considered as differentially expressed and were selected for further studies (Supplemental Tables S1 and S2).

Analysis of the *STM*-modified genes using Gene Ontology term enrichment revealed that there were no strong overrepresented functional categories among them (Supplemental Table S3). We observed, however, that *At1g50960*, which encodes a GA 2-oxidase7 involved in the catabolism of gibberellin, was significantly induced by both STM and STM-VP16 (Supplemental Tables S1 and S2). *IPT7*, which participates in cytokinin biosynthesis, was induced nearly three times by STM, although it did not pass the logit-T filter used for the analysis of the arrays (not shown). These observations are in agreement with previous results showing that STM increases cytokinin levels while reducing the gibberellins (Sakamoto et al., 2001; Hay et al., 2002; Jasinski et al., 2005; Yanai et al., 2005).

With the stringent selection criteria applied, 183 genes were induced by STM and 200 by STM-VP16 (Fig. 1B). Most of them (129 genes; Supplemental Table S4) were induced in both conditions. The higher activation capacity of STM-VP16, which is also correlated with the stronger leaf phenotypes observed, is likely responsible for the genes that are differentially expressed between *STM* and *STM-VP16* transgenic plants.

To validate our transcriptome analysis we turned to another inducible system where STM is fused to the glucocorticoid receptor (GR; Gallois et al., 2002). The STM-GR fusion protein is retained in the cytoplasm of transgenic plants, but moves into the nucleus once the cells are treated with dexamethasone (DEX). We selected 13 genes induced by both *STM* and *STM-VP16* (approximately 10% of the genes induced by both constructs) and tested their response to *STM-GR* ($Prom_{35S}$:*STM-GR* construct) by real-time quantitative PCR (RT-qPCR). We found that 10 out of 13 genes were also induced by this system after 12 h of DEX application (Fig. 1C; Supplemental Fig. S1; Supplemental Table S4). These results highlight at least a reasonable reproducibility of the microarray data.

Then, we decided to study in more detail the genes induced by both *STM* and *STM-VP16* (Fig. 1D). We classified these genes in three groups depending on their relative expression in *STM* and *STM-VP16* samples, using the criteria depicted above, variance $P >$ 0.05 (logit-T), and more than 2-fold change (GCRMA). Using this criteria, most genes (101 genes) were similarly induced by *STM* and *STM-VP16* (cluster no. 1), 25 genes were more expressed in *STM-VP16* than in *STM* (cluster no. 2), and only three genes were higher in *STM* than in *STM-VP16* (cluster no. 3).

As expected, *STM* was detected as significantly up-regulated in both samples. *STM* levels were, however, similar in *STM* and *STM-VP16* arrays (Fig. 1D, cluster no. 1), indicating that both plants express their transgenes at similar levels and differences between their transcriptomes are likely caused by the presence of the VP16 domain. That the VP16 activated version caused stronger phenotypic defects than STM alone, suggested that the group of genes moderately induced by STM but strongly by STM-VP16 (Fig. 1D, cluster no. 2; Supplemental Table S4) might be particularly related to the KNOXI pathway. *CUC1* (At3g15170) was included in this group and stood out as a particularly attractive candidate to study in more detail due to its known roles in the establishment of Arabidopsis meristem.

Specific Response of *CUC1* to *STM* Levels

The induction of *CUC1* by *STM* prompted us to study the effects on *CUC* expression of other transcription factors known to regulate the meristem function. First, we compared the induction of *CUC1* by *STM* with the ones caused by other meristem regulators such as *WUS* and *LFY* that were prepared as similar inducible versions (Leibfried et al., 2005). *STM* was able to induce *CUC1* levels 4-fold, while *STM-VP16* further enhanced the response to more than 100-fold in the microarray experiments (Fig. 2A). In contrast to *STM*, *LFY* and *WUS* were not able to induce *CUC1* (Fig. 2A). These transcription factors also failed to modify the levels of *CUC2* and *CUC3*, while *STM-VP16* caused a moderate up-regulation of *CUC3* (Fig. 2A).

We then tested the specificity of *CUC1* induction inside the *KNOXI* family of transcription factors, which comprises *STM*, *KNAT1*, *KNAT2*, and *KNAT6*, being *KNAT1* the more closely related to STM, as judged by phylogenetic analyses (Scofield and Murray, 2006). To test whether other *KNOXI* genes could activate *CUC1* expression we prepared ethanol-inducible transgenic lines harboring *KNAT1* and *KNAT2*. In contrast to *STM*, these other *KNOXI* genes failed to up-regulate *CUC1* (Fig. 2B). These results indicate that there is at least certain degree of specificity for its induction in planta.

We then prepared an activated version of KNAT1, by fusing to it the VP16 domain. In this case, we observed that the induction of *KNAT1-VP16* caused the activation of *CUC1* (Fig. 2B). It is known that KNOXI proteins can interact with different partners (for review, see Hake et al., 2004; Hay and Tsiantis, 2010). The enhanced activity of the VP16 fusions might indicate that other factors could be required in

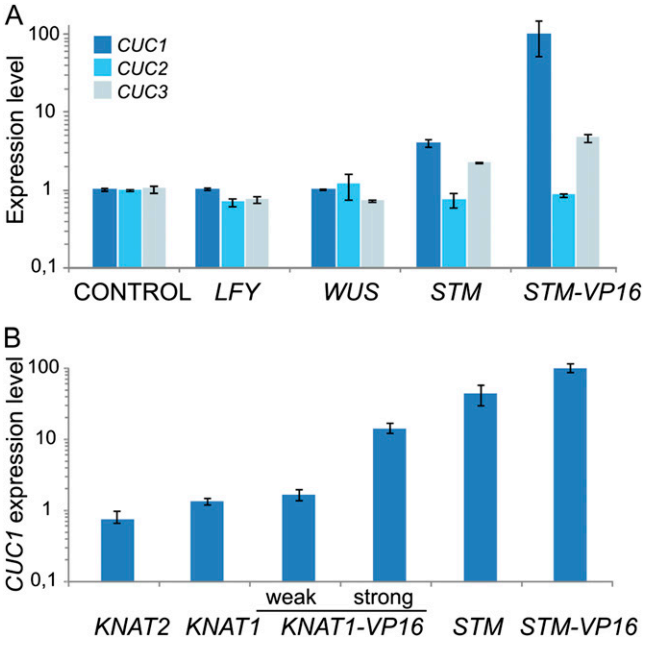

Figure 2. Specific response of *CUC1* to *STM* levels. A, Expression levels of *CUC1-3* after the induction of *LFY*, *WUS*, *STM*, and *STM-VP16* from Affimetrix microarrays (GCRMA normalization). Expression levels were normalized to plants carrying inducible *GUS* grown in the same conditions, which were used as a control. The data shown are mean of two biological replicates ± SEM. B, Regulation of *CUC1* expression by Arabidopsis *KNOXI* genes. The level of *CUC1* was determined in transgenic lines harboring an inducible version of *KNAT1*, *KNAT1-VP16*, *KNAT2*, *STM*, and *STM-VP16*, 12 h after ethanol treatment. *CUC1* levels were determined by RT-qPCR and expressed relative to plants expressing inducible *GUS* (Control). The data shown are mean of three biological replicates ± SEM. [See online article for color version of this figure.]

vivo for a maximum activation of *CUC1* by KNOXI proteins.

Direct Regulation of *CUC1* by STM

To test whether STM was directly regulating the expression of *CUC1*, we turned again to the *STM-GR* system. Direct targets of STM-GR should be induced after DEX treatment, even in the presence of the translational inhibitor, cycloheximide (CYC).

First, we crossed *STM-GR* transgenics to a *CUC1* reporter line ($Prom_{CUC1}$:*GUS*). We used 1.4-kb *CUC1* upstream sequences that have already been described to be sufficient to complement a *cuc1 cuc2* mutant when fused to its coding sequence (Baker et al., 2005). The *CUC1* promoter is normally expressed in the apical region, but DEX treatment during 24 h caused a strong induction in whole seedlings (Fig. 3A). As a control of the experimental approach, we observed that the supplemental addition of CYC largely prevented the burst of GUS protein activity, which is expected from the inhibition of the translational machinery (Fig. 3A). Note that these experiments were carried out under long induction and staining periods to ensure the saturation of the system.

We then analyzed the expression of *CUC* genes at the RNA level. As *CUC1* is posttranscriptionally regulated by miR164 in a quantitative way (Baker et al., 2005; Nikovics et al., 2006; Sieber et al., 2007), the potential induction caused by STM should overcome this repression to be detectable. We observed that 4 h of induction of *STM-GR* caused the up-regulation of *CUC1* (Fig. 3B). *CUC3* was also activated, but after 24 h. Supplemental addition of CYC prevented the induction of *CUC3*, while *CUC1* remained unaffected, confirming *CUC1* as a direct target of STM (Fig. 3B). We observed an effect on *CUC2* only 96 h after DEX treatment (Fig. 3B). The longer activation time, which is prevented by incubation with CYC suggests that both *CUC2* and *CUC3* are indirectly regulated by STM. That *CUC2* and *CUC3* lack obvious STM binding sites in their promoters is in agreement with this possibility.

In Vitro Binding of STM to the *CUC1* Promoter

Next, we searched for potential STM regulatory motifs by analyzing the promoters of genes up-regulated in the microarray data, as described previously (Schommer et al., 2008). We only found a potential candidate box when we analyzed genes induced at least 5-fold by STM-VP16, GTCACT (P = 0.06; Supplemental Table S5). Even though the enrichment of this site was not particularly high, it suggestively overlapped with the preferred binding site of STM, which has already been investigated in vitro and was found to be CTGTCA (Krusell et al., 1997; Smith et al., 2002; Viola and Gonzalez, 2006). These sequences share the minimal sequence recognized by KNOX homeodomains, a GTCA core (for review, see Hake et al., 2004).

Interestingly, both 6-mer sequences are present in the *CUC1* promoter in a narrow region at −135 (box1, CTGTCA and GTCACT) and −124 (box2, CTGTCA; Fig. 4A), which prompted us to perform a more detailed study. We tested whether a recombinant STM protein could recognize the *CUC1* promoter in vitro. Electrophoretic mobility shift assays (EMSAs) showed a strong and specific interaction between a promoter fragment and the STM homeodomain alone or the complete recombinant transcription factor (Fig. 4, B and C). Binding was competed by a 50-fold molar excess of the same unlabeled fragment but not by a similar amount of a different fragment, thus showing the specificity of the interaction (Supplemental Fig. S2). Mutating box1 caused a significant decrease in the binding efficiency and a further mutation in box2 almost completely abolished the interaction between the *CUC1* promoter and STM in vitro (Fig. 4, B and C).

We also analyzed the interaction between STM and the *CUC1* promoter in a yeast (*Saccharomyces cerevisiae*) one-hybrid assay. *STM* directed *CUC1* expression also in this system (Fig. 4D), expression which was lost when the putative binding box1 was mutated. In summary, these results confirmed that STM directly

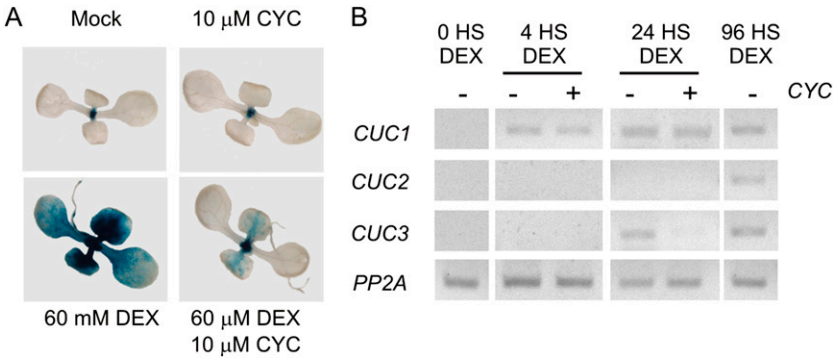

Figure 3. Direct regulation of *CUC1* by STM. A, GUS expression in seedlings of transgenic plants expressing Prom$_{CUC1}$:*GUS* crossed to *STM-GR* after 24 h treatment with or without DEX and CYC. B, *CUC1-3* expression levels determined by RT-PCR in *STM-GR* transgenics treated with or without DEX and CYC. The data shown is representative of at least three biological replicates. [See online article for color version of this figure.]

regulates *CUC1*, likely through these two specific binding boxes, and provide a mechanistic scheme for the regulation of these transcription factors.

CUC1 Expression in Plants

We then analyzed the expression of *CUC1* in the strong *stm-1* mutant. As expected, we found that the levels of *CUC1* were reduced in this mutant (Fig. 5A). We also crossed the Prom$_{CUC1}$:*GUS* plants to the weak *stm* allele *bum1-3* and found a reduction in the reporter expression in flowers (Fig. 5B). We tried to rescue the *stm-1* mutant by overexpressing a miR164-resistant version of *CUC1*. However, the expression of *CUC1* alone was not sufficient to complement the STM deficiency (not shown), as has been seen before when overexpressing a wild-type version of *CUC1* in *stm-1* mutants (Hibara et al., 2003).

To study the role of the STM-binding sites on *CUC1* transcription, we turned to reporters. Previously described transcriptional reporters for *CUC1* and *CUC2* are expressed in a broader domain inside the meristem while in situ hybridization assays have shown that *CUC* RNA accumulates in the boundaries (Nikovics et al., 2006; Sieber et al., 2007; Raman et al., 2008). This is at least partially achieved by the posttranscriptional repression carried out by miR164 (Sieber et al., 2007).

We analyzed the transcription of a wild-type reporter and two mutated versions where one or both STM-binding sites were removed. Mutations in the putative STM-binding sites quantitatively decreased its expression levels during vegetative development more than 2-fold by assaying seven independent transgenic lines for each construct (Fig. 5, C–E).

The expression of the mutated reporter was reduced 4-fold in inflorescences (Fig. 5C). Whole-mount stainings showed that the wild-type reporter was expressed at the base of the flowers and in the carpels (Fig. 5F), while the mutated version showed a significantly reduced staining in the carpels (Fig. 5G).

We then down-regulated *CUC1* and other miR164 targets by expressing *MIR164b* from different promoters. Expression of *MIR164b* from the *CUC1* promoter (Prom$_{CUC1}$:*MIR164b*) caused cotyledon fusions (20 out of 48 T1 plants; Fig. 6A) and most of them had severe stem-cauline leaf (Fig. 6B) and sepal fusions (Fig. 6C). In contrast, expression of *MIR164b* from the mutated *CUC1* promoter did not cause any cotyledon fusions and the defects during reproductive

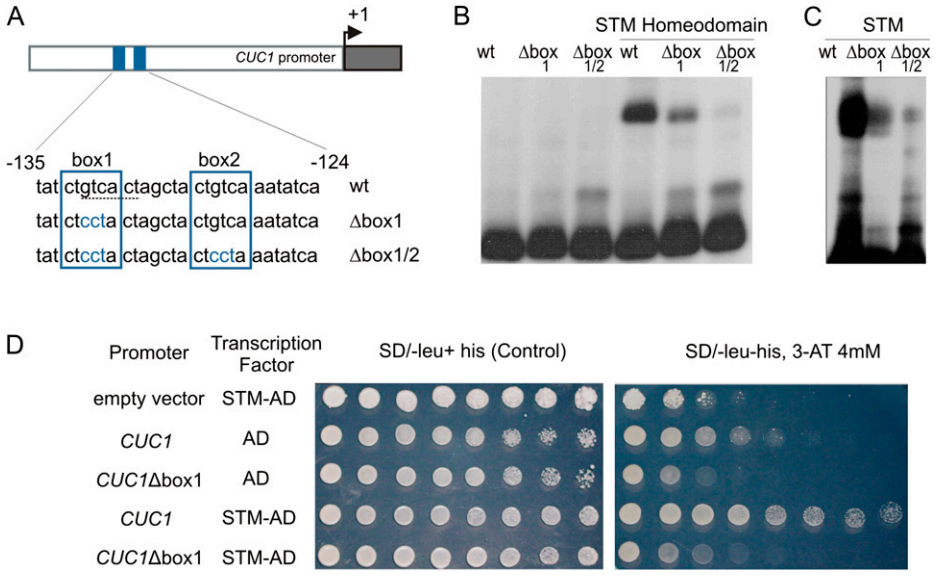

Figure 4. In vitro binding of STM to the *CUC1* promoter. A, Scheme representing the *CUC1* promoter. Putative STM-binding sites identified by SELEX are highlighted with blue squares and the sequence identified to be overrepresented in *STM-VP16* induced genes (Supplemental Table S5) is indicated with a dashed line. B and C, EMSA with *CUC1* promoter using recombinant STM homeodomain (B) or whole protein (C). D, One-hybrid experiment in yeast using wild-type and mutated *CUC1* promoter. Growth in the absence of His due to activation of the *HIS3* gene under the control of the *CUC1* promoter was monitored using serial dilutions of the corresponding yeast strains. 3AT, 3-Amino-1,2,4-triazole. [See online article for color version of this figure.]

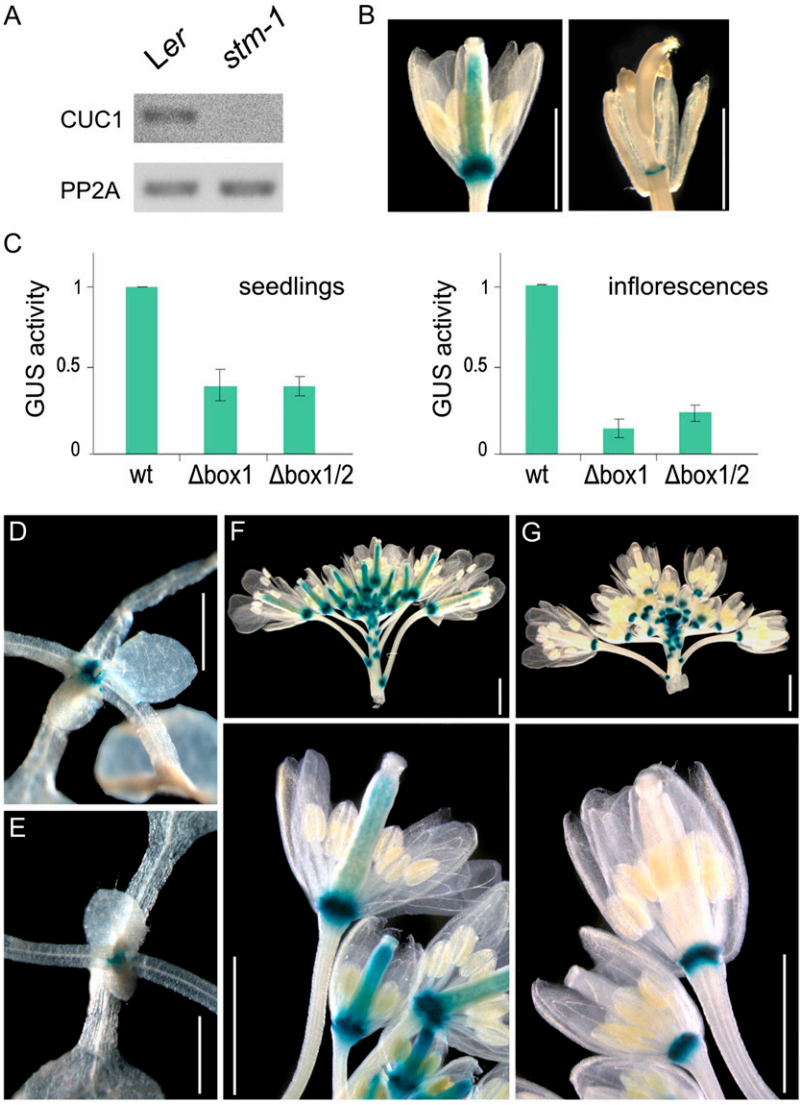

Figure 5. *CUC1* expression in plants. A, *CUC1* expression levels determined by RT-PCR in apices of wild-type (*Ler*) and *stm-1* plants grown for 6 d in Murashige and Skoog media. The data shown is representative of at least three biological replicates. B, *CUC1* reporter in a developing wild-type (left) or *bum1-3* (right) flower. C, GUS activity of transgenic plants expressing wild-type and mutant *CUC1* reporters in 12-d-old seedlings and inflorescences. The values correspond to the average of seven independent lines for each promoter version ± SEM. D to G, GUS expression in seedlings (D and E) and inflorescences (F and G) of transgenic plants harboring the wild-type (D and F) and mutant (Δbox1/2; E and G) *CUC1* reporter. Bars, 10 mm.

development were weaker (Fig. 6, A–C). Additionally, expression of *MIR164b* from a *STM* promoter ($Prom_{STM}$:*MIR164b*) also caused organ fusions (Fig. 6, D–F; see Supplemental Fig. S3 for $Prom_{STM}$:*GUS* stainings).

These developmental defects are similar to some of the phenotypes observed in $Prom_{35S}$:*MIR164* plants (Laufs et al., 2004; Mallory et al., 2004), but still highlight the importance of the STM-binding sites on the quantitative regulation of *CUC1* and the importance of CUC activity inside the STM domain. We also tried to complement the *cuc1/cuc2* double mutant with $Prom_{CUC1}$:*CUC1* and $Prom_{CUC1\Delta box}$:*CUC1* constructs. Unfortunately, the transgenes were silenced in the mutant background.

A Feedback Regulatory Loop Mediated by *MIR164a*

In many plant species with compound leaves, *KNOXI* transcription factors are expressed in the leaf primordia (Hareven et al., 1996; Bharathan et al., 2002; Hay and Tsiantis, 2006) where they interact with *CUC* genes (Blein et al., 2008). Therefore, we prepared Arabidopsis plants expressing *STM* from the *LEAFY* promoter ($Prom_{LFY}$:*STM*), which is active in the primordia of leaves and flowers (Blázquez and Weigel, 2000), and studied the effects on *CUC* activity.

$Prom_{LFY}$:*STM* transgenics had lobed leaves as expected from ectopic expression of a *KNOXI* gene in leaf primordia (Fig. 7A). Analysis of *CUC1* and *CUC2* reporters in $Prom_{LFY}$:*STM* plants revealed that they were both ectopically expressed in young leaves, especially at the base of leaf lobes (Fig. 7B; Supplemental Fig. S4) resembling the ectopic pattern of expression of *STM* itself (Fig. 7A, right). These plants have constitutively altered levels of *STM*, so the induction of *CUC2* is likely an indirect effect of STM as we have observed after 96 h of DEX treatment of STM-GR plants (Fig. 3B).

MIR164a has previously been implicated in the regulation of CUC activity during Arabidopsis leaf development (Nikovics et al., 2006; Hasson et al., 2010), so we

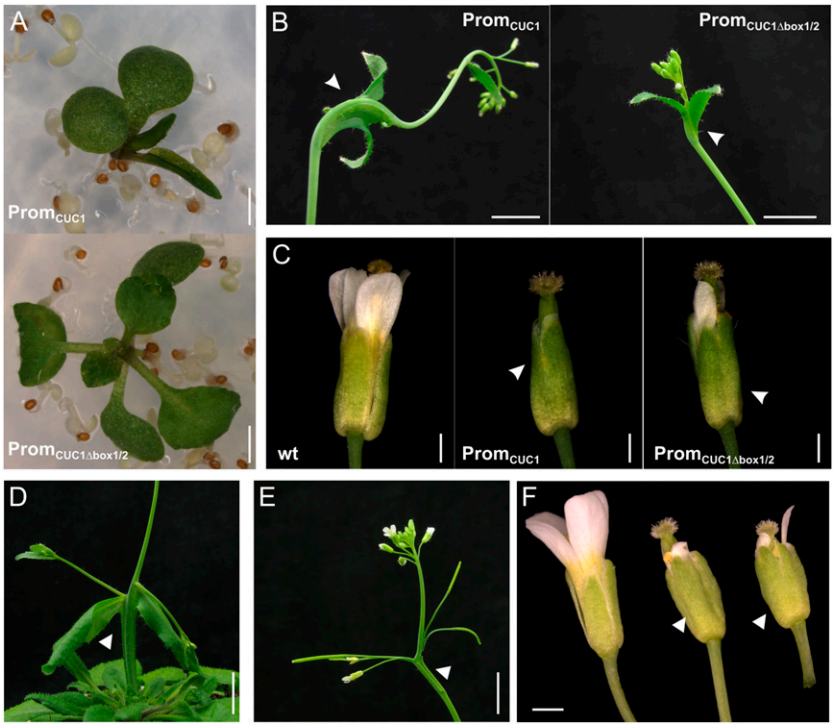

Figure 6. Misexpression of *MIR164b* using different promoters. A to C, Phenotypes of plants expressing *MIR164b* from a *CUC1* and *CUC1* Δ*box1/2* promoter. D to F, Phenotypes of Prom$_{STM}$:*MIR164b* transgenic plants. F, Wild-type (left) and Prom$_{STM}$:*MIR164b* flowers. Bars, 10 mm (A, C, F) and 1 cm (B, D, E). Organ fusions are indicated with arrowheads.

crossed Prom$_{LFY}$:*STM* plants to a *MIR164a* reporter. We found that *MIR164a* was also activated by *STM*, in a similar way to the *CUC* reporters (Fig. 7B; Supplemental Fig. S4).

To validate these results we performed sections of wild-type and Prom$_{LFY}$:*STM* developing leaves. We then determined the levels of *CUC* genes and miR164 by RT-PCR in the proximal and distal region of the organ. The levels of *CUC1-3* as well as miR164 were increased in the proximal part of the organ, as expected from the whole-mount staining (Fig. 7, C and D). We also determined the levels of the precursor of *MIR164a* and found that it was also activated, demonstrating its increased transcription is at least partially responsible for the elevated miR164 levels (Fig. 7D).

We then tested the short-term response of miR164 to STM levels. We measured miR164 12 h after the induction of STM (Fig. 7E). However, in this case we did not observe an obvious change in the levels of the miRNA.

These results suggest that *MIR164a* operates in a negative feedback loop to adjust the final *CUC* levels. The lack of change in miR164 levels when *STM* is transiently induced suggests that the activation of *MIR164a* is an indirect modification caused by *STM* and the consequence of the long-lasting expression of the *KNOXI* transcription factor in the leaf primordia.

We have also performed crosses between Prom$_{LFY}$:*STM* and *cuc1-1* mutants and observed that the plants still have lobes (Supplemental Fig. S5), which is in good agreement with the ability of Prom$_{LFY}$:*STM* to activate *CUC1*, *CUC2*, and *CUC3* in leaf primordia (Fig. 7C). These results also indicate that the activation of *CUC2* and *CUC3* by STM is independent of *CUC1*.

DISCUSSION

Targets of the KNOXI Transcription Factors

The class I of KNOX family of transcription factors comprises a small family of TALE homeobox genes that are widely distributed in plants. They regulate diverse developmental processes throughout the Arabidopsis life cycle. *KNOXI* transcription factors maintain the activity of the meristem, the boundaries between the stem and the meristem, and diverse aspects of flower development and leaf morphology (for review, see Hake et al., 2004; Hamant and Pautot, 2010; Hay and Tsiantis, 2010).

Despite their central role as developmental regulators, few downstream effectors of KNOXI activities are currently known. They regulate the levels of cytokinins and gibberelins (Sakamoto et al., 2001; Hay et al., 2002; Chen et al., 2004; Jasinski et al., 2005; Yanai et al., 2005) and the deposition of lignin (Mele et al., 2003). Still, precise mechanistic insights into KNOXI action are lacking in most cases. Here, we identified a direct target of STM in Arabidopsis. We show that STM directly activates the organ-boundary gene *CUC1* and characterize the process at the biochemical level.

Genetic analyses revealed that *KNOX* genes conforms a complex network exhibiting both overlapping and antagonistic activities (Hamant and Pautot, 2010).

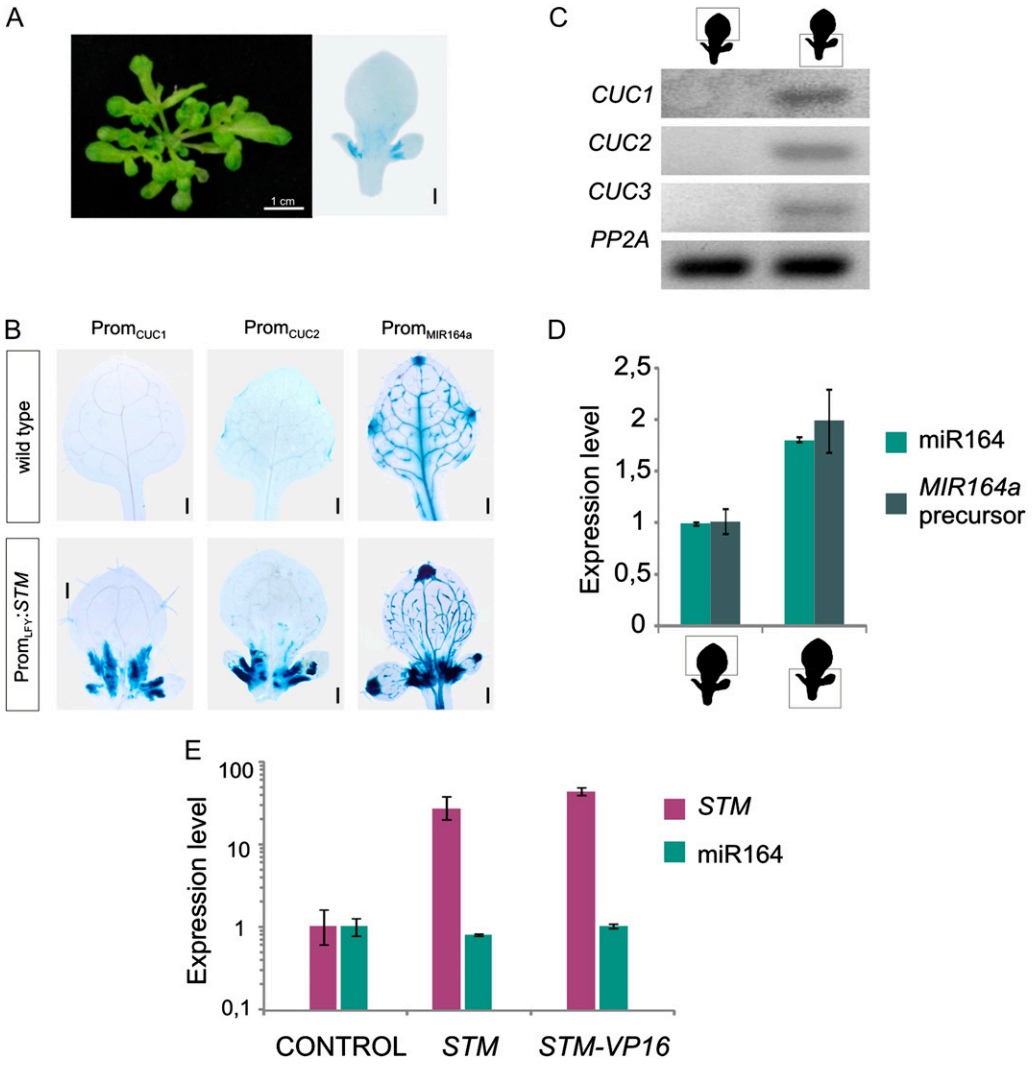

Figure 7. *CUC* and *MIR164* genes expression pattern in Prom$_{LFY}$:*STM* plants. A, Altered leaf shape of plants expressing *STM* under the control of the *LFY* promoter, Prom$_{LFY}$:*STM*. Right section: Prom$_{LFY}$:*STM* crossed to Prom$_{LFY}$:*GUS*, which indicates the domain of expression of *STM* in these transgenics. B, Expression of *CUC1*, *CUC2*, and *MIR164a* reporters in wild-type and Prom$_{LFY}$:*STM* plants. C, *CUC1-3* expression levels determined by RT-PCR in the proximal and distal parts of leaves 3 and 4 of Prom$_{LFY}$:*STM* plants. The data shown is representative of at least three biological replicates. D, miR164 and *MIR164a* precursor expression levels determined by RT-qPCR in Prom$_{LFY}$:*STM* leaves dissected as in C. The data shown are mean of three biological replicates ± SEM. E, Expression levels of miR164 determined by RT-qPCR after 12 h of induction of *STM* and *STM-VP16* with ethanol. Expression levels were normalized as described in Figure 1. The data shown are mean of three biological replicates ± SEM. Bars, 2 mm unless otherwise noted.

To add further complexity to the network, a recently discovered KNOX protein lacking the DNA-binding domain competes for interacting factors (Magnani and Hake, 2008). Our results indicate that the induction of *CUC1* is relatively specific to *STM* levels, although the closely related *KNAT1* could also rapidly induce *CUC1* when fused to the VP16 domain.

KNOXI transcription factors form complexes with BELL1-like proteins (for review, see Hake et al., 2004; Hay and Tsiantis, 2010). Our results cannot rule out that the activation of *CUC1* by STM requires additional factors in vivo, or that the STM-binding sites are also recognized by other TALE transcription factors in certain tissues. Actually, the higher activation capacity of STM and KNAT1 on *CUC1* levels conferred by the addition of VP16 might suggest that additional factors could be involved in plants.

It has been suggested that KNOXI transcription factors might exert their functions through the regulation of a few targets at least in certain situations (Hay and Tsiantis, 2010), as it has been shown for animal homeodomain transcription factors (Lovegrove et al., 2006). Genome-wide experiments will likely be required to understand the complex *KNOX* networks.

The transcriptome analysis that we have performed here might also aid in the identification of other *STM* regulated genes.

The *KNOXI-CUC* Regulatory Network

It has been shown that both *KNOXI* and *CUC* systems reinforce each other, and that the ectopic expression of *CUC1* induces *STM* (Aida et al., 2002; Hibara et al., 2003; Furutani et al., 2004; Kwon et al., 2006; Blein et al., 2008). The results obtained showing that STM directly induces *CUC1*, while indirectly activates *CUC2-3* and *MIR164a* provide further insights into this regulatory network.

That STM can directly activate *CUC1*, while indirectly affecting *CUC2-3* indicates that the *CUC* genes respond to different signals. Previous evidence has already shown that *CUC* genes can be regulated independently of each other by different factors. For instance, *CUC1-2* are differentially affected by *PIN1* (Aida et al., 2002), *CUC1-2* are targets of miR164 but not *CUC3* (Laufs et al., 2004; Mallory et al., 2004), and only *CUC2* is regulated by *SPLAYED* (Kwon et al., 2006). The regulation of redundant factors by different pathways might confer robustness to a biological process, such as the formation of the meristem.

The activation of *MIR164a* by *STM* that we observed, which is likely indirect, might also contribute to fine tune the levels of *CUC* expression as a part of a negative feedback loop. A general homeostatic function has been already proposed for miR159 as its targets, the GA-MYB transcription factors, might activate the expression of the miRNA (Achard et al., 2004).

KNOXI and *CUC* genes are also versatile developmental regulators whose functions go beyond the establishment and maintenance of the meristem. They are recruited during carpel and ovule development in Arabidopsis (Ishida et al., 2000; Pautot et al., 2001; Scofield et al., 2007) and the formation of complex leaves in many species (Bharathan et al., 2002; Blein et al., 2008; Berger et al., 2009). Specific relationships between *KNOXI*, *CUC*, and *MIR164* family members can be established during particular biological processes. The specific function of *MIR164c* in the regulation of petal number (Baker et al., 2005) and the role of *CUC2* and *MIR164a* in the formation of leaf serrations (Nikovics et al., 2006) are in good agreement with this possibility.

CUC1 and *CUC2* have diverged significantly within the Brassicaceae (Hasson et al., 2011). That *CUC1*, but not *CUC2*, responds directly to STM and has STM-binding sites in its promoter is also consistent with this data. The expression of STM also varies considerably in different species closely related to Arabidopsis. Many relatives express STM in the leaf primordia and have organs with more complex morphology (Piazza et al., 2010). Interestingly, the STM-binding sites are conserved in the *CUC1* promoter in several Brassicaceae species (S. Spinelli and J. Palatnik, unpublished data). It might be interesting to determine whether the direct regulation of *CUC1* by STM has a role in the formation of complex leaf morphologies within the Brassicaceae.

MATERIALS AND METHODS

Plant Material

Plants were grown in long days (16-h light/8-h dark) at 23°C. See Supplemental Table S6 for a list of transgenic lines and mutants. For Dex treatments, 2 week-old seedlings were transplanted to Murashige and Skoog plates containing 60 μM Dex or 60 μM Dex and 10 μM CYC. Control plates were treated in the same way without the addition of Dex or the translational inhibitor. Control plates treated only with CYC showed no significant differences with respect to untreated controls. CYC was also added before the DEX treatment as an additional control without any modification of the results. Seedlings were collected at different times after treatment for analysis.

Analytical Procedures

GUS stainings, microscopic observations, RNA extraction, and analysis by RT-qPCR was performed as described previously (Rodriguez et al., 2010). *PROTEIN PHOSPHATASE2* was used as a control to normalize the data (Czechowski et al., 2005). In cases where no expression was detected in the reference sample after 35 cycles, results were shown as semiquantitative data (*CUC1* was usually detected around cycles 26–29 with primers flanking the miRNA target site). GUS activity was assayed in protein extracts by a fluorescence method with 4-methylumbelliferyl glucuronide as a substrate (Jefferson et al., 1987). Mature miR164 levels were determined by stem-loop RT-qPCR (Chen et al., 2005). Primers are shown in Supplemental Table S7 and Supplemental Table S8 has a description of binary plasmids prepared for this study.

Microarray Analyses

Two-week-old seedlings were treated with 0.6% ethanol during 12 h. The shoot apex and the surrounding tissue was analyzed with Affymetrix ATH1 arrays (n = 2; E-MEXP-2550). Differentially expressed genes (Supplemental Tables S1 and S2) and overrepresented motifs (Supplemental Table S5) were identified as described before (Schommer et al., 2008).

DNA-Binding Assays

Proteins were obtained as fusions with glutathione *S*-transferase as described previously (Viola and Gonzalez, 2006, 2009). For EMSAs, purified recombinant proteins were incubated with 0.5 ng of labeled *CUC1* promoter fragments (−177/−84 respective to the transcription start site). Binding reactions (20 μL) contained 20 mM HEPES (pH 7.5), 50 mM KCl, 2 mM $MgCl_2$, 0.5 mM EDTA, 1.0 mM dithiothreitol, 0.5% Triton X-100, 22 ng/μL bovine serum albumin, 0.5 μg poly(dI-dC), and 10% glycerol. EMSAs were performed as described (Viola and Gonzalez, 2006). For the analysis of STM binding to the *CUC1* promoter in yeast (*Saccharomyces cerevisiae*), the *STM* coding sequence was cloned in pGADT7 (Clontech) and introduced into yeast strains constructed using the pHIS3NX/pINT1 vector and carrying the *CUC1* promoter inserted in the *PDC6* locus in front of the *HIS3* reporter gene preceded by its own minimal promoter.

Sequence data from this article can be found in the Array Express data libraries under accession number E-MEXP-2550.

Supplemental Data

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

Supplemental Figure S1. Expression levels of genes analyzed using the STM-GR system by RT-qPCR.

Supplemental Figure S2. Binding of STM to the CUC1 promoter fragment in vitro.

Supplemental Figure S3. Expression pattern of the STM reporter.

Supplemental Figure S4. Expression pattern of CUC1, CUC2, and MIR164a reporters in different backgrounds.

Supplemental Figure S5. Phenotypes of $Prom_{LFY}$:*STM* crossed to *cuc1-1* plants.

Supplemental Table S1. Genes modified by STM after 12 h of induction.

Supplemental Table S2. Genes modified by STM-VP16 after 12 h of induction.

Supplemental Table S3. Gene Ontology term enrichment for STM and STM-VP16 regulated genes.

Supplemental Table S4. Genes induced by both STM and STM-VP16.

Supplemental Table S5. Presence of putative STM-binding motifs in genes induced at least five times by STM-VP16.

Supplemental Table S6. List of previously described transgenic lines used in this article.

Supplemental Table S7. Relevant locus IDs and oligonucleotide primers used in RT-qPCR assays.

Supplemental Table S8. List of binary vectors prepared in this article.

ACKNOWLEDGMENTS

We thank Detlef Weigel, in whose lab at the Max Planck Institute for Developmental Biology (Tübingen) some of these experiments were started; Jan Lohmann for access to comparative microarray data; Carla Schommer, Edgardo Bresso, Juan Debernardi, Jean-Luc Gallois, and Detlef Weigel for discussions; Patrick Laufs for *CUC2* and *MIR164a* reporters; and Robert Sablowski for the Prom35S:*STM-GR* construct. S.S. and A.M. are Argentinian Research Council (CONICET) fellows, and J.P., I.L.V., and D.H.G. are members of CONICET.

Received April 5, 2011; accepted June 10, 2011; published June 17, 2011.

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