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Translational fusions with the *engrailed* repressor domain efficiently convert plant transcription factors into dominant-negative functions

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Received June 27, 2002; Revised and Accepted September 6, 2002

ABSTRACT

Evidence is provided that plant transcription factors can be efficiently reprogrammed to dominant-negative functions by the use of a repressor domain of the *engrailed* (*en*) gene from *Drosophila*. Ectopic expression of translational fusions between the *en*^{298} N-terminus and the complete coding regions of the *SHOOTMERISTEMLESS*, *APETALA3*, *PISTILLATA* and *KNAT1* transcription factors results in trans-dominant functions which phenocopy loss-of-function mutants. The combination of the dominant-negative *en*^{298}*-STM* function with the hormone-binding domain of the glucocorticoid receptor provides strong evidence that phenocopies rely on the incorporation of the chimeric protein into the nuclear compartment. By this dominant-negative approach *KNAT1* was rapidly identified to be encoded by the *BREVIPEDICELLUS* locus. Dominant-negative chimeric proteins may be of wide use to elucidate biological functions of plant transcriptional activators and may be suitable to study protein–protein interactions *in planta*.

INTRODUCTION

Plant transcription factors mostly belong to gene families containing numerous members. An example of how large such families can grow is the *myb* family comprised of at least 136 genes in *Arabidopsis* (1). Homeobox and MADS box genes are also found in multiple copies comprising several subfamilies in all plant species analysed so far (2,3). In genetic screens for loss-of-function alleles, only a few of these genes have been associated with a mutant phenotype. One explanation for numerous silent family members may be genetic redundancy; each gene-specific contribution may be subtle or dependent on special conditions. This fraction of silent genes in plants and in other genomes provides a major challenge in molecular biology because it is foreseen to oppose a functional analysis also in reverse genetic approaches.

To identify biological functions of transcription factors in *Drosophila* and amphibian development, an alternative method has been successfully used. A pioneering experiment was performed in *Drosophila* where the DNA-binding homeodomain of the Engrailed (En) gene product was replaced by its Fushi tarazu (Ftz) counterpart and expression of the chimeric gene controlled by a heat-shock promoter. After induction, the sequence-specificity of the Ftz homeodomain directed the strong transcriptional *en* repressor function to *ftz* target genes resulting in phenocopies of *ftz* loss-of-function alleles in transgenic progeny (4). In addition to the En^{298} repressor domain, the *Krüppel* associated box (5) is now used in animal systems where these repressor domains are combined frequently only with the DNA-binding domain of the transcription factor in question (6). Functional studies are subsequently performed either in transient gene expression experiments, e.g. tissue culture cell lines, where the c-MYB DNA-binding domain combined with the En^{298} domain causes G1 cell cycle arrest or apoptosis (7). In contrast, and more informative in respect to developmental questions, have been injections of chimeric RNA transcripts into oocytes, zygotes or developing embryos, a method which identified the Xenopus Brain Factor 2 (XBF-2) as a transcriptional repressor converting ectoderm into neural tissue (8).

Similar experiments in plants are restricted by the lack of functional repressor domains. However, plant transcription factors share a significant degree of similarity with their animal counterparts. Essential subunits of the transcription initiation complex are so similar that X-ray data originally obtained for the *Arabidopsis* TATA box binding protein (9) have been generalised to animal species (10). Also conserved are DNA-binding motifs like the homeodomain (2), the *myb*-motif (11) and the MADS box (3) or dimerisation motifs such as leucine zippers (12) or helix–loop–helix domains (13). In addition, transcriptional activation domains of the VP16 protein from Herpes simplex virus (14) and the GAL4 gene product from yeast (15) have been shown to be functional in plants. For the VP16 activation domain, direct interactions with TAFII31 (16) in the transcription initiation complex are known, and similar interactions can thus be implicated in plants. This obvious conservation of transcription factors and basic transcriptional machinery between plant and animal species made it tempting to test whether animal repressor domains might be functional in plants.

One criterion of functionality could be dominant-negative functions provided by chimeric gene products in analogy to the pioneering En-Ftz experiment in *Drosophila* (4). For an

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initial study we decided to test three genes: *SHOOT MERISTEMLESS* (*STM*) (17) an essential function in the shoot apical meristem (SAM), *APETALA3* (*AP3*) (18) and *PISTILLATA* (19) contributing to flower development. While *STM* belongs to the class of homeodomain proteins, the floral organ identity genes *AP3* and *PI* encode MADS-box proteins, which depend on heterodimerisation to achieve transcriptional control function (19). The three genes are not only members of different classes of plant transcription factors, but their mutant phenotypes affect different developmental stages, the early vegetative phase or rather late floral development.

Here, we describe ectopic expression experiments performed with *en*^{298}-*STM* and *en*^{298}-*AP3* or *en*^{298}-*PI* translational fusions, which result in transdominant phenocopies of *stm* or *ap3/pi* phenotypes in >75% of the primary transformants. The performance of the CHimeric Repressor Interference System (CHRIS) with unknown gene functions was tested with the *KNAT1* gene (20), a rather close relative to *STM*, which is also expressed in the SAM. Although at the time of this study *KNAT1* was not associated with any known mutant, the dominant-negative *en*^{298}-*KNAT1* fusion phenotype rapidly revealed the relationship to the *BREVIPEDICELLUS* locus.

MATERIALS AND METHODS

Chimeric gene constructions

As a precursor to all translational fusions a 929 bp *Afl*II–*Bam*HI fragment of the cDNA clone D_2B (21) was inserted between the CaMV 35S promoter and the poly(A) signal into *Not*I/*Bam*HI cleaved pRTΩNot-Asc vector (22). These cDNA sequences cover the natural translation start and encode the first 298 amino acid residues of the *Drosophila* Engrailed protein. Unique *Nco*I and *Bam*H1 sites were created behind the *en*^{298} N-terminus, resulting in the pCHRIS vector, and used to insert the complete protein coding regions which were amplified by PCR with appropriate restriction site additions. The *STM* coding sequences were kindly provided by Dr R. Simon, the *AP3* cDNA clone was a gift from Dr Zsuzsanna Schwarz Sommer (MPI für Züchtungsforschung) and the *PI* and *KNAT1* reading frames were isolated via RT–PCR. The entire open reading frames (ORFs) were fused to the *en*^{298} N-terminus, the ATG start being inherent in the *Nco*I site. The initial C-terminal deletions of *STM* made use of unique restriction sites in the *STM* coding sequence; all N-terminal deletions and subdomains were created via PCR. The engineering of a *Bam*HI site upstream from the *STM* TGA stop codon allowed the C-terminal fusion to the hormone-binding domain of the glucocorticoid receptor. The complete chimeric *en*^{298}-*STM* reading frame was transferred to the binary vector pBI-ΔGR (23). Generally, translational fusions and PCR products were verified by DNA sequencing. For complementation of the *bp-1* mutant the *KNAT1* coding region was inserted into the pRTΩNot-Asc vector (22).

Plant transformation, dexamethasone induction and analysis of transgenic plants

For transfer into the *Arabidopsis* genome the translational *en*^{298} fusions in pCHRIS were inserted into pGPTV-BAR *Asc*I (22) by use of the *Asc*I sites flanking the *CaMV 35S* promoter and poly-A signal. The complete binary vectors were transferred into *Agrobacterium tumefaciens* GV310 and the resulting strains were used for infiltration of *Arabidopsis* immature inflorescences (24). Transgenic plants were selected for BASTA resistence except *en*^{298}-*STM/GR* transgenics which carried the kanamycin-resistance marker. Hormone induction was performed by spraying wild-type control plants or transgenic *en*^{298}-*STM/GR* seedlings at the four- to six-leaf stage with 1 µM dexamethasone. Plants were photographed every day in the same orientation and magnification. Pictures were processed with the Adobe Photoshop 7.0 software on a Macintosh G4 computer.

Total RNA of *Arabidopsis* seedlings or inflorescences was extracted as described (25) and poly(A)$^+$ RNA enriched by oligo-dT chromatography. RNA samples were separated on formaldehyde agarose gels (1.2%) and transferred to Hybond N+ membranes (Amersham Buchler). Filters were hybridised overnight either at 42°C in 50% formamide, 6× SSPE, 5× Denhardt's, 0.5% SDS and 250 mg/ml CT-DNA or at 68°C in 0.7 M Na-phosphate pH 7.2, 7% SDS. Washing was performed at 68°C in 0.2× SSPE, 0.1% SDS. Probes labelled by random oligonucleotide priming were sufficient to visualise the transgene transcript in total or poly(A)$^+$ RNA. Single-stranded RNA probes were needed to confirm the endogenous *STM* transcript in poly(A)$^+$ RNA. First strand cDNA synthesis for RT–PCR was performed with the Superscript system (Gibco-BRL) primed with oligo-dT on total RNA (5 µg). PCRs generally contained 1 µM primer, 0.2 mM NTPs and 0.2 U *Taq* polymerase and were routinely run with 30 cycles. A unique forward primer CTCCCTAAAGAAGCTCGTC-AAC located in the *STM* coding region was combined with the *STM*-specific (CGCATAACAATAGAACACCAAAAGG) or the pCHRIS-specific (CCTTATCTGGGAACTACTCAC) reverse primer to discriminate between the native or chimeric transcript, respectively.

RESULTS

Chimeric gene constructions and plant transformation

For the construction of translational fusions between the *Drosophila* Engrailed (En) repressor domain and plant transcription factors a 929 bp cDNA fragment encoding 298 amino acids of the *en*^{298} N-terminus (21) was cloned behind the *CaMV 35S* promoter into the vector pRTΩNot (22). Two restriction sites, *Nco*I and *Bam*HI, inserted behind the repressor domain allowed the directional cloning of protein coding regions in the resulting pCHRIS vector. The ATG inherent in the *Nco*I site (CCATGG) allows in-frame fusions between the *en*^{298} coding sequences and the native methionine start codon. In this study, the *STM*, *AP3*, *PI* and *KNAT1* (17–20) coding regions were inserted. Translation of all chimeric proteins always starts from the translation start codon of the *Drosophila en* ORF.

To enable subsequent fusion with the hormone-binding domain of the rat glucocorticoid receptor in the vector pBI-ΔGR (23) a unique *Bam*HI site was introduced before the stop codon into the *STM* ORF. The C-terminal addition of the hormone-binding domain in the chimeric En^{298}-STM/GR protein should interfere with nuclear uptake until application of the dexamethasone hormone. All the different constructs schematically represented in Figure 1 were transferred into the

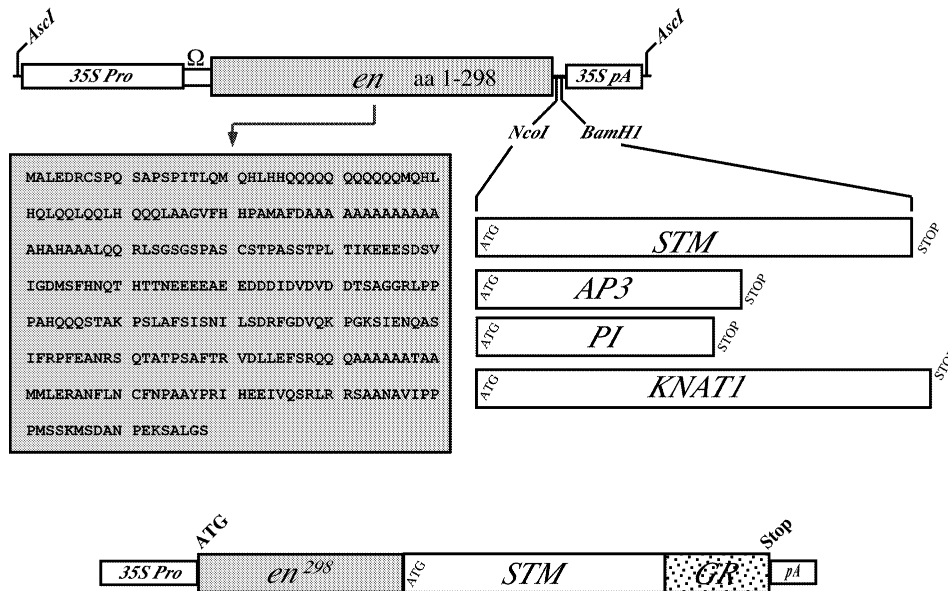

Figure 1. CHRIS expression cassette. The *TMV* Ω untranslated leader sequence and the *engrailed* N-terminal coding sequences (1–298 amino acids; grey box) were inserted between the *CaMV 35S* promoter and polyadenylation signal. The En^{298} protein sequence is indicated below the CHRIS cassette. The *STM*, *AP3*, *PI* and *KNAT1* coding regions (drawn to scale) were inserted directionally between the unique *Nco*I and *Bam*HI cloning sites of the pCHRIS vector. For comparison the *en*^{298}-*STM/GR* cassette in pBI-ΔGR is indicated below.

Arabidopsis genome via *A.tumefaciens* mediated T-DNA transfer.

Constitutive expression of chimeric *en*^{298}-*STM*, *en*^{298}-*AP3* or *en*^{298}-*PI* constructs mimics loss-of-function alleles

Abnormalities in the development of transgenic *en*^{298}-*STM* plants became evident during the earliest stages of seedling development. Compared to wild-type (Fig. 2A), where the SAM is always enclosed by several leaf primordia, the single leaf in the transgenic *en*^{298}-*STM* plant (Fig. 2B) originates between the petioles of both cotyledons. The close-up (Fig. 2C) shows that an apical dome is missing. Sometimes SAM activity in *en*^{298}-*STM* plants is completely terminated after the initiation of a single leaf. Most transgenic seedlings, however, cease development after four to seven leaf primordia, which all emerge in the absence of a detectable apical dome. The photograph in Figure 2D shows leaf 3 originating between the petioles of leaves 1 and 2. These trans-dominant alterations phenocopy weak *stm* alleles and have been reproduced in various independent large-scale experiments (>1000 transgenic T1 progeny) and are generally found in >75%, and sometimes up to 90%, of the primary transformants.

Concomitantly, or after a short break, axillary meristems are activated. The resulting secondary shoot axes may develop from normal rosettes, but functional deficiencies in these axillary meristems become evident during the inflorescence phase. A lack of internode elongation in most *en*^{298}-*STM* plants results in airborne rosettes of several cauline leaves (Fig. 2E). Characteristic is a cycling back and forth between elongating and non-elongating internodes resulting in multiple airborne rosettes. Exceptionally, such transgenic plants never flower, but most transgenic plants develop a few fertile flowers. The inheritance of phenocopies was analysed in 20 lines originating from primary *en*^{298}-*STM* transformants (T_1). BASTA resistant T_2 progeny were obtained from 18 T_1 plants and *stm* phenocopies were recovered in 16 families, although with variable strength. The absolute numbers of affected progeny plants varied between different families, however, none showed a 3:1 segregation of the BASTA resistance marker. The phenotypic variability therefore is presumably related to multiple T-DNA copies and the expression level of the transgene; low expression levels could also account for the failure to recover phenocopies in two of the 20 families.

To exclude silencing of the endogenous *STM* gene, transgenic *en*^{298}-*STM* phenocopy plants were subjected to RNA gel-blot and RT–PCR analysis. The chimeric transcripts derived from the *CaMV 35S* promoter were easily detectable in total or poly(A)$^+$ RNA probed with the *STM* coding regions (Fig. 2F). Although the high abundance of chimeric transcripts interfered with the detection of the shorter native mRNA in these gel-blot experiments, discrimination could be achieved by probing with the natural 3′-untranslated region (UTR) sequences that are lacking the *en*^{298}-*STM* transgene (Fig. 2F, right). The reduction in transcript level is not due to the absence of the *STM* transcripts in individual T_1 progeny, because the native transcripts could be detected by RT–PCR experiments in each individual phenocopy plant (Fig. 2G). The level of native *STM* transcripts is always significantly lower in transgenic seedlings than in wild-type, which coincides with the absence of an apical dome in the phenocopy plants.

In contrast to the phenotypes during vegetative and inflorescence development in *en*^{298}-*STM* transgenic plants, the phenocopies caused by ectopic expression of the *en*^{298}-*AP3* or *en*^{298}-*PI* construct were exclusively restricted to the flower. In 76% (*en*^{298}-*AP3*: 109/143) or 83% (*en*^{298}-*PI*: 159/183) of the primary transformants obtained with each chimeric

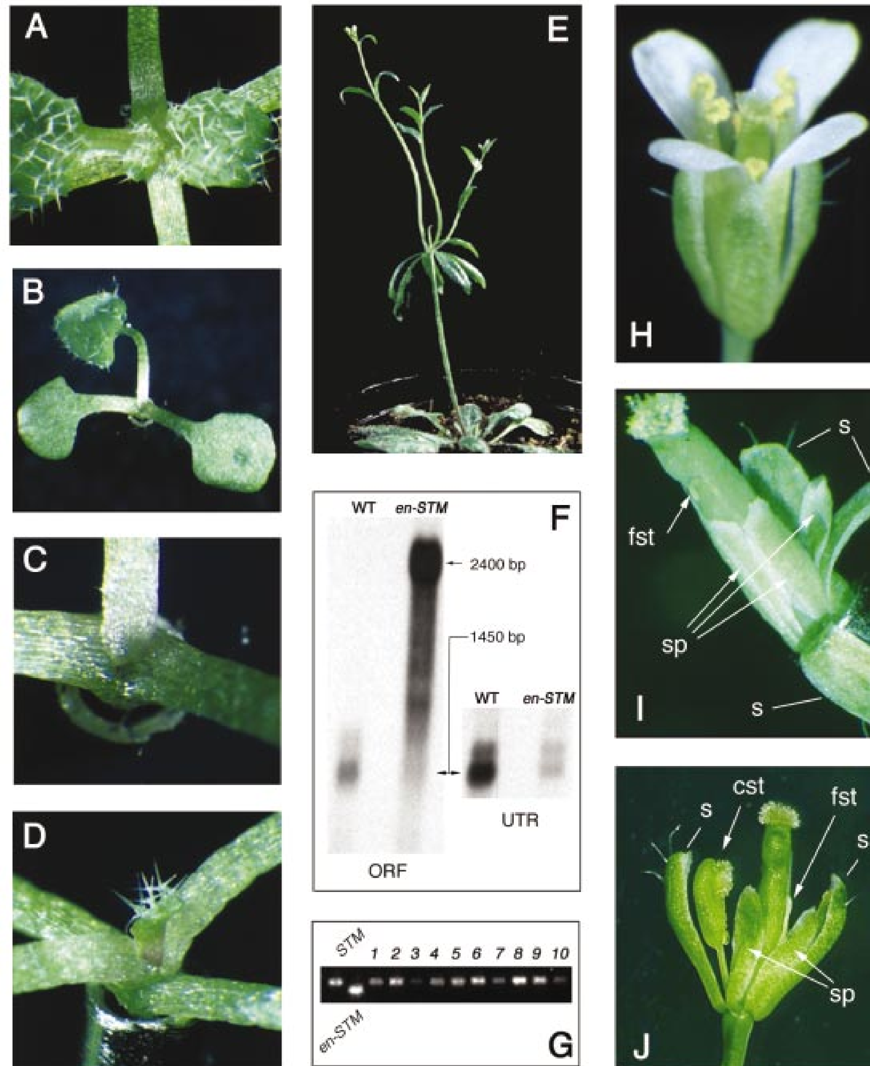

Figure 2. Analysis of *en^{298}-STM*, *en^{298}-AP3* or *en^{298}-PI* transgenic plants. (**A**) Six-leaf stage wild-type seedling. Note the small leaflets enclosing the wild-type SAM in comparison with the *en^{298}-STM* transgenic seedling (**B**), with only a single leaf emerging between the two cotyledons. (**C**) Close-up of the apex of the same transgenic seedling as in (B) showing the absence of an apical dome. (**D**) Emerging third leaf in a weaker *stm* phenocopy. (**E**) Inflorescence emerging from an axillary meristem. Note the airborne rosette comprised of several cauline leaves and three secondary inflorescences initiated from axillary meristems after the primary inflorescence meristem has lost activity. Most of these secondary inflorescences often develop a few fertile flowers. (**F**) RNA gel-blot experiment performed with 5 µg of poly(A)$^+$ RNA pooled from 10 individual seedlings. The size of the transcripts is indicated on the margin. The same filter was hybridised with a probe of the *STM* coding region (ORF, left) or the 3′-UTR (right), which is absent in the chimeric *en^{298}-STM* transcript. (**G**) RT–PCR experiment to confirm transcriptional activity of the native *STM* gene in individual transgenic seedlings (1–10). The native amplicon (*STM*) was discriminated from the chimeric product (*en^{298}-STM*) by combining a unique forward primer with reverse primers specific to the 3′-UTR of the native *STM* transcript or the pCHRIS vector. Single-tube RT–PCR experiments with all three primers failed to detect the endogenous transcripts because the unique forward primer achieved saturation in excess of the chimeric amplicons. (**H**) Wild-type *Arabidopsis* flower with four sepals, four petals (white), six stamens and two fused central carpels. (**I**) Transgenic *en^{298}-AP3* flower with sepaloid petals (sp) and filamentous stamen (fst) in whorls 2 and 3. The first and fourth whorl, sepals(s) and carpels, respectively, are unaffected. A single sepal has been removed for better detection of the homeotic transformations in whorls 2 and 3. (**J**) Slightly more phenotypic variation is observed in transgenic *en^{298}-PI* flowers in addition to sepaloid petals (sp) and filamentous stamen (fst): carpeloid stamens (cst) frequently replace the filamentous appendages in whorl 3.

construct, respectively, petals were converted to sepals in the second floral whorl and filamentous or carpeloid structures (*en^{298}-PI*) or the complete absence of organs replaced stamens in the third whorl (Fig. 2, compare wild-type in H with transgenic flowers depicted in I and J). Both homeotic transformations are characteristic for either *ap3* or *pi* mutant alleles. A higher phenotypic variability was observed in *en^{298}-PI* transgenic plants, which reflects the range of phenotypes from weak to strong *pi* alleles and thus presumably indicates quantitative differences in the expression level of the transgene. Ectopic expression of the En^{298}-AP3 or the En^{298}-PI chimeric protein therefore phenocopies the loss of B-function in flower development. Back-crossing of *en^{298}-AP3* transgenic plants to wild-type showed inheritance of phenocopies to subsequent generations always strictly co-segregating with the BASTA resistance marker. Therefore, the constitutive expression of three translational fusion proteins comprised of the *Drosophila* En^{298} transcriptional repressor

domain and the entire STM, AP3 or PI polypeptides results in very specific phenocopies of loss-of-function alleles.

Phenocopies rely on the incorporation of the chimeric En^{298}-STM protein into the nuclear compartment

To address the molecular mechanism the chimeric En^{298}-STM polypeptide was expressed in a C-terminal fusion with the hormone-binding domain of the glucocorticoid receptor in transgenic *Arabidopsis* plants. Due to this addition, the resulting En^{298}-STM/GR protein should accumulate in the cytoplasm. A linkage between the dexamethasone treatment and alterations in SAM activity thus should depend on nuclear import of the protein and strongly argues against homology-based post-transcriptional gene silencing (26,27).

Although some of the primary *en*^{298}*-STM/GR* transformants (T_1) exhibited weak *stm* phenocopies, several primary transformants showed wild-type development. To test for dexamethasone inducibility, we focused on one T_2 family with phenotypically normal kanamycin resistant seedlings. After wild-type development to the four- to five-leaf stage, 10 T_2 transgenic seedlings were sprayed with dexamethasone and photographed daily to document the developmental progress. As controls, wild-type plants sprayed with dexamethasone or untreated transgenic progeny were analysed concomitantly. In the 6 day interval depicted in Figure 3, five to six new leaves emerged in the control plants, in contrast to transgenic *en*^{298}*-STM/GR* plants, where only a single leaf appeared after spraying with dexamethasone. The hormone treatment also affected growth of pre-existing leaves: the petioles hardly elongated and the leaf blades remained closely attached to the shoot axis. The result depicted in Figure 3 is representative for 7 out of 10 transgenic progeny. The remaining three transgenic progeny showed no response to the hormone application. However, RNA gel-blot analysis showed the absence of the *en*^{298}*-STM/GR* transcript which was detected at high levels in the responsive progeny (data not shown). The arrest in SAM activity therefore was strictly correlated with transcription of the transgene and the three non-responding escapes are presumably caused by insufficient transgene expression. One line carrying a single T-DNA insertion has since been propagated for five generations and whilst heterozygous progeny develop normally, homozygous seedlings show mild *stm* phenocopies in the absence of the hormone. This resembles the mild phenotypes observed among T_1 *en*^{298}*-STM/GR* primary transformants carrying multiple transgene copies (data not shown) and indicates that at higher concentrations the chimeric En^{298}-STM/GR protein may act outside the plant cell nucleus.

Squelching versus transcriptional repression

As an alternative to transcriptional repression, excessive chimeric En^{298}-STM/GR protein in the cytoplasm could interact with potential partner proteins and thus deplete the native STM protein from its essential interaction partners—a mechanism frequently referred to as squelching. Consistent with this assumption, the specificity of floral phenocopies in *en*^{298}*-AP3* and *en*^{298}*-PI* transgenic plants might be explained by the obligatory dimerisation of AP3 and PI monomers to the active AP3/PI heterodimer if an excess of En^{298}-AP3 titrates the native PI protein or vice versa. To further investigate the molecular mechanism we created C- and N-terminal deletions of the STM protein and expressed these deletion polypeptides either fused or unfused to the En^{298} repressor domain in transgenic *Arabidopsis* plants. The whole series of deletion constructs is shown in Table 1A relative to conserved domains in the STM protein. The phenocopy frequency during vegetative development for each construct was determined 3 weeks after sowing (seven-leaf stage in wild-type).

Results with chimeric En^{298}-STM deletion polypeptides that lack the DNA-binding homeodomain support squelching as a mechanism. For example, the frequency of phenocopies obtained with the C-terminal deletion polypeptide that lacks the homeodomain (En^{298}-STMΔHD) is as high as that obtained with the complete *en*^{298}*-STM* coding region. Overall, the phenocopy frequency shows some direct correlation to the length to the *STM* cDNA fragment, but a major contribution is provided by the KNOX or MEINOX domain (28). Consistent with this assumption, 55% of the primary transformants obtained with the En^{298}-$KNOX_{(STM)}$ fusion showed the characteristic dominant-negative phenotype. A further increase (68%) is observed in the presence of the ELK domain (En^{298}-KNOX/ELK), which might contribute a nuclear localisation signal (29). Smaller KNOX subdomains did not cause any detectable phenotype (data not shown). These phenocopies strictly depend on the translational fusion between the En^{298} and the $KNOX_{(STM)}$ domains, as a frameshift version (En^{298}-fs-$KNOX_{(STM)}$), uncoupling translation in the $KNOX_{(STM)}$ domain does not affect plant development, similar to control plants transformed with the empty pCHRIS vector. Phenocopies, therefore, are strictly dependent on the expression of the chimeric En^{298}-$KNOX_{(STM)}$ protein.

To test for the contribution of the En^{298} repressor domain, transgenic plants were raised expressing only the $KNOX_{(STM)}$ or $KNOX/ELK_{(STM)}$ polypeptides (Table 1B). Surprisingly, in the absence of the En^{298} repressor domain, all except 2–3% of the transgenic plants obtained with both constructs exhibited pronounced phenotypes. Severe phenocopies with only one to three leaves were observed in 74% of the *35S::KNOX/ELK* transgenic plants and an additional 24% of plants showed internode elongation of rosette phytomers (Fig. 4A) but mostly maintained SAM activity (Fig. 4A and B). The ratio between phenocopies and elongated internodes is reversed in transgenic plants expressing the KNOX domain, where 16% of plants phenocopied a loss of *stm* function and 79% exhibited elongated internodes, with approximately 1/4 of these additionally showing a late block in SAM activity. This elongation of internodes is a novel phenotype not seen in En^{298} fusions and associated with alterations in phyllotaxy, e.g. changing from a spiral to a distichous pattern (Fig. 4C and D). This high frequency of phenotypes (97 or 98%) depends on the translation of the $KNOX_{(STM)}$ domain, as only 25 or 23% of *stm* phenocopies are observed in transgenic plants expressing frameshift versions of the $KNOX_{(STM)}$ or $KNOX/ELK_{(STM)}$ coding regions (Table 1B). The low, but similar, percentage of phenotypic plants observed with both frameshift versions contrasts with results with the *en*^{298}*-fsKNOX*$_{(STM)}$ construct, where we have not seen any evidence for co-suppression. However, it indicates that homology-dependent silencing of the endogenous *STM* gene may contribute a minor fraction of the total frequency dependent on the construct. Although expression of the $KNOX_{(STM)}$ or $KNOX/ELK_{(STM)}$ domain provides dominant-negative functions, the associated novel

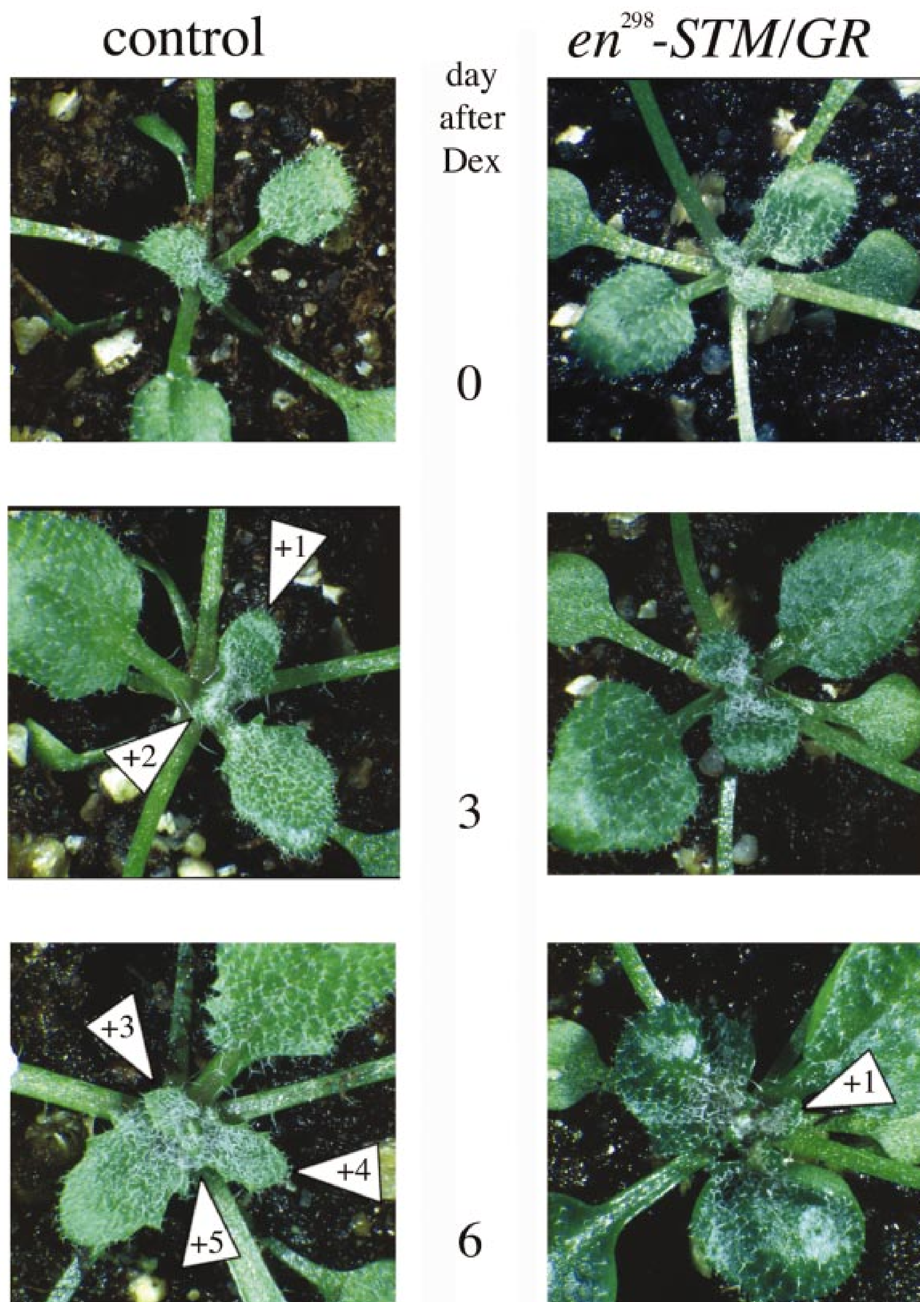

Figure 3. Dexamethasone-dependent arrest of SAM activity. Wild-type (control, left) or transgenic *en*^{298}*-STM/GR* seedlings (right) were sprayed with dexamethasone at the four- to five-leaf stage and photographed daily. While five new leaves emerge in the control seedling (numbered +1 to +5), only a single new leaf is detectable in the transgenic *en*^{298}*-STM/GR* seedlings in the depicted 6 day interval. Development in untreated transgenic *en*^{298}*-STM/GR* siblings was also comparable to wild-type and resulted in four to five leaves.

phenotypes, e.g. internode elongation, indicate that the translational fusion with the En^{298} repressor domain largely contributes to the specificity of phenocopies.

Expression of the En^{298}-KNAT1 polypeptide phenocopies the *brevipedicellus* mutant

To further investigate the specificity, we exchanged the *STM* coding region against the *KNAT1* ORF. The *KNAT1* expression pattern in the SAM is very similar to that of *STM*, but, in addition, the *KNAT1* gene is transcribed in the cortical layers of the inflorescence and floral pedicel. Transgenic plants expressing *en*^{298}*-KNAT1* exhibited normal vegetative development (compare Fig. 5A and B), but in 75.6% of the primary transformants (508/672) a phenotype was observed in the inflorescence: the length of the internodes and the floral pedicel was reduced and the pedicels bent downwards, so that the orientation of flowers or siliques was opposite to that of the wild-type (compare Fig. 4C and D). This phenotype is reminiscent of the *brevipedicellus* (*bp*) mutant (30) which specifically affects inflorescence development. Although the

Table 1. Deletional analysis of the STM protein

A

Construct	*stm* phenocopy [%]	Total[a]
STM (KNOX, ELK, HD)		
En^{298}-STM	74.3	369
En^{298}	0.0	77
En^{298}-STMΔHD	79.0	238
En^{298}-STMΔC2	1.3	77
En^{298}-STMΔC3	0.0	97
En^{298}-STMΔN1	65.3[b]	75
En^{298}-STMΔN2	45.4	185
En^{298}-STMΔN3	30.1	322
En^{298}-STMΔN4	4.6	174
En^{298}-KNOX/ELK	68.1	144
En^{298}-KNOX	55.2	406

B

Construct (KNOX, ELK)	*stm* phenocopy [%]	elongated internodes [%]	Total[a]
En^{298}-KNOX[c]	55.2	0.0	406
En^{298}-fsKNOX	2.7	0.0	556
KNOX	16.8	79.6[d]	328
fsKNOX	25.3	0.0	572
KNOX/ELK	73.6	24.1[e]	440
fsKNOX/ELK	23.0	0.0	506

100 aa

[a]Total number of analysed transgenic plants.
[b]48% of transgenic plants show a *stm* phenocopy and lobed leaves.
[c]See also (A).
[d]23.2% of transgenic plants have elongated internodes and show a *stm* phenocopy.
[e]3% of transgenic plants have elongated internodes and show a *stm* phenocopy.

bp-1 allele is in the Landsberg *erecta* (L*er*) ecotype, the expression of *en^{298}-KNAT1* in the Columbia background leads to very similar alterations in the angle of side shoots or floral pedicels and in pedicel length relative to the corresponding wild-type background (Table 2). The increased number of secondary inflorescences (Fig. 5E) indicates that apical dominance is reduced in *en^{298}-KNAT1* transgenic and *bp* mutant plants. The elongation of internodes, which is reduced by 68% in *bp-1*/L*er* is only reduced to 30% in *en^{298}-KNAT1*/Col transgenic plants, and this is reflected in the total height of *en^{298}-KNAT1* plants. The number of flowers is unaffected in *en^{298}-KNAT1* transgenic and *bp* mutant plants. We also raised transgenic plants expressing the $KNOX_{(KNAT1)}$ or $KNOX/ELK_{(KNAT1)}$ domains fused to the En^{298} repressor domain. Strong *bp* phenocopies with flowers and siliques oriented downwards were observed only with the *en^{298}-KNOX/ELK$_{(KNAT1)}$* construct. In the absence of the ELK domain, flowers or siliques emerged, at most, perpendicular to the inflorescence axis. We did not observe an alteration in the SAM function with any of the chimeric *KNAT1* genes.

KNAT1 and *bp* both map to chromosome 4 and the *bp* phenocopies in *en^{298}-KNAT1* transgenic plants suggested that *bp* may in fact represent a loss of *KNAT1* function. To substantiate this assumption the *KNAT1* ORF was expressed behind the constitutive *CaMV 35S* promoter in the *bp-1* mutant background. A resulting *35S::KNAT1* transgenic plant in the *bp-1*/L*er* background is shown in Figure 5G and demonstrates a rescue of the mutant phenotype (compare

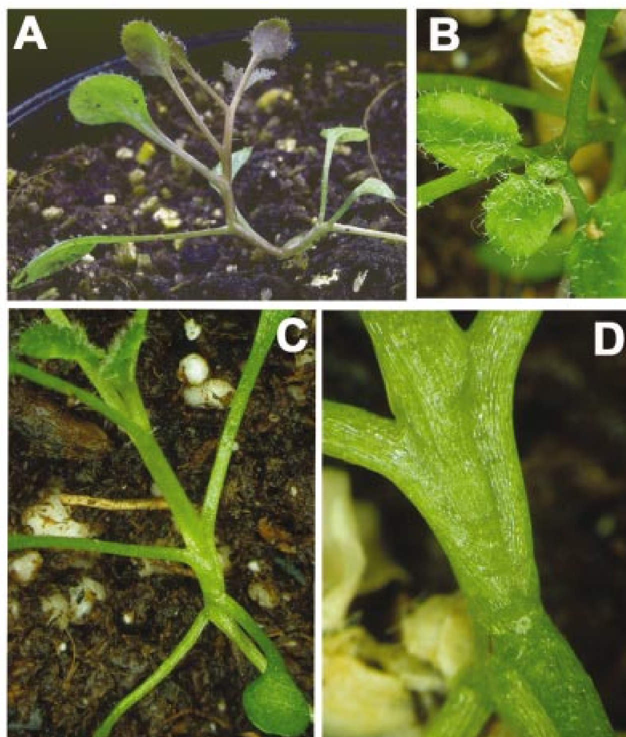

Figure 4. Phenotypes in transgenic *35S::KNOX*$_{STM}$ and *35S::KNOX/ELK*$_{STM}$ plants. (**A**) Elongated internodes in a *35S::KNOX*$_{STM}$ plant, small leaflets enclose the active SAM (**B**). Internodal elongation is often associated with alterations in phyllotaxy (**C**), a distichous pattern with two leaves emerging from a single node is depicted in (**D**).

Fig. 5G and F). The internode and the pedicel are elongated in the *35S::KNAT1* transgenic *bp-1*/L*er* plant compared with the *bp-1* mutant and the flowers/siliques point upwards or are oriented at least perpendicular to the stem axis. The strong lobing of rosette leaves is typical of *KNAT1* overexpression from the 35S promoter and is due to ectopic activity in the leaves. Both the *bp* phenocopy in *en*^{298}*-KNAT1* transgenic plants and the complementation of the *bp-1* mutant in *35S::KNAT1* plants thus argue that *KNAT1* is encoded by the *BP* locus. A similar conclusion was recently achieved by two other groups (31,32), together with the suggestion that the *bp* phenotype may largely depend on the *erecta* mutant background. However, this assumption is not supported by our data for trans-dominant *en*^{298}*-KNAT1* phenocopies in the Columbia background comprising a wild-type *ERECTA* function.

DISCUSSION

The experimental data demonstrate that ectopic expression of N-terminal fusion proteins between the *en*^{298} N-terminus and the complete *STM*, *KNAT1*, *AP3* or *PI* coding regions results in dominant-negative functions which mimic loss-of-function alleles. With all four test genes the efficiency is high and the frequency of characteristic phenocopies in primary transformants always exceeds 75%. The trans-dominant phenocopies are specific and distinct from phenotypes observed in overexpression experiments with *AP3*, *PI*, *STM* or *KNAT1* (33,34) The homogeneity of the resulting phenotypes and their high frequency in primary transformants provide rapid and reliable information about the contribution of each gene during plant development. Although *STM*, *AP3* and *PI* were chosen as test genes because of their predictable loss-of-function phenotypes, the association between *KNAT1* and the *BREVIPEDICELLUS* locus became apparent during these studies and is consistent with parallel efforts recently published by other groups (31,32).

The question of the molecular mechanism was addressed in various ways. In combination with the hormone-binding domain of the glucocorticoid receptor, the activity of the En^{298}-STM/GR fusion polypeptide is under control of the dexamethasone hormone, which allows the incorporation of the chimeric protein into the nuclear compartment (23). The inducibility of the En^{298}-STM/GR fusion protein provides evidence that the main mechanism is protein-based, although post-transcriptional gene silencing (26,27) may contribute to the total frequency at a low level. Based on frameshift nonsense versions, e.g. *fsKNOX* or *fsKNOX/ELK*, the co-suppression frequency is two to four times lower than that observed with the corresponding sense constructs (see Table 1B). When a frameshift (+1) is introduced C-terminally to the En^{298} domain and in front of the KNOX domain (*en*^{298}*-fsKNOX*), thus uncoupling translation of the repressor domain from the *STM* sequences, no phenotypic alterations are detectable. The fraction of phenocopy plants attributable to co-suppression therefore depends on the construct: there may be a low contribution of co-suppression, but this cannot account for the high phenocopy frequency.

Besides hormone control in *en*^{298}*-STM/GR* transgenic plants, the most compelling argument for a protein-based mechanism is provided by results for *KNOX*$_{(STM)}$ or *KNOX/ELK*$_{(STM)}$ transgenic plants. More than 97% of the primary transformants expressing the sense constructs exhibit phenotypic alterations compared with <25% with the nonsense frameshift versions. Most of the frequency therefore depends on the translation of the KNOX domain, which may interact with protein partners and deplete native endogenous proteins including STM from essential interaction partners. Squelching could thus account for the dominant-negative function due to KNOX$_{(STM)}$ overexpression, and resembles results for the removal of the bZIP domain in the tobacco transcription factor TGA1a which interferes with the formation of the ASF-1 complex (35,36). All effects on the SAM are specific to the KNOX$_{(STM)}$ or KNOX/ELK$_{(STM)}$ domains, since the expression of the corresponding KNAT1 subdomains has no detectable consequence for SAM activity.

Remarkably, the internode elongation in transgenic plants expressing KNOX$_{(STM)}$ or KNOX/ELK$_{(STM)}$ polypeptides is not observed in fusions with the En^{298} repressor domain. All the En^{298} fusions result in a single phenotype, which mimics a loss of *stm* function, with only the frequency of phenocopies being variable. The same specificity is observed with the En^{298}-AP3, the En^{298}-PI or the En^{298}-KNAT1 fusions. In each case, the N-terminal fusion of the En^{298} repressor domain to the full-length protein results in specific dominant-negative functions, which rapidly uncover the biological contribution of the corresponding gene product. Neomorphic phenotypes, e.g. internode elongation as obtained with the KNOX$_{(STM)}$ or the KNOX/ELK$_{(STM)}$ domain, are missing in the presence of

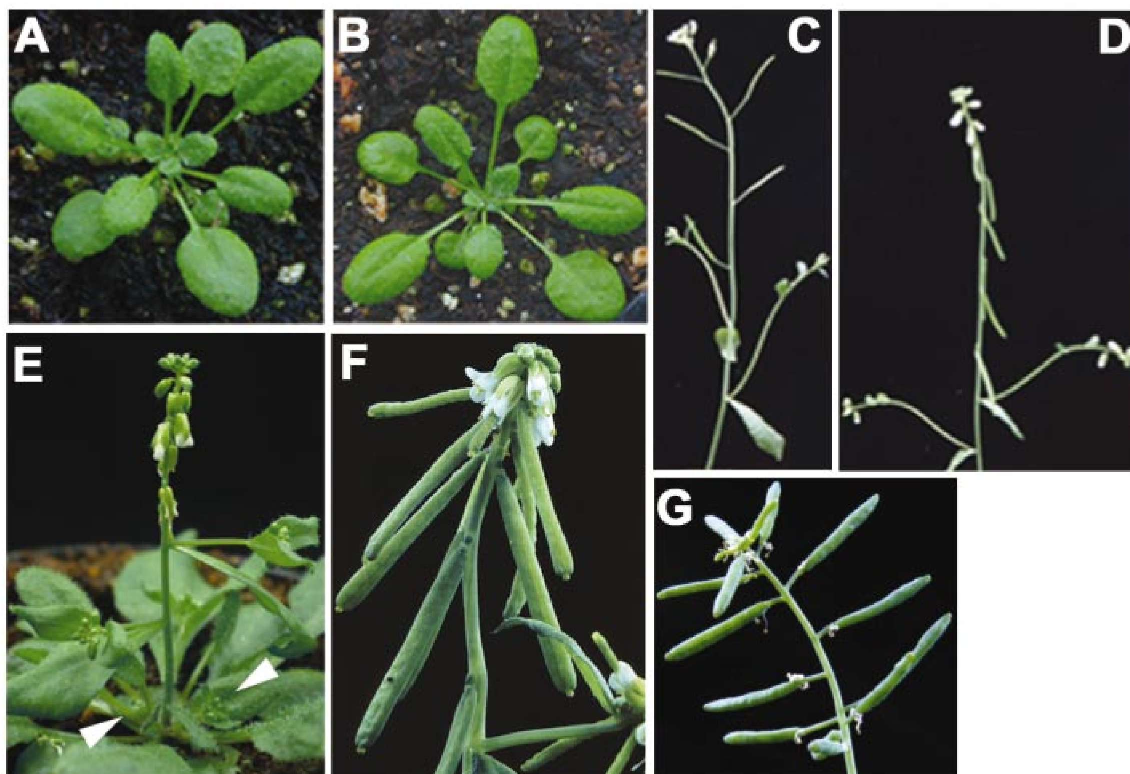

Figure 5. Transgenic *en*^{298}-*KNAT1* plants phenocopy *bp* mutants. Vegetative development in transgenic *en*^{298}-*KNAT1* plants is normal [compare wild-type (**A**) with the transgenic plant (**B**)]. The wild-type inflorescence (**C**) is compared with the inflorescence of a transgenic *en*^{298}-*KNAT1* plant (**D**). (**E**) Secondary inflorescences (arrows) emerging in a *en*^{298}-*KNAT1* plant. (**F**) Close-up of the *bp* inflorescence. (**G**) Partial complementation of the *bp* mutant phenotype by *35S::KNAT1* expression. Homozygous *bp-1* mutant plants were transformed with the *35S::KNAT1* transgene and selected for BASTA resistance.

Table 2. Comparison between *en*^{298}-*KNAT1*/Col and *bp-1*/L*er* plants

Genotype	Height (cm)	Number of flowers	Average internode length (cm)	No. of secondary inflorescences	Angle (°)		Pedicel length (cm)
					Side shoot	Pedicel	
L*er*	17.7 ± 0.2	28.3 ± 0.2	0.56 ± 0.01	0.14 ± 0.03	53 ± 2	56 ± 2	0.52 ± 0.05
bp-1/L*er*	5.9 ± 0.2	29.1 ± 0.2	0.18 ± 0.01	1.8 ± 0.1	92 ± 3	132 ± 3	0.09 ± 0.01
Δ	11.8/67%		0.38/68%				0.43/83%
Col	35.5 ± 0.3	43.9 ± 0.3	0.66 ± 0.03	0.04 ± 0.01	62 ± 2	77 ± 1	0.69 ± 0.1
en^{298}-*KNAT1*/Col	23.6 ± 0.4	45.2 ± 0.5	0.46 ± 0.04	2.2 ± 0.2	92 ± 1	127 ± 1	0.20 ± 0.1
Δ	11.9/34%		0.2/30%				0.49/71%

Plants ($n \geqslant 20$; *en*^{298}-*KNAT1*: $n = 60$) were measured 50 days after sowing. The average internode length is determined for the first 3 cm (*bp-1*/L*er*) or 10 cm (*en*^{298}-*KNAT1*/Col) of the inflorescence starting at the point where the first flower arises. Values given are means ± SE.

the En^{298} repressor domain which therefore highly increases the specificity of phenocopies.

Contribution of the En^{298} repressor domain

The 298 amino acid residues of the *en* gene product fused with the plant sequences here span the *eh1* domain conserved in numerous homeodomain repressor proteins (34). The *eh1* motif is known to interact with *Groucho* (37), a transcriptional co-repressor, which is recruited to DNA by a variety of DNA-binding proteins. *Groucho* homologues have been cloned from several animal species and the corresponding protein in yeast Tup1 is thought either to position nucleosomes over the core promoter (38) or to directly inhibit the RNA–polymerase initiation complex (39). Recalling the similarity in the basal transcriptional machinery between plants and animals (15,40,41) it cannot be excluded that *Groucho* homologous co-repressor functions exists in plants, although solely based on sequence homology these are not rapidly identified in the *Arabidopsis* genome. One possibility, therefore, seems that the chimeric En^{298} fusion proteins act as transcriptional repressors. Expressed from the strong *CaMV 35S* promoter, their vast excess may displace the native gene products from target gene promoters thus explaining trans-dominance over the functional endogenous gene.

Although the dexamethasone response in *en*^{298}*-STM/GR* transgenic plants implicates the cell nucleus as the target compartment, this does not imply that the chimeric protein exhibits an active repressor function. Trans-dominant phenocopies with STM deletion polypeptides are obtained in the absence of the DNA-binding homeodomain. However, a homeodomain-deleted Fushi tarazu (Ftz) polypeptide incapable of binding DNA can control segmentation in *Drosophila*, through interactions with other proteins (42). In analogy, En^{298}-STM deletion derivatives might still be recruited to target gene promoters with the help of interacting proteins.

Whether the En^{298} N-terminal sequences actively repress transcription of target genes in the plant cell nucleus remains an open question, because all test proteins presumably rely on partner proteins. In contrast to active repression, competitive modes can be envisioned. Non-functional En^{298} fusion proteins included into heterodimeric or multimeric protein complexes may displace functional native complexes from target genes, or excessive En^{298} fusion proteins may interfere with the assembly of functional complexes or their nuclear uptake. Independent of whether the dominant-negative functions rely on transcriptional repression of target gene promoters or are based on competition, the underlying mechanism has to explain how the ubiquitous expression of *en*^{298}*-STM*, *en*^{298}*-AP3*, *en*^{298}*-PI* or *en*^{298}*-KNAT1* constructs can result in precise phenocopies of loss-of-function alleles. Transcribed from the constitutive *CaMV 35S* promoter, organ specificity associated with the dominant-negative functions cannot reside in transcriptional activity. An attractive assumption is obligatory and cell- or organ-specific protein–protein interactions, which have been shown for the AP3 and PI gene products (43). Although the En^{298}-AP3 or En^{298}-PI fusion proteins are expressed ubiquitously from the *35S* promoter, the essential interaction partner is still confined to the second and third floral whorl, thus confining activity to the flower. We cannot discriminate whether excess of the En^{298}-PI or En^{298}-AP3 fusion protein binds the essential AP3 or PI partner protein in non-functional complexes, or whether the En^{298} fusion is recruited to target genes as heterodimers. In both scenarios, target promoters dependent on the activation via AP3/PI heterodimers would be negatively affected, resulting in a specific B-function phenotype. As there is no evidence for STM or KNAT1 homodimers (M. Cole and W. Werr, unpublished results) the specificity of phenocopies in *en*^{298}*-STM* or *en*^{298}*-KNAT1* transgenic plants indicates that protein partners may be confined to the SAM or the inflorescence, respectively. This assumption gains further support since the isolated KNOX domains of STM and KNAT1 cause phenocopies either exclusive to the SAM or the inflorescence. Therefore, the phenocopy specificity most likely relies on compatible protein interactions which are confined to individual cell types or plant organs.

Applications

Dominant-negative functions may provide a rapid method to elucidate the biological function of plant transcription factors. The opposite approach, use of the strong VP16 activation domain fused to the meristem identity gene *LEAFY*, uncovered transcriptional activation of the organ-identity gene *AGAMOUS* (44). After completion of the genome-sequencing project the sequence of the whole set of transcription factors in *Arabidopsis* is known, but for most of these genes biological functions and contributions to regulatory networks remain to be elucidated. As efficient gene disruption systems based on homologous recombination are lacking in plants, this deficiency is circumvented by laborious PCR-based screens for insertion alleles in large transposon or T-DNA populations, so-called gene machines. However, any success in reverse or true genetic screens depends on a unique gene function. Dominant-negative functions as demonstrated here with *AP3*, *PI*, *KNAT1* and *STM* may not only be more rapid, but also superior in the case of redundancy. At the protein level, excess of the chimeric gene product might compete not only with its native counterpart, but simultaneously with other family members that possibly mask a desired gene function.

In contrast to gene machines, which will remain restricted to a few model plants only, dominant-negative functions are applicable to all species susceptible to transformation. As the *AP3* function in *Arabidopsis* for example can be partially complemented by the homologous *DEFICIENS* gene from *Antirrhinum* (45), it is likely that dominant-negative functions would also be informative in comparisons between species. Since the evolution of regulatory networks probably accounts for the majority of variation between different plant species, the central position of transcription factors in gene regulation makes them an interesting subject for such functional comparisons, both from academic and applied perspectives. On the applied side, dominant-negative functions may be suitable to transfer knowledge elaborated in model systems like *Arabidopsis* to crop species.

In conclusion, the data described here provide evidence that plant transcription factors can be reprogrammed to dominant-negative functions by fusion to the *en*^{298} N-terminus. Although we cannot distinguish between active transcriptional repression and a competitive mode of action, dominant-negative functions may be of wide use in elucidating biological functions of plant transcriptional factors. Reproducibly, with four transcription factors, we observed ~75% phenocopies of loss-of-function alleles in primary transformants. The N-terminal addition of the En^{298} repressor domain to the complete coding region of plant transcription factors therefore provides a rapid and efficient method to visualise a biological contribution of a gene of interest.

ACKNOWLEDGEMENTS

We thank Professor Michael Hoch (Universität Bonn) for the gift of the *engrailed* cDNA clone D_2B, and Petra Comelli and Melanie Cole for excellent technical assistance. The *STM* cDNA clone was kindly provided by Dr Rüdiger Simon (Universität zu Köln), whom we also thank for experimental suggestions and critical reading of the manuscript. The *AP3* cDNA clone was kindly provided by Dr Zsuzsanna Schwarz-Sommer (MPI für Züchtungsforschung, Köln). Part of this work was supported through GENOPLANTE (project number NO1999013) and the Deutsche Forschungsgemeinschaft WE-1262/3-1. H.M. was funded by the Graduiertenkolleg ‘Molekulare Analyse von Entwicklungsprozessen’ at the University of Cologne.

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