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**Activity of transcription factor JACKDAW is essential for
SHR/SCR-dependent activation of *SCARECROW* and *MAGPIE* and is
modulated by reciprocal interactions with MAGPIE, SCARECROW and
SHORT ROOT**

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Abstract

Two GRAS family transcription factors, SHORT-ROOT (SHR) and SCARECROW (SCR), are required for ground tissue and quiescent center formation in *Arabidopsis* roots. The action of SHR and SCR is regulated by two INDETERMINATE DOMAIN (IDD) family proteins, JACKDAW (JKD) and MAGPIE (MGP). Although the reciprocal interaction of these transcription factors is considered to be involved in the modulation of SHR and SCR action by JKD and MGP, the underlying mechanism remains unclear. In this study, we use a transient assay with *Arabidopsis* culture cells to show that the physical interaction of these transcription factors modulate their transcriptional activity. Transient expression of LUC reporter genes with the proximal sequences upstream from the ATG codon of *SCR* and *MGP* in protoplasts were activated by JKD. Moreover, promoter activities were enhanced further by the addition of SHR and SCR to JKD, but not by the combination of SHR and SCR in the absence of JKD. Yeast one-hybrid analysis showed that JKD binds to the *SCR* and *MGP* promoter sequences, indicating the existence of another binding sequences of JKD different from the previously determined IDD binding sequence. Our findings suggest that JKD directly regulates SCR and MGP expression in cooperation with SHR, SCR and MGP.

Keywords

JACKDAW, MAGPIE, SHORT-ROOT, SCARECROW, IDD family, roots

Introduction

Root cells display a radial pattern of concentric epidermal and ground tissue layers surrounding a central stele. The radial pattern of the ground tissue is the result of stereotypical divisions of cortex/endodermis initial (CEI) cells. The CEI cell first divides transversely, generating a new initial cell and a daughter cell. The CEI daughter (CEID) cell then divides longitudinally, generating the first cells of the endodermal and cortical cell lineages (Fig. 1a). In *Arabidopsis*, two GRAS family proteins, SCARECROW (SCR) and SHORTROOT (SHR) are involved in the radial pattern formation of the ground tissue (Di Laurenzio et al. 1996; Helariutta et al. 2000; Heidstra et al. 2004; Sena et al. 2004).

SHR mRNA is found exclusively in the stele (Fig. 1a), but SHR protein moves outward to cells adjacent to the stele (Helariutta et al. 2000; Nakajima et al. 2001). *SCR*

is expressed in cells of the endodermis, CEI and the quiescent center (QC), where it has been shown to prevent stem cells differentiation in adjacent cells (Di Laurenzio et al. 1996). In *scr* mutant roots, ground tissue consists of a single layer with mixed endodermal and cortical identity, the QC is not properly specified, and SHR protein movement is not restricted to the endodermis (Di Laurenzio et al. 1996; Sabatini et al. 2003; Heidstra et al. 2004; Sena et al. 2004; Nakajima et al. 2001). These results suggest that *SCR* is required for asymmetric periclinal ground tissue division, maintenance of QC identity, and localization of SHR to endodermal cells. The *SCR* gene was shown to be a direct target of the SHR transcription factor, and the SCR protein binds to its own promoter in the presence of SHR (Levesque et al. 2006; Cui et al. 2007), demonstrating that *SCR* is controlled by a SHR/SCR-dependent feed-forward loop. Although *scr* mutants form a single layer of ground tissue with endodermal characteristics, in *shr* mutants the single layer of ground tissue has only cortical identity (Helariutta et al. 2000; Benfey et al. 1993). This demonstrates that endodermis specification is controlled through SHR targets independent of the SHR-SCR complex.

A recent meta-analysis approach identified two *INDETERMINATE DOMAIN (IDD)* family genes, *MAGPIE (MGP)* and *NUTCRACKER (NUC)* as direct targets of SHR (Levesque et al. 2006). Another IDD family protein, JACKDAW (JKD) was also identified as an important factor that acts in a SHR-dependent feed-forward loop to regulate the range of action of SHR and SCR. JKD and MGP were also shown to interact physically with both SCR and SHR (Welch et al. 2007).

Here we investigated the effect of interactions of JKD, MGP, SHR and SCR transcription factors on the transcriptional activity of these transcription factor complexes by transient assay using an *Arabidopsis* culture cell system. Our data demonstrate that, of these transcription factors, only JKD activates transcription from the promoters of *SCR*, *MGP* and *JKD* by itself. Moreover, we show activation of transcription from the *SCR* and *MGP* promoters by the combination of SCR and SHR proteins, only when JKD is present.

Materials and methods

Construction of vectors for transient assay

Effector vectors with the DNA-binding domain of the yeast GAL4 transcription factor (GAL4BD) were constructed by introducing sequences for fusion proteins (JKD, MGP, SCR and SHR with GAL4BD) to downstream of cauliflower mosaic virus (CaMV) 35S

promoter. First, ORFs of these transcription factor genes were amplified by PCR using primer sets listed in Table1: for amplification of full length ORF coding sequences of JKD, MGP, SCR and SHR, primer sets of JKD full F/R, MGP F/ R, SCR F/R and SHR F/R, respectively, were used. Amplified fragments were cloned into pCR-Blunt (Invitrogen) and cut by EcoRI and BamHI or EcoRI and SalI. The fragments were cloned in to EcoRI and BamHI or EcoRI and SalI site of pGBT9 (Clontech) in frame with GAL4BD. The sequence for fusion proteins were amplified by PCR using the primer pair 5'-GCATACAATCAACTCCAAGC-3' and 5'-GAAATTCGCCCGGAATTAGC-3', and cloned into smaI site downstream of CaMV35S promoter in pUC19 vector (35S:pUC19, Maeo et al. 2009). To construct effector vectors without GAL4BD, PCR-amplified ORFs of these transcription factors were directly cloned into the SmaI site downstream of CaMV35S promoter of 35S:pUC19.

To construct various LUC reporter plasmids, the 1.5 kb, 3.5 kb, 2.5 kb and 3.5kb sequence regions upstream of the ATG codon of SCR, MGP, SHR and JKD, respectively, were amplified by PCR using primer sets listed in Table 1, and amplified DNA sequences were introduced upstream of the luciferase reporter gene (LUC) in pBI221(pBI221-luc, Maeo et al. 2009). For detection of the activity of GAL4BD fusion proteins, pGLL, whose expression was driven by a chimeric promoter containing four copies of the GAL4 binding site fused upstream of a minimal 35S promoter (-98) fragment, was used. As an internal control, 35S:hRLUC was used (Maeo et al. 2009).

Transient assay

Protoplasts prepared from the *Arabidopsis* T87 suspension-cultured cells were transfected with plasmid DNA according to a methods by Maeo et al.(Maeo et al. 2009). A suspension of protoplasts (150 μ l; 10^5 protoplasts per ml) was co-transfected with 10 μ g each of the LUC reporter and the effector plasmid DNAs, and 0.5 μ g of the 35S:hRLUC internal control plasmid. To adjust the amount of total DNA, empty vector (35S:pUC19) was used (total amount of effector vectors were adjusted to 40 μ g). The protoplasts were incubated at 22 °C for 20h before collection and measurement of reporter activities. The LUC and hRLUC activities were measured using the Dual-Luciferase Reporter Assay system (Promega). The LUC activity was normalized according to the hRLUC activity in each assay, and the relative ratio was determined by comparing this ratio with that obtained with the empty vector. The mean relative ratios were calculated from three to four independent experiments.

Yeast one-hybrid assay

The yeast one-hybrid assay was performed using the MATCHMAKER One-Hybrid system (Clontech). For amplification of JKD and MGP, the same primer pairs used for construction of effector vectors were used. For amplification of the N terminal half of JKD and C terminal half of JKD, primer sets of JKD full F/JKD ND R and JKD CD F/JKD full R, respectively, were used (Table 1).

The amplified ORF regions were cloned in frame after the transcriptional activation domain of the yeast GAL4 transcription factor (without DNA binding domain) in pGAD424. For construction of reporter plasmids, we amplified the promoter sequence of *SCR* (SCRp-up: from -1500 to -500bp, SCRp-down: from -1000 to -1bp from the start codon) and *MGP* (MGPp-3.5-3.0: from -3.5 to -3.0 kb, MGPp-3.0-2.5: from -3.0 to -2.5 kb and MGPp-2.5-2.0: from -2.5 to -2.0 kb) by PCR from *Arabidopsis* (Columbia) genomic DNA. Primer pairs used for amplification were SCRp1500F and SCRp500R for SCRp-up, SCRp1000F and SCRpR for SCRp-down, MGPp3500F and MGPp3000R for MGPp-3.5-3.0, MGPp3000F and MGPp2500R for MGPp-3.0-2.5 and MGPp2500F and MGPp2000R for MGPp-2.5-2.0 (Table 1).

Amplified PCR products were cloned upstream of the *LacZ* reporter gene in pLacZi vector (Clontech), and then, the promoter sequences and reporter genes were transferred to the genomic DNA of yeast strain BY5444 according to the manufacturer's manual.

The vectors in pGAD424 were introduced into yeast which carry *SCR* or *MGP* promoter sequences, and X-gal activities were detected by filter assay according to the manufacturer's manual (Clontech).

Results

Transcriptional activity of JKD and MGP

In wild-type *Arabidopsis* roots, expression of JKD and MGP overlap in the endodermis and CEI, whereas in the QC and cortex, only JKD is expressed (Fig. 1 a and b, Welch et al. 2007; Levesque et al. 2006)). JKD and MGP are reported to interact with each other, and they each interact with SCR and SHR as well (Welch et al. 2007). To assess whether the interaction of any or all of these transcription factors influences the transcriptional activity of JKD and MGP, we performed transient assays using *Arabidopsis* culture cells. JKD and MGP were fused to the GAL4 DNA binding domain

(GAL4BD) gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter to construct effector vectors GBD JKD and GBD MGP (Fig. 2a). We also made constructs where JKD, MGP, SCR and SHR are under the control of the 35S promoter (JKD, MGP, SCR and SHR, Fig. 2a).

When GBD JKD was transferred into protoplasts prepared from *Arabidopsis* T87 cells together with a luciferase reporter gene that contained four copies of the GAL4-responsive element (pGLL), a low level of activity was detected (Fig. 2b). By contrast, GBD MGP showed no transcriptional activity (Fig. 2c). Effectors without GAL4BD showed no activity by themselves (data not shown).

To determine whether addition of other transcription factors influence the level of JKD transcription activity, effector vectors without GAL4BD were transformed into the cells together with GBD JKD. Expression of reporter genes was about three times higher when GBD JKD was co-transformed with SHR than with GBD JKD by itself. On the other hand, addition of SCR showed only a slight increase in the activity of GBD JKD and transcriptional activity was slightly reduced with MGP. Maximum JKD activity was observed in the presence of both SHR and MGP. Interestingly, the increased GBD JKD activity observed by addition of SHR was severely attenuated when SCR was added as well (Fig. 2b).

Next, we examined the influence of additional effectors on the activity of MGP. Similar to what was found with JKD, the activity of GBD MGP was more than three times higher when SHR was added. In contrast with the results observed when MGP was added to GBD JKD, the addition of JKD to GBD MGP increased the activity to similar level of SHR. Addition of both SCR and SHR greatly increased the activity of MGP (about 30 times higher activity than MGP by itself). However, the addition of JKD along with SCR and SHR reduced the highest activity to basal levels (Fig. 2c). These results suggest that various combinations of transcription factors can influence transcriptional activity in both positive and negative directions.

Transcriptional activity of SCR and SHR

Because we observed the influence of protein interactions on the transcriptional activity of JKD and MGP, we also examined the activity of SCR and SHR. We constructed GBD SCR and GBD SHR effector plasmids in the same way as for GBD JKD and GBD MGP (Fig. 2d and e).

Significant activity for SCR by itself was not detected, and activity was not increased by the addition of any single factor. Elevated activity (about 3-fold) was observed when

both JKD and MGP were added together. However, this increased activity was abrogated by the addition of SHR with JKD and MGP (Fig. 2d).

On the other hand, among the four transcription factors tested, SHR showed highest activity by itself, with about a 15-fold higher level than background (Fig. 2e). Because a large portion of SHR protein is excluded from the nucleus of plant cells in the absence of SCR (Sena et al. 2004; Cui et al. 2007), SHR might not show considerable transcriptional activity by itself in cells. In these experiments, the nuclear localization signal from the yeast vector most likely shuttles SHR protein to the nucleus. Interestingly, addition of any of the other factors, except for MGP, reduces the activity of SHR (Fig. 2e). Although SHR and SCR are considered to activate their target genes as a complex, the activity of both GBD SCR and GBD SHR were reduced in the presence of SHR and SCR, respectively.

Transcriptional activity of JKD, MGP, SCR and SHR on the *SCR* promoter

Expression of *SCR* is regulated directly by SHR and SCR (Levesque et al. 2006; Heidstra et al. 2004). Recent data showed that JKD is also required for maintenance of *SCR* expression particularly in the QC (Welch et al. 2007). To analyze the transcriptional regulation of *SCR* by these transcription factors, the 1.5kb promoter region of *SCR* driving the luciferase gene was introduced and used as a reporter construct in transient assays in plant cells. This 1.5 kb sequence upstream of ATG was shown to be sufficient for accurate *SCR* expression when fused to a yellow fluorescent protein (YFP) reporter gene and transferred to *Arabidopsis* (Aida et al. 2004). We used effectors without GAL4BD hereafter.

Individually, SHR, SCR and MGP did not activate transcription from the *SCR* promoter, while JKD alone showed a slight increase in transcription from the *SCR* promoter (about five times higher than empty vector). Surprisingly, while the combination of SCR and SHR did not activate transcription from *SCR* promoter, JKD with SHR showed approximately three times the transcriptional activity of JKD alone (Fig. 3a). The combination of JKD, SCR and SHR did not significantly increase LUC activity compared to just JKD and SHR, but transcriptional activity was increased significantly when all four transcription factors are introduced (Fig. 3a). The localization of these factors is observed in CEI cells and endodermis (Fig. 1b), and these results suggest that the high expression of *SCR* in these tissues is activated by the combined action of these factors. In this experiment, LUC activity was detected only when JKD was present, indicating that feed-forward activation of *SCR* transcription by

SHR and SCR requires JKD.

Transcriptional activity of JKD, MGP, SCR and SHR on the *MGP* promoter

Meta-analysis identified *MGP* as one of the putative direct targets of SHR (Levesque et al. 2006), and SHR and SCR were found to bind to the promoter region of *MGP*. A Chromatin immunoprecipitation-polymerase chain reaction (ChIP-PCR) assays and promoter scanning indicated that both SHR and SCR bind to a region approximately 3.2kb upstream of the translation start site of *MGP* (Cui et al. 2007). In addition, a 3.5kb region upstream of *MGP* was determined to be sufficient for correct expression of *MGP*, when the sequence was fused to GFP (Miyashima et al. 2009). Therefore a 3.5 kb sequence upstream of the *MGP* start codon was used as a reporter sequence in transient assay.

When individual transcription factors were added, only JKD showed increased transcription activity from the *MGP* promoter, with a seven-fold increase over basal levels (Fig. 3b). The combination of SCR and SHR showed a slight but insignificant increase in activity (Fig. 3b). Although it has not been reported previously, this result indicates that JKD is a direct regulator of *MGP*. Interestingly, the addition of SCR, SHR or MGP suppressed JKD activity to basal level. Moreover, the highest level of activity from the *MGP* promoter occurs when the trio of JKD, SCR and SHR were used in the assay with transcription levels increased to nearly 10-fold higher than basal, and slightly higher than JKD by itself (Fig. 3b). In marked contrast to the result with the SCR promoter, the addition of MGP completely eliminated the high transcriptional activity mediated by JKD, SHR and SCR together (Fig. 3b). These results indicate MGP itself may mediate a feed-back inhibition of its own promoter.

We obtained similar results to that with the 3.5 kb promoter assay above when the transient assay was carried out with the 1.5kb sequence (from -3.5 to -2.0kb from the *MGP* ATG) containing a putative binding site of SHR and SCR (data not shown). This result shows that the region from -3.5 to -2.0kb from ATG is sufficient for the regulation of MGP expression by these factors.

Transcriptional activity of JKD, MGP, SCR and SHR on the *JKD* and *SHR* promoter

JKD expression is not affected by SCR and SHR in early stages of embryo development, but in post-embryonic stages, *JKD* expression in the QC and ground tissue stem cells is enhanced by SCR and SHR (Welch et al. 2007). A 2.8kb region upstream of JKD is

sufficient to drive a reporter gene expression in a pattern that matches the *in situ* hybridization pattern (Welch et al. 2007). We used a slightly longer sequence, 3.5kb upstream of the *JKD* start codon, to construct a reporter for a transient assay. A low level of activity, about two-fold above basal, was detected when *JKD* alone was added to the assay, and the addition of other transcription factors by themselves had no effect on transcription from the reporter (Fig. 3c). In addition, the combination of *JKD* with any other factor did not reduce or increase activity levels relative to *JKD* alone. These results indicated that there is a feed-forward mechanism for *JKD* expression. Moreover, we did not observe regulation of *JKD* by *SCR* and *SHR* together (Fig. 3c). This result suggests that the expression of *JKD* might be regulated by factors other than the transcriptional regulators examined here.

We also tested whether *SHR* expression was controlled by these transcription factors by using a 2.5kb region upstream of *SHR* fused to luciferase gene as a reporter. A previous study demonstrated that the *SHR* promoter is contained within this 2.5 kb region (Nakajima et al. 2001). None of these transcription factors, including *SHR* itself, had a large effect on transcription from the *SHR* promoter (Fig. 3d). These results agree with the fact that *SHR* is expressed exclusively in the stele, where the *SCR*, *JKD* and *MGP* transcription factors are not co-expressed (Fig. 1a). This finding also supports a previous report that *SHR* expression is not altered in *scr* or *shr* mutants (Helariutta et al. 2000).

***JKD* binds to the *SCR* and *MGP* promoter sequences**

Since *JKD* by itself is able to activate the promoters of the *SCR* and *MGP* genes and transcription from its own promoter, we examined the direct interaction between *JKD* and the promoter region of *SCR* and *MGP* using a yeast one-hybrid assay.

Two regions upstream of *SCR*, a sequence -1.5 to 0.5 kb from the ATG (*SCR*_{p-up}) and the 1.0 kb sequence upstream of the ATG (*SCR*_{p-down}) were placed upstream of *LacZ* gene to create reporter constructs, and integrated into the yeast genome (Fig. 4a). For prey vectors, full length *JKD*, the N-terminal half of *JKD* and C-terminal half of *JKD* were fused with the yeast GAL4 activation domain (GAL4 AD). The prey vectors were introduced into the yeast carrying the reporter genes, and DNA binding activity was estimated by X-gal staining assay.

Full-length *JKD* protein was found to interact with the 1kb region of *SCR* immediately upstream of the ATG, but no interaction was evident with the sequence from -1.5 to -0.5kb (Fig. 4b). These results suggested that *cis*-acting elements recognized by *JKD* are

present within a region 0.5kb from the translation start site. Chip-qRT-PCR experiments indicated that SHR binding site is present between 209-528 bp upstream from ATG of *SCR* (Levesque et al. 2006). Therefore the JKD binding site may be close to that of SHR.

When JKD was divided into two parts, the N-terminal peptide was sufficient to bind the *SCR* promoter (Fig. 4b). Because JKD binds to the SCR promoter though N-terminal half, most of which consists of ID domain (IDD), we tested whether MGP also bind to *SCR* promoter. Although MGP did not activated transcription from SCR promoter, it does appear to bind to the *SCR* promoter in this assay (Fig. 4b).

To analyze the binding of JKD to the MGP promoter, we used a 1.5kb sequence (-3.5 to -2.0kb from the ATG), because we found that the entire 3.5kb is too long to be effective in a yeast one-hybrid assay, and our preliminary data showed that JKD activated the reporter gene expression when the 1.5kb region was used in transient assays (data not shown). The 1.5kb region was further divided into three parts, sequences -3.5 to -3.0 kb, -3.0 to -2.5 kb and -2.5 to -2.0 kb from ATG, and these sequences were placed upstream of *LacZ* gene to create reporter construct (MGPp-3.5-3.0, MGPp-3.0-2.5 and MGPp-2.5-2.0).

When MGPp-3.5-3.0 was used as a reporter, we could detect a faint signal with full length of JKD and MGP, and the N-terminal region of JKD in X-gal staining assay. Chip-qRT-PCR experiments indicated that SHR bind to around -3.2kb upstream of translational start site of *MGP* gene (Cui et al. 2007), indicating JKD binding site may be close to that of SHR as same as in the case of *SCR* promoter.

Previously, we showed that two zinc fingers of the maize ID1 protein, as well as another maize ID1 homolog, bind to an 11bp consensus sequence (Kozaki et al. 2004). Here we observed that JKD and MGP also bind to the 11 bp ID1-binding consensus sequence in yeast (Fig. 4b). However, we could not find this 11bp sequence in the 0.5 kb promoter region of *SCR* or *MGP*, indicating that JKD and MGP recognize sequences other than the 11bp.

Discussion

Current research suggests that transcriptional regulators often form protein complexes and act in combination with each other to mediate tissue specific expression of numerous genes and to modulate the levels of gene transcripts (Stobe et al. 2009). However, the molecular mechanisms that specify how complexes of transcription factors

control target gene expression are poorly understood (Pan et al. 2010). Several ID family proteins, JKD (AtIDD10), MGP (AtIDD3) and NUC (AtIDD8) recently were shown to play important roles in radial pattern formation in the *Arabidopsis* root meristem by interacting with the GRAS family transcriptional regulatory proteins, SHR and SCR (Welch et al. 2007). In this report, we investigated the transcription activities of these putative regulatory factors and the effects of their reciprocal interaction and activities by transient assay in *Arabidopsis* culture cells.

A previous study showed that SHR and SCR cooperatively regulate several downstream genes, including *SCR* and *MGP* (Levesque et al. 2006; Cui et al. 2007). Transient assays with *SCR* and *MGP* promoters showed that even in the presence of both SCR and SHR proteins, there was little or no apparent transcription from either the *SCR* promoter or the *MGP* promoter. Here we find that enhanced activity is observed only when JKD is present, presumably due to interaction between these factors (Fig. 3 and 5). These results agree with another finding that showed *SCR* expression is not detected in the QC in *jdk* mutants (Welch et al. 2007). However, low levels of SCR mRNA were still detected in the ground tissue of *jdk* mutants (Welch et al. 2007), thus indicating that other factors are involved in controlling *SCR* expression in this region of the root.

Transcription activity from the *SCR* promoter was enhanced when SHR and JKD were added together and feed-forward regulation by SCR on the *SCR* promoter was observed only when MGP was present along with SHR and JKD, presumably acting in a ternary complex (Fig. 3a).

On the other hand, whereas JKD alone activated transcription from the *MGP* promoter, both SHR and SCR each individually suppressed activity from the *MGP* promoter. Only the combination of both SHR and SCR together along with JKD caused activity to be enhanced slightly compared to transcription mediated by JKD alone (Fig. 3b). These results agree with the report that *MGP* expression is eliminated in both *scr* and *shr* mutants (Levesque et al. 2006). The combination of SHR, SCR and JKD, which exist in the QC where MGP is not expressed (Welch et al. 2007), activated the *MGP* promoter to the highest level in this system. The expression of *MGP* is probably strongly repressed in the QC by other unknown factors. The highest activity by the combination of SHR, SCR and JKD was suppressed to basal levels by the addition of MGP, indicating that feedback regulation is in effect (Fig. 5). The combination of these four factors is observed in the CEI and endodermal cells, where *MGP* is highly expressed (Welch et al. 2007). It is possible that MGP expression needs to be maintained at an adequate level by feedback regulation.

In contrast to what was observed with the *SCR* and *MGP* promoters in this system, neither the *JKD* nor *SHR* promoters were regulated by SCR and SHR. *JKD* transcription is reported to be initiated early in ground tissue development by a SHR- and SCR-independent mechanism; later, maintenance of *JKD* expression becomes dependent on SHR and SCR (Welch et al. 2007). Our results show that JKD itself has a slight positive effect on its own promoter (Fig. 3c). Although the 3.5 kb sequence upstream of *JKD* was shown to be sufficient for *JKD* expression in the QC and ground tissues (Welch et al. 2007), 5' sequences further upstream might be required for regulation by SCR and SHR, or unknown factors might be required for SHR/SCR-dependent regulation of the *JKD* promoter as in the case of the *SCR* and *MGP* promoters. Recently, it was reported that SHR and SCR together activate the transcription of micro RNAs (miRNA) 165 and 166 (miR 165/166) in the endodermis. miR165 and miR166 suppress the expression of the Class III HD-ZIP transcription factor PHABULOSA (PHB), which suppress the expression of JKD (Miyasima et al. 2011; Carlsbecker et al. 2010). These results indicate that SHR and SCR indirectly regulate the expression of JKD. Further analysis will clarify the regulation of *JKD* expression. The *SHR* promoter was not activated by any of the transcription factors described here (Fig. 3d). This finding was not unexpected because *SHR* is expressed in the stele where SCR, MGP and JKD are not present (Fig. 1a). Moreover, SHR does not appear to regulate expression from its own promoter.

The binding of JKD to the *SCR* and *MGP* promoters was demonstrated by transient assay and yeast one-hybrid analysis (Fig. 3a,b and 4b,d). MGP also interacted with the *SCR* and *MGP* promoter in yeast (Fig. 4b), but it did not activate transcription from the *SCR* or *MGP* promoters (Fig. 3a). Binding to the *SCR* and *MGP* promoter was mediated by the N-terminal half of the JKD protein, which contains nearly the entire ID domain including all four zinc fingers (Fig. 4b). This suggests that the ID domain is involved in binding of sequences upstream of *SCR* and *MGP*. We previously demonstrated that IDD family proteins bind to an 11 bp of consensus sequence via two internal zinc fingers of the ID domain (Kozaki et al. 2004). This 11bp sequence is not present in the 1.5 kb *SCR* or *MGP* promoter sequence used here, suggesting that IDD proteins have the potential to bind other sequences.

JKD was also shown to affect intracellular SHR localization in the QC (Welch et al. 2007). GFP-SHR was found to localize to the cytoplasm in root cells of *jdk* mutants, in contrast to the nuclear-localization of GFP-SHR in the QC of wild-type root cells. This was explained as mislocalization of SHR protein resulting from reduced SCR expression due to the absence of JKD (Welch et al. 2007). In transient assays, transcription activity

of JKD was markedly enhanced by the presence of SHR (Fig. 2b and 3a), further supporting the idea that JKD directly controls the nuclear localization of SHR, even in the absence of SCR.

In contrast to JKD, MGP alone showed no appreciable transcriptional activity (Fig. 2c and 3a), which may explain why the *mpg* single mutant does not show a visible phenotype (Welch et al. 2007). Although MGP did not show transcription activity by itself, it regulates the transcription of downstream genes through interaction with other transcription factors, in different way from JKD.

Major phenotypes observed in ground tissue of *jkd* mutants include an increase in cell numbers within layers of the cortex and endodermis, and ectopic periclinal divisions in cortical cells. The ectopic periclinal divisions found in cortical cells of the *jkd* mutant were not observed in *jkd scr* or *jkd shr* double mutants. Further, increased numbers of cortical and endodermal cells in the *jkd* mutant was complemented by suppression of *MGP* expression, indicating that JKD counteracts a MGP-dependent cell division-promoting activity that is also dependent on SHR and SCR (Welch et al. 2007). The opposing effects of JKD and MGP were clearly observed in the GBD MGP experiment described here; i.e., the activity of GBD MGP increases dramatically in the presence of both SHR and SCR, and activity is reduced to basal levels by the addition of JKD (Fig. 2C). Moreover, the high transcription activity level shown by SHR and SCR together is markedly decreased in the absence of either SHR or SCR. If cell division-promoting genes are under this type of regulation, their gene expression is usually repressed by JKD such that, in the absence of JKD, gene expression and cell division are enhanced by the presence of both SCR and SHR (Fig. 5).

It is a challenge to analyze how a complex of several transcription factors can control specific target genes because one transcription factor could activate or repress several signaling pathways and, in turn, could be regulated by a complicated network of inter-related signaling pathways. In this context, the transient assays used here is an effective tool in dissecting the mechanisms of gene regulation by multimeric transcription factor protein complexes of interest because other factors that might otherwise confound this analysis are excluded.

In *Arabidopsis*, trichome cell fate determination is known to utilize a lateral inhibition mechanism that relies on the interplay of several transcription factors. GLABRA1 (GL1), an R2R3 MYB transcription factor (Oppenheimer et al. 1991), GLABRA3 (GL3), a basic helix-loop-helix (bHLH) transcription factor (Payne et al. 2000), and TRANSPARENT TESTAGLABRA1 (TTG1), a WD40 protein (Walker et al. 1999), are believed to form an activator complex that controls the transcription of *GLABRA2* (*GL2*),

a homeodomain protein (Rerie et al. 1994), which in turn specifies trichome formation in shoots (Ishida et al. 2007). A recent study using the *Arabidopsis* mesophyll protoplast transfection assay revealed that in addition to the protein-protein interaction between GL1-GL3, concurrent binding of GL1 and GL3 to the promoter of *GL2* via their own DNA-binding domains is probably required to activate *GL2* (Wang and Chen 2008).

Studies of Hox transcription factors, which play fundamental roles in controlling morphogenetic diversity along the anterior-posterior body axis in animals by regulating distinct sets of target genes (Mann and Morata 2000), revealed how Hox proteins regulate target gene expression in cooperation with other transcription factors. Although Hox proteins are active in a large number of cells, individual Hox proteins control cell differentiation and pattern formation and, consequently, target gene expression in a highly localized manner, sometimes even only in a single cell (Hersh et al. 2007). In *Drosophila*, a recent study of the regulation of the cell death gene *reaper* (*rpr*) by the Hox protein Deformed (Dfd) revealed that local activation of *rpr* expression in the anterior part of the maxillary segment is achieved through combinatorial interaction of Dfd with at least eight functionally diverse transcriptional regulators on a minimal enhancer for the Hox response elements (HREs). The authors suggest that a large number of transcription factors could dictate transcriptional output in combination with the respective Hox protein by binding selectively and independently to cis-regulatory sequences within HREs of target genes. Additionally, their model explains how Hox proteins can act as repressors in one context and as activators in another, because the combined transcriptional output is dependent on the regulatory activity of all transcription factors assembled on the enhancer (Stobe et al. 2009).

Our results support the model described above. We show that reciprocal interaction of transcription factors JKD, MGP, SCR and SHR differentially control target genes, depending on specific promoters. We found also that these transcription factors can act as both activator and repressor depending upon its interaction partner (or partners) and the promoter targets.

Taken together, it is important to elucidate how these transcription factors interact with promoter sequences and other transcription factors to clarify the mechanism used by these transcription factors to regulate the expression of their respective target genes. Understanding how the SCR-SHR pathway controls the ground tissue patterning will help define some of the principles involved in multiple transcription factor gene regulation.

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Table1. Primers used in this study

	Name	Sequence
Effector		
JKD	JKD full F	5'-GCTCTAGAGAATTCATGCAGATGATTCCAGGAG-3'
	JKD full R	5'-GCCTCGAGTCAACCCAATGGAGCAAACC-3'
	JKD ND R	5'-GGATCCCCCCTCGCTCCTTCCTCAGTTAATG-3'
	JKD CD F	5'-GAATTCATGAGTTCACCTTAGTAAC-3'
MGP	MGP F	5'-GAATTCATGACAACTGAAGATCAGAC-3'
	MGP R	5'-GGATCCTCAAATCCATCCATTGATAG-3'
SCR	SCR F	5'-GAATTCATGGCGGAATCCGGCGATTTC-3'
	SCR R	5'-GGATCCCTAAGAACGAGGCGTCCAAG-3'
SHR	SHR F	5'-GAATTCATGGATACTCTCTTTAGACTA-3'
	SHR R	5'-GTCGACTTACGTTGGCCGCCACGCACT-3'
Reporter		
JKD promoter	JKDp3500F	5'-AAGCTTCTGCAGGGATGATAATAGAAGACTG-3'
	JKDpR	5'-GGATCCCCCGGGCGGATCGGAGCTAAAGAAC-3'
MGP promoter	MGPp3500F	5'-CTGCAGAATTGGAGACGATATATG-3'
	MGPproR	5'-CAGCTGGTCTTCTTCTTGGACAAAAGTTTTGTTG-3'
	MGPp3000R	5'-CTCGAGAAGCTTAAAATAAAATGCAATAAACGAC -3'
	MGPp3000F	5'-AAGCTTCTCGAGTGGTATGGATCTAACCTTTG-3'
	MGPp2500R	5'-GTCGACGAACTCACATATTACCCCA-3'
	MGPp2500F	5'-AAGCTTCTCGAGGGGGTAATATGTGAGTTTC-3'
	MGPp2000R	5'-GTCGACATTTGGTCTATCCCGCTC-3'
	MGPp1500R	5'-AAGCTTACAAAAGTCTAAAAATTG-3'
SCR promoter	SCRp1500F	5'-AAGCTTACAAAAGTCTAAAAATTG-3'
	SCRpR	5'-CCATGGCTCGAGGGAGATTGAAGGGTTGTTG-3'
	SCRp500R	5'-CTCGAGTGTGTTTGACGTCTTCCG-3'
	SCRp1000F	5'-AAGCTTAAATTGCAAGTTGGTGCC-3'
SHR promoter	SHRp2500F	5'-AAGCTTGGGACTTTTCCTAGAATGC-3'
	SHRpR	5'-CCATGGCTCGAGGGAGACCCACAATATCTAAG-3'

Fig. legends

Fig. 1. Localization of JKD, MGP, SCR and SHR transcripts and protein in *Arabidopsis* roots. (a) Schematic of the longitudinal section, and RNA localization of *JKD*, *MGP*, *SCR* and *SHR* in *Arabidopsis* roots. S:Stele, E:Endodermis, C:Cortex, CEI:Cortex/Endodermis Initial, QC:Quiescent Center (b) Diagram illustrating JKD, MGP, SCR and SHR protein localization and co-localization patterns in *Arabidopsis* roots.

Fig. 2. Transcriptional activity of JKD, MGP, SCR and SHR, in combination with other factors in an *Arabidopsis* protoplast system. (a) Schematic diagram of the reporter and effector plasmids used in transient assays. The GAL4-responsive reporter construct, pGLL, contains four copies of the GAL4 binding site and a minimal CaMV 35S promoter (35Smin), the gene for luciferase (LUC), and a nopaline synthase (NOS) terminator. Each effector construct contains a GAL4 DNA binding domain (GAL4BD) and either the *JKD*, *MGP*, *SCR* or *SHR* genes, driven by the CaMV35S promoter. Effector constructs without GAL4 DNA binding domain contained *JKD*, *MGP*, *SCR* or *SHR* genes driven by the CaMV 35S promoter.

Transcriptional activity of (b) JKD fused with GAL4BD (GBD JKD), (c) GBD MGP, (d) GBD SCR and (e) GBD SHR in combination with other factors. As a control, an empty vector was used and all LUC activities are expressed relative to the control (value set at 1). Values shown are averages of results from three or four independent experiments. The error bars represent SD. Bars with asterisks are significantly different from control (*, $0.05 > P > 0.01$; **, $P < 0.01$).

Fig. 3. Activation of transcription from promoters of the *JKD*, *MGP*, *SCR* or *SHR* genes by JKD, MGP, SCR and SHR transcription factors alone or in various combinations with other factors. Transactivation of (a) *SCR* promoter, (b) *MBP* promoter, (c) *JKD* promoter and (d) *SHR* promoter, by JKD, MGP, SCR and SHR in combination with other factors. Schematic diagram of the reporter plasmid used in each transient assay is shown under each graph.

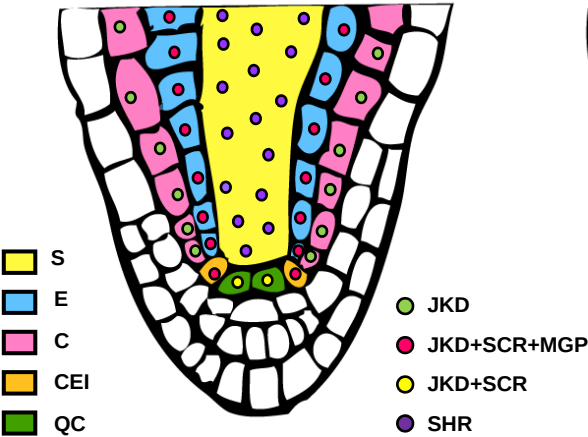
As a control, an empty vector was used and all LUC activities are expressed relative to the control (value set at 1). Values shown are averages of results from three or four independent experiments. The error bars represent SD. Bars with asterisks are significantly different from control (*, $0.05 > P > 0.01$; **, $P < 0.01$).

Fig. 4. JKD and MGP bind to the *SCR* and *MGP* promoter. (a) *SCR* promoter region from -1000 to -1bp (SCRp-down) and -1500 to -500bp (SCRp-up) were placed upstream of *LacZ* gene. IDD binding sequence contains four copies of the 11pb IDD binding sequence (TTTGTCGTATT) in tandem placed upstream of *LacZ* gene. (b)(d) X-gal staining assay. (c) *MGP* promoter region from -3.5 to -3.0kbp (MGPP-3.5-3.0), from -3.0 to -2.5.kbp (MGPP-3.0-2.5) and from -2.5 to -2.0kbp (MGPP-2.5-2.0) were placed upstream of *LacZ* gene. empty, empty vector pGAD424; JKD F, full length JKD; JKD N, N-terminal half of JKD; JKD C, C-terminal half of JKD; MGP, full length MGP.

Fig. 5. Model illustrating action of JKD, MGP, SCR and SHR on promoters of *SCR*, *MGP* and a putative target gene. JKD directly binds to promoters of *SCR* and *MGP*, and activates these genes. SCR and SHR enhance the transcription of *SCR* and *MGP* only when JKD is present. MGP enhances the *SCR* promoter along with other factors. On the other hand, MGP suppresses the *MGP* transcription activated by other factors. The combination of MGP, SCR and SHR will activate the putative target gene (*X*) which promotes cell division, while JKD will suppress the activated transcription of the putative target gene.

Figure 1

A



B

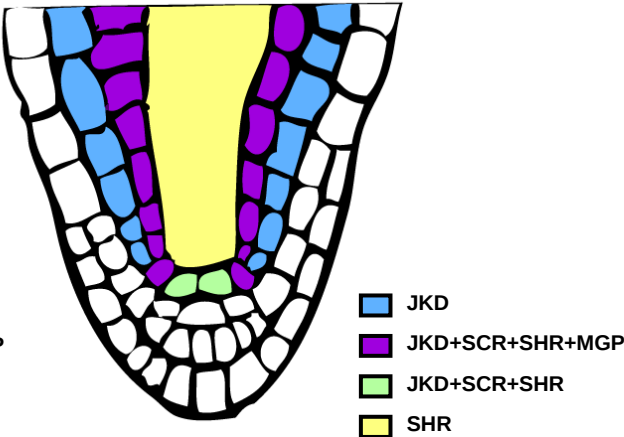
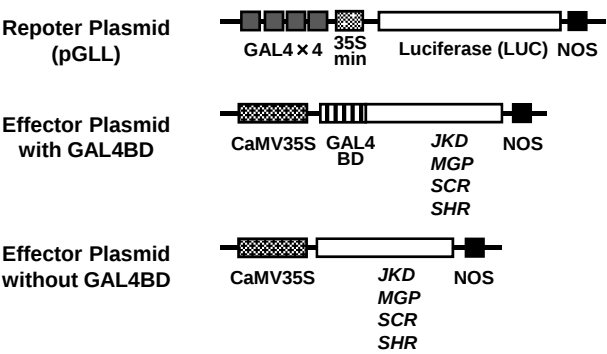
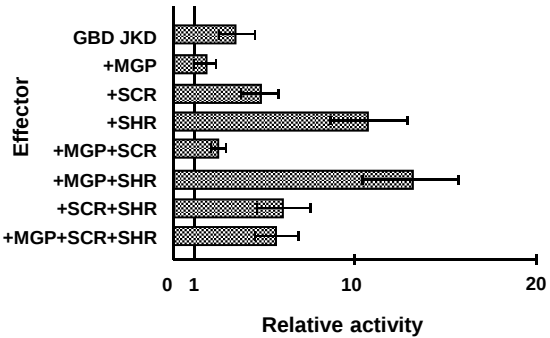


Figure 2

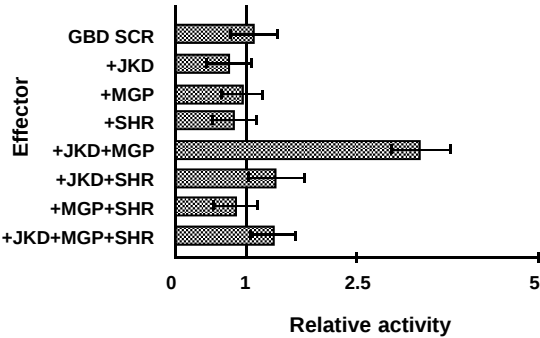
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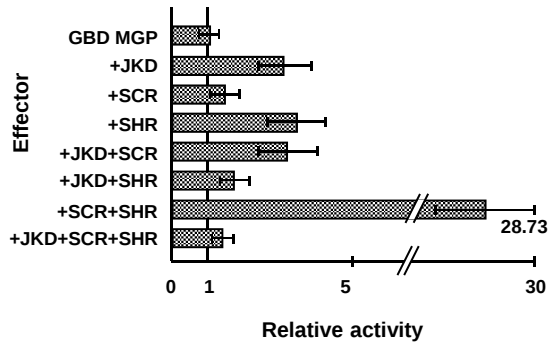
B



D



C



E

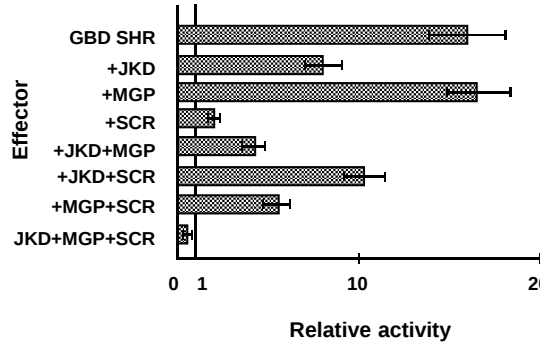


Figure 3

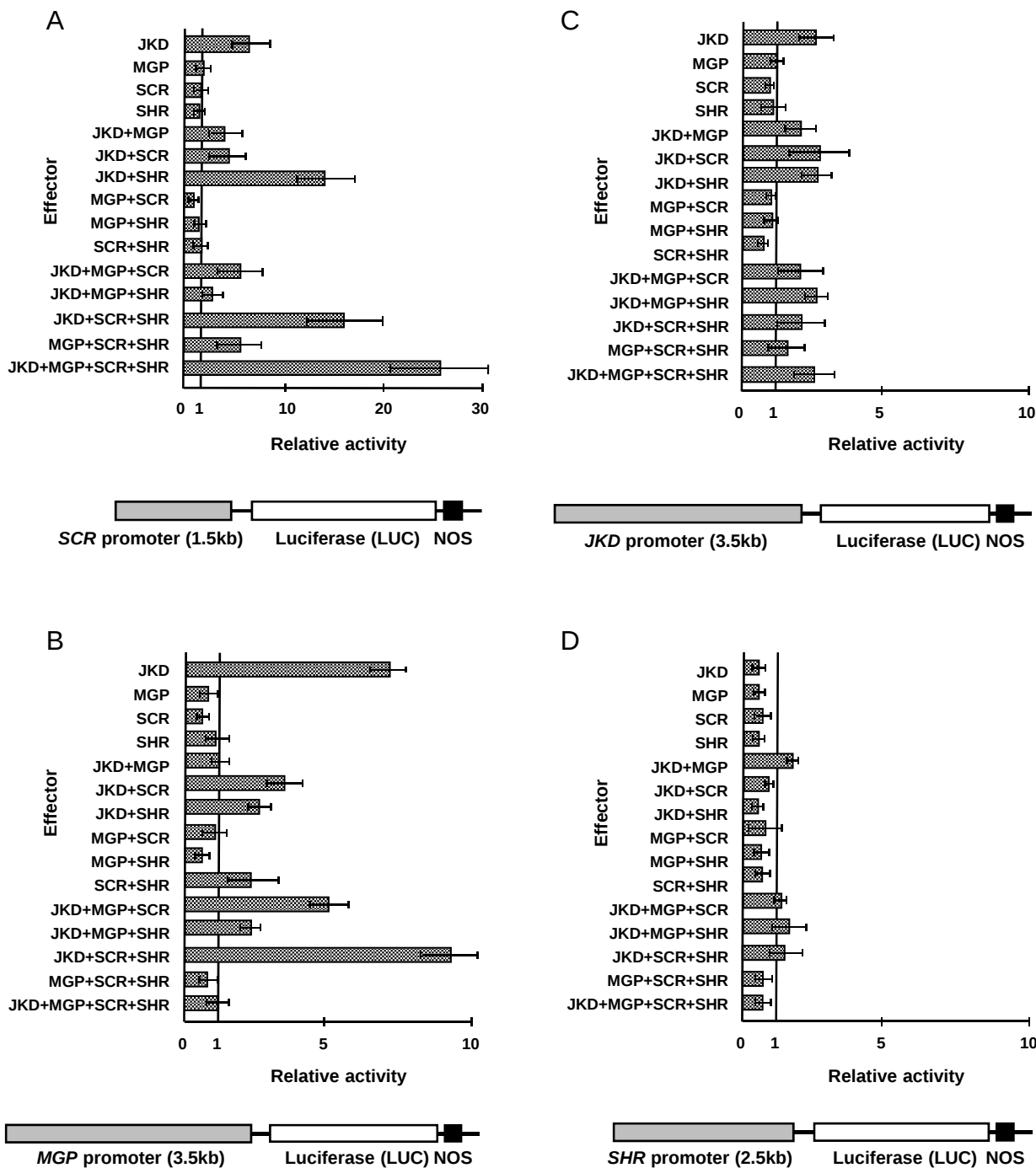


Figure 4

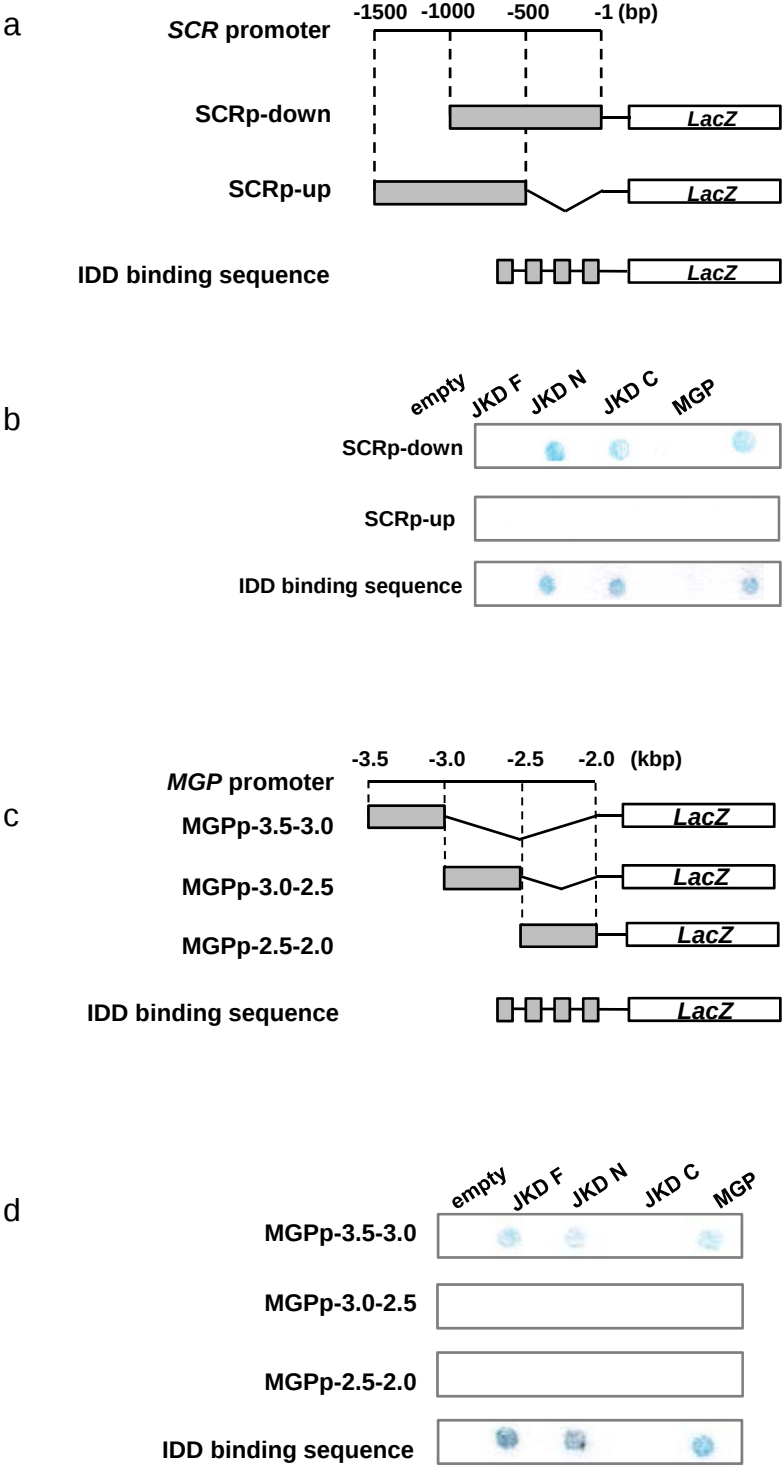


Figure 5

