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Identification of the minimal repression domain of SUPERMAN shows that the DLELRL hexapeptide is both necessary and sufficient for repression of transcription in *Arabidopsis*

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Abstract

We reported previously that the carboxy-terminal 30 amino acids of SUPERMAN (SUPRD) function as a repression domain in *Arabidopsis*. In this study, we identified the peptide sequences in SUPRD that is both necessary and sufficient for repression of transcription. To our surprise, the hexapeptide DLELRL was sufficient, by itself, to confer the ability to repress transcription on a DNA-binding domain. A database search revealed that there are 32 TFIIIA-type zinc finger proteins in the *Arabidopsis* genome that contain a hexapeptide sequence similar or identical to that of DLELRL. These peptides acted as repression domains, suggesting that these zinc finger proteins might function as active repressors. Further mutational analysis within DLELRL revealed that an amphiphilic motif composed of six amino acids (XLxLXL) with preferences at the first and fifth positions is necessary and sufficient for strong repression. An assay of positional effects suggested that GAL4DB–DLELRL might function as a short-range repressor. A possible mechanism of the DLELRL-mediated repression is discussed.

Keywords: Transcriptional repressor; EAR-like motif; Amphiphilic XLxLXL motif; SUPERMAN; Repression domain; *Arabidopsis*; Gene expression; Transcription factor; ERF; TFIIIA-type zinc finger protein

Repressors of transcription can be considered to be either passive or active, and active repressors, unlike passive repressors, include an independent repression domain [1]. A large number of transcriptional repression domains that can be fused to heterologous DNA-binding domains have been identified in eukaryotes. These domains vary in terms of both length and sequence, and they exhibit no significant homology to one another at the amino acid level. They can, however, be loosely categorized according to their amino acid content, being defined, for example, as alanine-rich, proline-rich, or charged [1].

In plants, we reported previously that the repression domains of class II ethylene responsive element-binding factors (ERFs) and TFIIIA-type zinc finger repressors of transcription contain a strongly conserved amino acid sequence (L/FDLNL/FxP) in their respective carboxy-terminal regions, which we designated the ERF-associated amphiphilic repression (EAR) motif [2]. This motif is essential for repression and mutations within this repression domain of the tobacco ERF3 protein (ERF3RD; 35 amino acid residues) eliminate the repressive activity of the protein [2]. We also reported that the carboxy-terminal domain (SUPRD; 30 amino acid residues) of SUPERMAN, a TFIIIA-type zinc finger protein that contains an EAR-like motif, functions as a repression domain [3]. When we compared the repression by ERF3RD and SUPRD in transient-expression

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assays, we found that SUPRD had approximately 5-fold more activity than ERF3RD ([2,3], Fig. 1B). Reflecting these results, transgenic plants expressing the gene for a chimeric repressor, in which the ETHYLENE-INSENSITIVE3 protein (EIN3) was fused to SUPRD (*35S::EIN3SUPRD* plants), seemed to be more insensitive to ethylene than *35S::EIN3RD* (=*35S::EIN3ERF3RD*) plants (see Fig. 1B in [4]).

In the present study, to identify elements that are both necessary and sufficient for repression of transcription, we examined the activity of derivatives of SUPRD of various lengths and with various amino acid sequences in transient-expression assays in *Arabidopsis*. We found that a sequence of only six amino acid residues, DLELRL, was necessary and sufficient to confer the capacity for the repression of transcription on a heterologous DNA-binding domain. Moreover, analyses of the effects of mutation and the position of DLELRL suggested a possible mechanism for DLELRL-dependent repression.

Materials and methods

Construction of effector and reporter plasmids. Effector plasmids include the GAL4DB-coding region fused to the coding sequence for a variety of derivatives of SUPRD, in-frame, under control of the cauliflower mosaic virus 35S promoter (−800 to +8; CaMV35S) [5]. The appropriate coding DNA fragments that had been generated by annealing individual pairs of chemically synthesized complementary DNAs were inserted into the *Sma*I and *Sal*I sites of the 35S-GAL4DB plasmid to generate effector plasmids [6]. The reporter gene 35S-GAL4-TATA-LUC-NOS, which was inserted into pUC19, was described previously [3]. This gene consisted of a CaMV35S enhancer (−800 to −46; CaMV35S′), a TATA box (−45 to +8), five GAL4 binding sites, a translational enhancer sequence from tobacco mosaic virus (Ω), the firefly gene for luciferase (LUC), and a nopaline synthase terminator (NOS). The reporter genes corresponding to GAL4-35S-TATA-LUC-NOS, 35S-TATA-GAL4-LUC-NOS, and 35S-TATA-LUC-NOS-GAL4 were constructed by appropriate ligation of these elements in the orders indicated.

Transient expression. Details of analysis of transient expression in *Arabidopsis* leaves after particle bombardment were described previously [3,6]. In most co-transfection assays, 1.6 μg of the reporter construct and 1.2 μg of the effector construct were used for each bombardment. Luciferase (LUC) assays were performed with the Dual-Luciferase Reporter Assay System and a luminescence reader (TD-20/20; Promega, Madison, WI, USA). For normalization of values after each transfection, 0.4 μg of plasmid pPTRL, which includes a LUC gene from *Renilla* under control of the CaMV35S promoter, was co-bombarded as an internal control.

Computerized search for putative repressors. Genes that encode putative TFIIIA-type zinc finger repressors in the *Arabidopsis* genome were searched using our original computer program written by "Perl." This program is available upon request. The genomic sequences of *Arabidopsis* were retrieved from the internet (ftp://ftp.arabidopsis.org/home/tair/; version "ATH1_pep_20030417," released in July 2003). Putative zinc finger motifs were defined as the amino acid sequence Cx(1–5)Cx(10–16)Hx(2–6)H (where "x" can be any amino acid) in the present study. All proteins containing this putative zinc finger motif and an LxLxL sequence located within the carboxy-terminal region of 30 amino acids were listed and proteins that were obviously not TFIIIA-type zinc finger proteins were eliminated manually.

Results

The minimum functional unit of SUPRD is the DLELRL hexapeptide

We confirmed previously that the carboxy-terminal regions of 30–60 amino acids of class II ERFs and of SUPERMAN that contain an EAR motif or an EAR-like motif can act as a repression domain when fused to a heterologous DNA-binding domain [2,3]. However, the EAR-like motif repression domain of SUPERMAN (SUPRD) was about five times as active as the typical EAR-motifs found in class II ERFs in our experimental conditions ([2,3], Fig. 1B). In order to characterize in further detail the functions of EAR-like motifs that mediate the strong repression of transcription, we attempted to identify the minimal functional unit of SUPRD that is both necessary and sufficient for repression of transcription. We set up a transient-expression assay in *Arabidopsis* in which an effector plasmid that encoded a truncated version of SUPRD (RD) was fused downstream of the coding sequence for the DNA-binding domain of the GAL4 protein (GAL4DB) from yeast, under the control of the CaMV35S promoter (35S-GAL4DB-RD). The reporter gene consisted of the enhancer sequence of the CaMV35S promoter and five copies of the GAL4 binding site (35S-GAL4-TATA-LUC-NOS). We coexpressed the effector and reporter in *Arabidopsis* leaves (Col-0) after particle bombardment of leaves (Fig. 1A). As we reported previously by the other reporter plasmid [2,3], SUPRD showed approximately 5-fold more activity than ERF3RD in this reporter system (Fig. 1B). Assays with a series of deleted SUPRD constructs revealed that the hexapeptide DLELRL had repression activity similar to that of SUPRD (Fig. 1B). Further deletion of the first Asp residue or the last Leu residue of DLELRL resulted in loss of repression activity (Fig. 1B), indicating that DLELRL is the minimal functional unit for repression of transcription.

Two Leu residues in DLELRL are necessary for repression

We attempted to determine which amino acid residue(s) might be essential for activity and/or replaceable by others without any effects on activity. We generated several mutant versions of DLELRL fused to GAL4DB by site-directed mutagenesis and examined the resultant effector plasmids in transient-expression assays. As shown in Fig. 2A, whenever any individual residue within DLELRL was replaced by Ala, the mutated peptide had reduced or no ability to repress transcription. In particular, each of the Leu residues appeared to be more essential than the other three hydrophilic residues since replacement of any of the three Leu residues by Ala

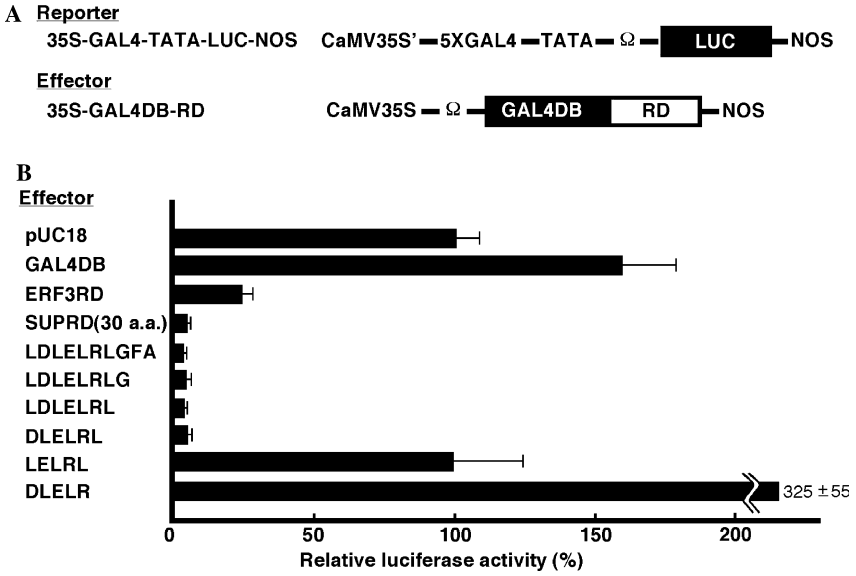


Fig. 1. Mapping of the minimal repression domain of SUPRD. (A) Schematic representation of the constructs used in bombardment experiments. The GAL4-responsive reporter, 35S-GAL4-TATA-LUC-NOS, was constructed as described in Materials and methods. Each effector construct contained GAL4DB and part of the coding region of SUPRD or ERF3RD (RD) under control of the CaMV35S promoter. (B) Relative LUC activities after co-bombardment of *Arabidopsis* leaves with the 35S-GAL4-TATA-LUC-NOS reporter plasmid and the GAL4DB fusion effector plasmids. pUC18 and GAL4DB were used as controls, and the details on the left indicate the RD portion of each effector plasmid. All LUC activities are expressed relative to values obtained after co-bombardment with the reporter plasmid and pUC18 (with the value for pUC18 set arbitrarily at 100%). The values cited are averages, with standard deviations, of results from a minimum of three independent experiments.

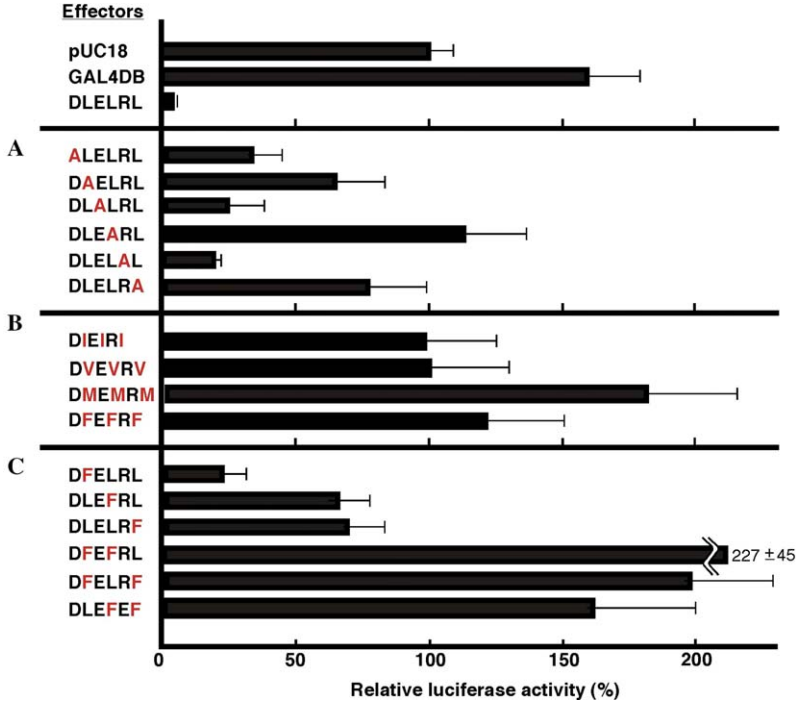


Fig. 2. Mutational analysis of the DELERL minimal repression domain. The reporter and the effector plasmids were the same as described in Fig. 1. The RD portion of each effector plasmid is shown on the left. All of the replaced amino acid residues are indicated by red letters. (A) Replacement of amino acid residues in DLELRL by Ala. Each amino acid residue within DLELRL was replaced sequentially by Ala, as indicated. (B) Replacement of Leu by other hydrophobic amino acids. The three Leu residues of DLELRL were replaced by Ile, Val, Met or Phe, as indicated. (C) Replacement of Leu by Phe. One or two Leu residues were replaced by Phe. All LUC activities are expressed relative to values obtained after co-bombardment of the reporter plasmid and pUC18 (with the value for pUC18 set arbitrarily at 100%). The values cited are averages, with standard deviations, of results from a minimum of three independent experiments.

resulted in a considerable reduction in or completely loss of repression activity. When any of the three Leu residues within DLELRL was replaced by another hydrophobic residue, such as Ile, Val, Met, or Phe, the resultant peptides had no repression activity (Fig. 2B). The derivatives of DLELRL in which each Leu in turn was changed to Phe (DFELRL, DLEFRL, and DLELRF) functioned weakly as repression domains. However, when two of the three Leu residues within DLELRL were replaced by Phe in any combination (DFEFRL, DFELRF, and DLEFEF), the resultant peptides lost their repression activity completely (Fig. 2C). These results indicated that the Leu residues cannot be replaced by other hydrophobic amino acids if strong repressive activity is to be required and, moreover, that at least two Leu residues are indispensable for repression.

The DLELRL hexapeptide is conserved in the carboxy-terminal region of TFIIIA-type zinc finger proteins in Arabidopsis

We searched the *Arabidopsis* genome for putative repressor proteins similar to SUPERMAN (SUP) that had a TFIIIA-type zinc finger DNA-binding domain and a sequence that resembled DLELRL in the carboxy-terminal region, as described in Materials and methods. We found 32 candidate proteins, including SUPERMAN (Fig. 3). The number of zinc finger domains in these proteins varied from one to three but proteins with one zinc finger were dominant. Eight proteins contained a complete DLELRL sequence, while the others had sequences that included different residues in place of the Asp, Glu, and Arg residues of DLELRL. To assess the importance of the hydrophilic residues other than Leu in DLELRL, we constructed 18 effector plasmids that encoded the derivatives of DLELRL from all of the zinc finger proteins shown in Fig. 3 and investigated their repression activities in transient-expression assays. As shown in Table 1, DLDLRL and DLTLRL had repression activity similar to that of DLELRL (the level of expression of the reporter gene was reduced by >90%), while DLSLRL, DLSLKL, DLSLSL, DLSLHL, DLTLKL, CLDLRL, SLDLHL, SLDLRL, NLNLKL, and CLDLSL had moderate activity that was similar to that of a typical EAR-motif repression domain (the level of expression of the reporter gene was reduced by 65–85%) ([2,3], Fig. 1B). By contrast, SLSLSL had weak activity (the level of expression of the reporter gene was reduced by only about 2%), while DLKLEL, DLHLSL, CLDLDL, and SLSLKL appeared to function as activation domains. The results for DLSLHL, SLDLHL, and DLHLSL indicated that the particular combination of amino acid residues at the first, third, and fifth positions is important for repression activity. When we compared the results for DLDLRL and CLDLRL and for DLTLRL and DLTLKL, for example, we found that Asp at the first position and Arg at the fifth position

Gene ID#		Zinc finger motif	RD motif	Length
At3g23130	(SUPERMAN)	1 CSFCKREFRSAQALGGHMNVH	GLINESEQDLDLELRLGFA	204
At2g37740	(AtZFP10)	1 CSFCRREFKSAQALGGHMNVH	KHKGDRFEDLDLELRLGTDPPKGI	304
At2g42410	(AtZFP11)	1 CSFCRREFRSAQALGGHMNVH	MSLRNPNQVLDLELRLGYL	214
At3g09290		1 CSFCIRGFSNAQALGGHMNIH	SSHHRDIEVLDLELRLGQSVVKKKTT	172
At3g53820		1 CEFCERGFSNAQALGGHMNIH	KALSQSADVIDLELRLGLDPYKKTTSTST	142
At4g17810		1 CNFCRREFRSAQALGGHMNVH	EATGTSVDELDLELRLGHHPP	180
At5g06070		1 CSFCGREFKSAQALGGHMNVH	SEINGHHEELDLELRLGADPPKVN	226
At5g43540		1 CTFCKRGFTNAQALGGHMNIH	ENVVVEGNEIDLELRLGL	137
At3g23140		1 CDICKRGFTNPQALGGHNNIH	GGSQPQDGDLDLDLRLGGHRHH	172
At1g24625	(ZFP7)	1 CNYCRRKFYSSQALGGHQNAH	GGVDNNSSKPDLTLRL	209
At1g04445		1 CAVCKRVFLSSHQLISHYNAAH	ENGGANSSPIDLSLRL	172
At5g14010		1 CQYCPRKFYTSQALGGHQNAH	VMEEDEPLDLDLSLRL	161
At5g57520	(ZFP2)	1 CNYCQRKFYSSQALGGHQNAH	LDQDQEKSHLDLSLRL	150
At5g01860		1 CTFCKKEFSTSQALGGHQNAH	EADPCKDSDIDLSLKL	215
At5g10970		1 CNYCQRTFYSSQALGGHQNAH	PAEEEKQKNLDLSLKL	272
At5g25160	(ZFP3)	1 CNYCQRTFYSSQALGGHQNAH	TSHHEEQQKLDLSLKL	235
At5g27880		1 CHFCKKGFSTSQALGGHQNAH	EEEKTKSGRIDLSLSL	278
At5g48890		1 CLFCSRKFHSSQALGGHQNAH	NSDKGKDQCLDLSLHL	173
At1g66140	(ZFP4)	1 CNYCQRKFYSSQALGGHQNAH	QAMDMESSLPDLTLKL	260
At1g80730		1 CNYCQRKFYSSQALGGHQNAH	QEDHNQFKKIDLTLKL	228
At4g35280		3 CNICFRVFSSGQALGGHMRCH	DLSTSDTSGCCLDLRLGL	284
At1g10480	(ZFP5)	1 CQYCGKEFANSQALGGHQNAH	SRSDQMRSINSLDLHLGFAGDAA	211
At2g41940		1 CHYCFRNFPTSQALGGHQNAH	LMKPNVQDHVSLDLHL	257
At3g58070		1 CHYCFRNFPTSQALGGHQNAH	GLSPNVQDHVSLDLHL	253
At2g17180		3 CNICSRVFSSGQALGGHMRCH	ATSSDTTLGCSLDLRLGL	270
At5g06650		1 CHYCFRNFPTSQALGGHQNAH	ESKKNVPDHVSLDLRL	191
At5g59820	(ZAT12)	2 CPICGVEFPMGQALGGHMRRH	CLDLSLGMVDNLNLKLELGRTVY	162
At5g05120		1 CKYCPRKFDKTQALGGHQNAH	AEKENDGSSLSLSLKL	201
At2g28710		2 CPICGAEFAVGQALGGHMRKH	DLNLTPLENEDLKLELGRFIF	156
At1g67030	(ZFP6)	1 CQYCCREFANSQALGGHQNAH	GIKLENGIGLDLHLSLGP	197
At1g68360		1 CQYCCREFGNSQALGGHQNAH	GTTFDDGLGLDLHLSLAPAGH	244
At3g46090	(ZAT7)	2 CPICGVKFPMGQALGGHMRRH	KKFSSGKRVACLDLDLDSMESLVNWKLELGRTISWS	168

Fig. 3. Alignment of amino acids in the zinc finger domains and the carboxy-terminal regions of putative TFIIIA-type zinc finger transcriptional repressors. The number on the left of each of the zinc finger motif indicates the number of zinc finger domains found in each protein. For zinc finger proteins, with more than one, only one is included. In the column labeled RD motif, for repression domain motif, red letters indicate putative repression domains, and the regions of the hexapeptides that overlap each other are indicated by dark-red letters. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

Table 1
Repression activities of the hexapeptides similar to DLELRL

	Relative LUC activity (%)[c]
Hexapeptide[a]	
DLELRL	6±1
DLDLRL	6±3
DLTLRL	10±3
DLSLRL	20±3
DLSLKL	18±7
DLSLSL	25±9
DLSLHL	24±6
DLTLKL	36±10
CLDLRL	18±3
SLDLHL	35±10
SLDLRL	17±5
NLNLKL	15±9
CLDLSL	19±1
SLSLSL	98±16
DLKLEL	174±16
DLHLSL	117±4
CLDLDL	446±16
SLSLKL	289±30
Hexapeptide[b]	
DLNLRL	4±2
DLQLRL	1±1
DLRLRL	4±3
ELELRL	4±3
NLELRL	7±2
QLELRL	36±15
DLELEL	127±18
DLELDL	92±17
DLELNL	21±8
DLELQL	51±11
SLELRL	7±2
TLELRL	21±10
DLELTL	9±3
DLELSL	26±9
DLHLRL	13±4
NLNLNL	110±23
Control (pUC18)	100

[a] The peptides from the *Arabidopsis* TFIIIA-type zinc-finger proteins listed in Fig. 3.
[b] The peptides designed artificially and from the *Zea ramosa* TFIIIA-type zinc-finger protein.
[c] Relative LUC activities to control (pUC18, set as 100%).

were indispensable for strong repressive activity, whereas the residue at the third position seemed to be more tolerant to variations. To confirm that the motif DLxLRL is essential for strong repression, we prepared another 16 effector plasmids that contained 15 custom-designed derivatives of DLELRL and the DLQLRL motif from the TFIIIA-type zinc finger protein of *Zea ramosa* (CAD23294), respectively, and then we analyzed their activities (Table 1). The results and those described above indicated that Arg at the fifth position is important but Thr is also tolerated at this position, while the first Asp residue is replaceable by Glu, Asn or Ser.

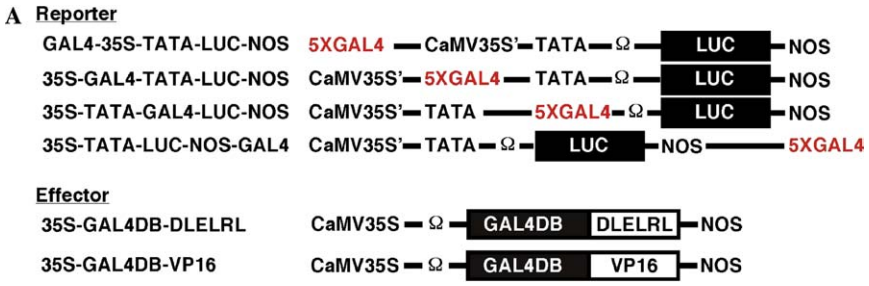
The results of our transient-expression assays revealed that the amphiphilic motif composed of six amino acids (XLxLXL) with preferences at the first (X = D/N/Q/S) and fifth positions (X = R/T) is necessary and sufficient for strong repression, and that the combination of residues at the first and fifth positions is important if the peptide is to act as a repression domain with strong repression activity as described above.

GAL4DB–DLELRL functions as a short-range repressor and is active immediately upstream of the TATA box

To characterize the mechanism of repression that is mediated by the DLELRL repression domain, we examined whether the position of the target site with which the GAL4DB–DLELRL repressor interacts might affect the repression. We prepared four different reporter genes, in which the target site (5xGLA4) was located, respectively, upstream of the CaMV35S enhancer, between the CaMV35S enhancer and the TATA box, downstream of the TATA box, and downstream of the NOS terminator (Fig. 4A). To compare the effects of position on DLELRL-dependent repression to those for activation of the well-characterized VP16 activation domain of *Herpes simplex* virus [7], we used 35S-GAL4DB-VP16 [8] as the effector plasmid. Fig. 4B shows that GAL4DB–DLELRL effectively repressed the expression of the reporter gene when the target site was located upstream of the TATA box but was less effective in the case of other targeted sites. These results indicated that GAL4DB–DLELRL functions as a short-range repressor. It is noteworthy that transcriptional activation by GAL4DB-VP16 occurred only when the target was upstream of the TATA box, and it functioned as a weak repressor when the target was at other sites. These results suggest that the mechanism of DLELRL-dependent repression might be similar to that of VP16-dependent activation and might involve interaction(s) with general transcription factors [9]. When GAL4DB or fusion proteins that included GAL4DB were targeted to sites downstream of the TATA box or downstream of the NOS terminator, they seemed to function as passive repressors because the extent of repression was similar for all the GAL4DB-containing effector plasmids (Fig. 4B).

Discussion

In this study, we showed that the minimum sequence of the repression domain of SUPERMAN is the DLELRL hexapeptide, which is both necessary and sufficient for repression when fused to a heterologous DNA-binding domain. We also demonstrated that the three Leu residues in DLELRL are important for repression in a series of substitution experiments (Fig. 2). Tiwari et al. [10] reported that the LxLxL motif within domain I of AUX/IAA proteins is important for repression and noted its similarity to EAR and EAR-like motifs. They also



B

Effector \ Reporter	GAL4-35S-TATA-LUC-NOS	35S-GAL4-TATA-LUC-NOS	35S-TATA-GAL4-LUC-NOS	35S-TATA-LUC-NOS-GAL4
pUC18	100 (%)	100	100	100
GAL4DB	92 ± 13	97 ± 8	81 ± 5	59 ± 23
DLELRL	58 ± 15	8 ± 2	55 ± 8	84 ± 7
VP16	55 ± 6	325 ± 12	45 ± 6	86 ± 12

Fig. 4. Positional effects of DLELRL-dependent repression of transcription. (A) Schematic representation of the constructs used in bombardment experiments. Construction of the reporter and the effector plasmids is described in Materials and methods. The position of 5xGAL4, the target site for the GAL4DB-RD and GAL4DB-VP16 effectors, is indicated in red letters. (B) LUC activities in *Arabidopsis* leaves that had been co-bombarded with reporter and effector plasmids. All LUC activities are expressed relative to values obtained after co-bombardment of leaves with each reporter plasmid and pUC18 (with values for pUC18 set arbitrarily at 100%). The values cited are averages of results from a minimum of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

noted the importance of the Leu residues in the LxLxL motif, which is consistent with our results. However, the minimal sequence that acts as a repression domain within the domain I of AUX/IAA proteins has not been determined [10].

In case of the LxLxL motif within domain I of AUX/IAA proteins, replacement of the amino acid residues that corresponded in terms of position to Asp, Glu, and Arg in DLELRL by Ala did not critically affect repression [10]. By contrast, in our study, replacement of Asp, Glu or Arg of DLELRL by Ala resulted in a major reduction in repression. These conflicting results may be due to differences in the activities of repression between protoplast and leaves, in the length of the repression domain that were used for each transient assay, or in the strength of repressive activity between DLELRL and the domain I of AUX/IAA proteins. The latter had repressive activity similar to that of the typical EAR-motif repression domain of AtERF4 (AtERF4RD) [10], whereas DLELRL was approximately five times more active as a repressor than AtERF4RD in transient-expression assays (data not shown). Strong repression may require more strictly conserved sequences of amino acid residues even at the ''non-Leu'' positions in the amphiphilic XLxLXL motif. Some hexapeptides that we examined had no repressive activity even when the three Leu residues were conserved at their proper positions (Fig. 3).

To summarize: the three Leu residues in the minimal hexapeptide repression domain are critical, as indicated in the LxLxL motif of AUX/IAA proteins by Tiwari et al. [10], but the other residues are also important for repression. It is clear, moreover, that appropriate combinations of amino acid residues in the amphiphilic XLxLXL motif are required for repression.

In the *Arabidopsis* genome, we found 32 TFIIIA-type zinc finger proteins that contained DLELRL or a derivative in the carboxy-terminal region and the hexapeptides from 27 of those proteins had repressive activity. The various TFIIIA-type zinc finger proteins with a hexapeptide repression domain might function as repressors of transcription in vivo since they resemble SUPERMAN not only in terms of the repression domain but also in terms of their entire structure, including the TFIIIA-type zinc finger domains. In particular, the AtZFP11 protein contains the DLELRL sequence in its carboxy-terminal region and the importance of this sequence for the action of AtZFP11 was reported by Dinkins et al. [11].

Minimization of the repression domain is the first step towards elucidation of the mechanism of repression. In this study, we identified a sequence of six amino acid residues, DLELRL and its derivatives, as the minimal repression domain. This domain is, to our knowledge, smallest repression domain identified to date. Many mechanisms for active repression have been reported in eukaryotes [9], involving interactions with general transcription factors, the chromatin remodeling complex, and other macromolecules. Therefore, the identification of the factors that interact with the

minimal repression domain is now of prime importance. Our results indicated that the GAL4DB–DLELRL functioned as a short-range repressor and that the mechanism of DLELRL-dependent repression might be similar to that of VP16-dependent activation that involves general transcription factors [9]. The DLELRL repression domain might interact with the general transcriptional machineries. Because the repression domain of AUX/IAA protein (IAA17) functions as a short-range repressor [10], it might also interact with the general transcriptional machineries.

In the murine zinc finger-homeodomain transcriptional repressor, δEF1, the PLDLSL sequence is essential for repression of transcription and for interactions with corepressors, namely, carboxy-terminal binding protein 1 (CtBP1) and CtBP2 [12]. This sequence is also conserved among adenovirus E1A proteins as a CtBP-binding motif [12]. Because of the similarity between the CtBP-binding motif and the minimal repression domain identified in this study, we examined whether the *Arabidopsis* homolog of CtBP might affect repression that is mediated by the DLELRL repression domain. We performed transient-expression assays using *Arabidopsis* plants with a mutant *ANGUSTIFOLIA* gene, which is the gene for the plant ortholog of CtBP [13,14], but we detected no reduction in the extent of repression by DLELRL (data not shown). These results suggest that ANGUSTIFOLIA is not likely to be involved in the mechanism of DLELRL-dependent repression of transcription.

Repression motifs that resemble the EAR-like motif (=the amphiphilic XLxLXL motif) of the TFIIIA-type zinc finger family can be found at least in four families of plant transcription factors. They are found in members of the ERF family and the TFIIIA-type zinc finger family (in which the EAR motif is L/FDLNL/FXP) [2]; in members of the AUX/IAA family (the LxLxL motif) [10]; and in members of subgroup 4 of Myb family (pdLNLD/ElxiG/S) [15], which can be included in this category. Some members of each family have been shown to function as repressors or corepressors [3,10,16]. Many transcriptional repressors and corepressors in other families of proteins that contain the repression motifs described above might be identified in the future. Our observations move us a little closer towards elucidation of mechanisms of transcription and the identification of the full complement of the repressors and corepressors of transcription in plants.

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References

[1] W. Hanna-Rose, U. Hansen, Active repression mechanisms of eukaryotic transcription repressors, Trends Genet. 12 (1996) 229–234.
[2] M. Ohta, K. Matsui, K. Hiratsu, H. Shinshi, M. Ohme-Takagi, Repression domains of class II ERF transcriptional repressors share an essential motif for active repression, Plant Cell 13 (2001) 1959–1968.
[3] K. Hiratsu, M. Ohta, K. Matsui, M. Ohme-Takagi, The SUPERMAN protein is an active repressor whose carboxy-terminal repression domain is required for the development of normal flowers, FEBS Lett. 514 (2002) 351–354.
[4] K. Hiratsu, K. Matsui, T. Koyama, M. Ohme-Takagi, Dominant repression of target genes by chimeric repressors that include the EAR motif, a repression domain, in *Arabidopsis*, Plant J. 34 (2003) 733–739.
[5] R.X. Fang, F. Nagy, S. Sivasubramaniam, N.H. Chua, Multiple cis regulatory elements for maximal expression of the cauliflower mosaic virus 35S promoter in transgenic plants, Plant Cell 1 (1989) 141–150.
[6] M. Ohta, M. Ohme-Takagi, H. Shinshi, Three ethylene-responsive transcription factors in tobacco with distinct transactivation functions, Plant J. 22 (2000) 29–38.
[7] S.J. Triezenberg, R.C. Kingsbury, S.L. McKnight, Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early gene expression, Genes Dev. 2 (1988) 718–729.
[8] S.Y. Fujimoto, M. Ohta, A. Usui, H. Shinshi, M. Ohme-Takagi, *Arabidopsis* ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression, Plant Cell 12 (2000) 393–404.
[9] S.G. Roberts, Mechanisms of action of transcription activation and repression domains, Cell. Mol. Life Sci. 57 (2000) 1149–1160.
[10] S.B. Tiwari, G. Hagen, T.J. Guilfoyle, Aux/IAA proteins contain a potent transcriptional repression domain, Plant Cell 16 (2004) 533–543.
[11] R.D. Dinkins, C. Pflipsen, G.B. Collins, Expression and deletion analysis of an *Arabidopsis SUPERMAN*-like zinc finger gene, Plant Sci. 165 (2003) 33–41.
[12] T. Furusawa, H. Moribe, H. Kondoh, Y. Higashi, Identification of CtBP1 and CtBP2 as corepressors of zinc finger-homeodomain factor δEF1, Mol. Cell. Biol. 19 (1999) 8581–8590.
[13] U. Folkers, V. Kirik, U. Schobinger, S. Falk, S. Krishnakumar, M.A. Pollock, D.G. Oppenheimer, I. Day, A.S. Reddy, G. Jurgens, M. Hulskamp, A.R. Reddy, The cell morphogenesis gene *ANGUSTIFOLIA* encodes a CtBP/BARS-like protein and is involved in the control of the microtubule cytoskeleton, EMBO J. 21 (2002) 1280–1288.
[14] G.T. Kim, K. Shoda, T. Tsuge, K.H. Cho, H. Uchimiya, R. Yokoyama, K. Nishitani, H. Tsukaya, The *ANGUSTIFOLIA* gene of *Arabidopsis*, a plant *CtBP* gene, regulates leaf-cell expansion, the arrangement of cortical microtubules in leaf cells and expression of a gene involved in cell-wall formation, EMBO J. 21 (2002) 1267–1279.
[15] H.D. Kranz, M. Denekamp, R. Greco, H. Jin, A. Leyva, R.C. Meissner, K. Petroni, A. Urzainqui, M. Bevan, C. Martin, S. Smeekens, C. Tonelli, J. Paz-Ares, B. Weisshaar, Towards functional characterization of the members of the *R2R3-MYB* gene family from *Arabidopsis thaliana*, Plant J. 16 (1998) 263–276.
[16] H. Jin, E. Cominelli, P. Bailey, A. Parr, F. Mehrtens, J. Jones, C. Tonelli, B. Weisshaar, C. Martin, Transcriptional repression by AtMYB4 controls production of UV-protecting sunscreens in *Arabidopsis*, EMBO J. 19 (2000) 6150–6161.