The petunia floral pathway: target genes of ALF, DOT and EVG

Elske Kusters, Rob Castel, Alexandra Rebocho,
Mattijs Bliek and Ronald Koes

The key findings described in this chapter were incorporated into Souer et al. (2008) and Rebocho et al. (2008).
Abstract

The specification of floral identity of meristems in petunia requires floral meristem identity genes, *ABERRANT LEAF AND FLOWER (ALF)*, *DOUBLE TOP (DOT)* and *EVERGREEN (EVG)*. In order to further characterize the role of these genes in inflorescence and flower development, we employed two complementary approaches to find their early target genes. We compared the gene expression profiles of single, double and triple mutants, and we constructed post-translationally inducible forms of the three corresponding proteins. As *ALF, DOT* and *EVG* encode very different types of proteins that are expressed in distinct patterns, we expected to find three different sets of target genes. Here we show that the *ALF, DOT* and *EVG* pathways converge earlier than expected: at the control of *ALF* activity. We found that the transcriptomes of *alf* and *dot* mutants are very similar and that DOT is a direct activator of ALF target genes, supporting the idea that DOT is involved in the post-translational activation of ALF. DOT is strongly down regulated in *evg* mutants and the flowers reappear when DOT is constitutively expressed in an *evg* mutant background. This led to the idea that EVG indirectly promotes floral identity in petunia by relieving the repression of DOT. We conclude that *alf, dot* and *evg* mutants lack flowers for one common reason, namely the failure to generate an activated ALF protein that can trigger the transcription of downstream genes. Altogether, these data point towards a mechanism of floral meristem and floral organ identity specification with only a limited number of steps and involved genes.
Target genes of ALF, DOT and EVG

Introduction

The development of plants can be divided into a vegetative and a reproductive phase. During the vegetative phase only leaves are produced, and when the plant switches to flowering specialized leaves (bracts) and flowers are developed. The part of the plant that bears the flowers is called the inflorescence. When and where flowers are formed is determined by groups of stem cells in the tips of shoots, called meristems. When flowering commences floral meristem identity genes are expressed in the meristems that become flowers, but if these floral meristem identity genes are mutated, shoots develop instead of flowers. This phenotype was observed in three petunia (Petunia hybrida) mutants: aberrant leaf and flower (alf), double top (dot) and evergreen (evg). The corresponding genes were cloned and found to encode a LEAFY (LFY)/FLORICAULA-like transcription factor, an F-box protein and a homeodomain transcription factor, respectively (Coen et al., 1990; Rebocho et al., 2008; Souer et al., 2008; Souer et al., 1998; Weigel et al., 1992).

alf mutants bear inflorescence shoots where wild type petunia develop flowers (Fig. 1A-B). When ALF or its Arabidopsis thaliana homolog LFY is ectopically expressed in Arabidopsis, flowering is induced extremely early and the shoot apical meristem (SAM), which normally remains undifferentiated, is precociously converted into a terminal flower (Souer et al., 2008; Weigel and Nilsson, 1995). In petunia, however, ubiquitous expression of ALF (homologs) does not trigger this early conversion of shoots into flowers: surprisingly, petunia plants expressing LFY or ALF from the cauliflower mosaic virus 35S promoter (35S:LFY or 35S:ALF) develop wild type (Souer et al., 2008).

dot, like alf, develops shoots instead of flowers (Fig. 1C). However, in dot these shoots occasionally terminate in a carpel (Fig. 1C, inset). DOT is homologous to Arabidopsis UNUSUAL FLORAL ORGANS (UFO) and snapdragon (Antirrhinum majus) FIMBRIATA (Ingram et al., 1995; Levin and Meyerowitz, 1995; Simon et al., 1994). The encoded F-box proteins are the target-specifying component of SCF ubiquitination complexes. Proteins that are ubiquitinated by these complexes are usually targeted for proteasome breakdown (Skowyra et al., 1997). DOT and UFO bind to ALF and LFY, but instead of targeting them for breakdown, the F-box proteins are thought to activate ALF and LFY by this ubiquitination (Chae et al., 2008; Souer et al., 2008).

In contrast to the flowerless petunia dot mutants, Arabidopsis ufo mutants only have a mild flower-to-shoot phenotype, and the main characteristic of ufo is the formation of petalless and stamenless flowers (Ingram et al., 1995; Levin and Meyerowitz, 1995). The contrast between the phenotypes of 35S:DOT (or 35S:UFO) in petunia and in Arabidopsis further emphasizes the divergent roles of these genes.
in their cognate hosts species: ectopic expression of DOT in petunia causes severely early flowering and the formation of ectopic flowers, whereas 35S:DOT in Arabidopsis merely causes the production of supernumerary petals and stamens. Although DOT is fully dependent on functional ALF for the early flowering effect in petunia (Souer et al., 2008), ALF is thought to have functions independent of DOT, for example because the carpels seen in dot mutants do not appear in alf. Furthermore, when combined with extrapetals, hermit or veggie mutations alf gives a distinct and stronger phenotype than dot (Chapter 4) (Castel, 2009; Roobeek, 2011).

Figure 1. Phenotypes of alf, dot and evg.
(A) W138 wild type inflorescence (B) alf inflorescence. (C) dot inflorescence. The inset shows an inflorescence shoot that terminates in a carpel, which regularly appear in dot inflorescences. (D) evg inflorescence with typical fasciated stems (arrow). (E-F) Typical flower in evg. Note the extremely supernumerary petals and stamens and the double carpels in the evg flower (B) compared to wild type (A).
Target genes of ALF, DOT and EVG

ev mutants are generally flowerless, like alf and dot, but they sometimes spontaneously develop flowers with supernumerary organs on the otherwise flowerless stems. The main distinction with alf and dot is that in ev the stems originating from the apical and lateral (sympodial) meristems grow fused together (Fig. 1D). EVG encodes a WOX homeodomain protein and is homologous to Arabidopsis STIMPY (STIP) and STIP-LIKE (STPL), which are redundantly involved in meristem growth and maintenance (Wu et al., 2005). EVG mRNA is expressed strongly, but briefly in one half of the meristem dome at the moment that the newly emerging sympodial meristem has grown out to about half of this dome (Castel et al., 2010; Rebocho et al., 2008). At this moment, the floral and sympodial meristems are morphologically similar, and because the mutant lacks most of the flowers, it was intuitively supposed that the dome that expresses EVG would be the floral meristem. However, with double label in situ hybridizations with an ALF and an EVG antisense probe, and detailed analysis of the phylotactic patterns in the petunia inflorescence, it became clear that the dome that expresses EVG is the sympodial meristem (Castel et al., 2010; Rebocho et al., 2008). The fascination of the stems in ev is thought to be due to failure in proper outgrowth of the sympodial meristem. Separation of floral and sympodial meristem is probably achieved by a similar mechanism as the one by which Arabidopsis homologs STIP and STPL promote meristem outgrowth (Wu et al., 2005). As EVG is expressed exclusively in the sympodial meristem, it can only perform this function there.

In order to further characterize ALF, DOT and EVG, we set out to determine their direct (or relatively direct) target genes. We used two parallel methods, one based on inactivation of the genes by comparing the expression profiles of the mutant meristems by cDNA-Amplified Fragment-Length Polymorphism (cDNA-AFLP) (Bachem et al., 1996), and the second based on activation of the genes by use of post-translationally inducible glucocorticoid receptor (GR) fusion proteins. We found that the expression profiles of alf, dot and evg mutants are very similar and that the only genes expressed differently are DOT and members of the ABCDE-type MADS-box genes, which are involved in floral organ identity specification (Vandenbussche et al., 2003). Nevertheless, the phenotypes of double and triple mutants and the expression profiles suggest that in evg floral development is arrested at an earlier stage than alf, and that alf is arrested at an earlier stage than dot. Furthermore, the expression of at least some floral organ identity MADS-box genes can be induced by post-translationally activating DOT, without the need to synthesize intermediate regulators. As we did not find other target genes, we suspect that the indirect activation of DOT is the major role of EVG, and that ALF and DOT together directly activate floral organ identity MADS-box genes, which points towards a floral pathway with a very limited number of involved steps.
Results

Comparison of \textit{alf}, \textit{dot} and \textit{evg} mutants

\textit{alf}, \textit{dot} and \textit{evg} mutants appear very similar (Fig. 1A-D) (Souer \textit{et al}., 2008; Souer \textit{et al}., 1998). However, whereas the \textit{alf} mutant is completely flowerless, \textit{dot} mutant inflorescences occasionally develop carpels and \textit{evg} mutants regularly produce flowers on the otherwise flowerless, fasciated stems.

The \textit{evg} allele used for the initial experiments was a \textit{dTph1} transposon-induced allele in petunia line W138 background. To assess whether the occasional flowers in \textit{evg} resulted from transposon excisions, a stable \textit{evg} allele (\textit{evg}\textsuperscript{d2299}) was isolated in which the \textit{dTph1} element had excised and created an 8-bp footprint that disrupted the reading frame. This stable \textit{evg} mutant indeed produced significantly less flowers than its transposon induced predecessor, but nevertheless flowers still appeared spontaneously on occasion. The flowers on \textit{evg}\textsuperscript{d2299} have extremely supernumerary organs and appear to consist of multiple, fused flowers, which, just like the \textit{evg} shoots, seem to develop from fasciated meristems (Fig. 1E-F) (Rebocho \textit{et al}., 2008). As the number of flowers was significantly reduced in the \textit{evg}\textsuperscript{d2299} mutant, we used this stable allele for all subsequent experiments.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Analysis of \textit{alf}, \textit{dot} and \textit{evg} single, double and triple mutants. (A-D) inflorescence apices of \textit{alf dot} (A); \textit{alf evg} (B); \textit{dot evg} (C) and \textit{alf dot evg} (D). Compare to the wild type and \textit{alf}, \textit{dot} and \textit{evg} single mutants in Figure 1. Note the fasciated stems (arrows) in (B-D). (E) Ethidium bromide stained gel showing RT-PCR analysis of \textit{ALF}, \textit{DOT} and \textit{EVG} in \textit{alf}, \textit{dot} and \textit{evg} single, double and triple mutant inflorescence apices. Note that \textit{ALF} is down regulated in all genotypes containing \textit{alf} and that \textit{DOT} is down regulated in all genotypes containing the \textit{evg} mutation. \textit{ACTIN} was used as a constitutively expressed positive control. Triple, \textit{alf dot evg} mutant; -, no-template negative control.}
\end{figure}
To further characterize alf, dot and evg, we created double and triple mutants. alf dot double mutants (Fig. 2A) appear identical to alf (Fig. 1B), and do not have the carpels that regularly appear in dot mutants, suggesting that alf is epistatic over dot. alf evg, dot evg and alf dot evg triple mutants (Fig. 2B-2D) have fasciated stems and look identical to evg single mutants, suggesting that the evg mutation is epistatic over alf, dot or alf dot. However, in contrast to evg single mutants, flowers were never observed in alf evg, dot evg or alf dot evg mutants, indicating that, as expected, alf and dot are necessary for the production of the flowers, also in an evg background.

Since ALF, DOT and EVG encode proteins that have no obvious mechanistic link and are expressed in different patterns, we anticipated that the corresponding mutants would only differ in the expression of genes relatively directly downstream of ALF, DOT or EVG, whereas far downstream genes, such as genes involved in flower color production, are expected to be down-regulated to a similar extent, and thus not differentially expressed in these mutants. By comparing the expression profiles in the inflorescence apices of single, double and triple mutants via cDNA-AFLP (Bachem et al., 1996), we aimed to find the target genes of ALF, DOT and EVG.

We isolated PolyA+ RNA from carefully dissected flowerless apices (2-3mm) from two independent plants of each of the seven genotypes. Of evg single mutants, only the tops that did not contain any visible flowers were taken. We made double stranded cDNA (ds-cDNA) to be used for transcript profiling by cDNA-AFLP and first assayed these ds-cDNA samples for the expression of a few known genes by RT-PCR (Fig. 2E).

These experiments revealed that ALF mRNA is expressed at lower levels in all plants that contain the alf mutation. The lower abundance of ALF mRNA in alf can be explained by the position of the transposon. It is located in the first exon and causes a premature termination codon, which typically leads to nonsense-mediated mRNA decay (Belostotsky and Sieburth, 2009).

EVG mRNA is found at low, but equal levels in all genotypes, independently of the evg mutation. This can be interpreted in two ways: (i) either the expression of EVG mRNA is not affected by any of the mutations, or (ii) EVG mRNA is reduced to an equal extent by all 3 mutations. The observation that alf dot evg triple mutants express equal amounts of EVG mRNA as single evg mutants is more easily explained by the first hypothesis than by the second. To clearly distinguish between the two hypotheses EVG mRNA levels in mutants should ideally be compared to the expression in wild type. However, this cannot reliably be done by RT-PCR approaches, as the widely divergent anatomy of the wild and the mutants will affect the amount of mRNA expressed in an entire inflorescence apex. However, the finding that EVG
mRNA is easily detectable in alf, dot and evg inflorescences by in situ hybridization suggests that none of these mutations have drastic effects on EVG mRNA expression (Rebocho et al., 2008).

Surprisingly, we found DOT mRNA to be expressed at lower levels in all genotypes with the evg mutation. This indicates that DOT expression is regulated by EVG, or that evg mutants do not reach the developmental stage at which DOT is expressed.
EVG promotes DOT expression indirectly

To examine whether the deficiency in DOT expression in evg mutants could explain the homeotic transformation of flowers into shoots in evg, we decided to transform evg mutants with 35S:DOT (Sourer et al., 2008). However, when the evgD2299 allele from line W138 was crossed into transformable line W115 we observed to our surprise that only part of the evgD2299 progeny were flowerless (Fig. 3A), whereas other evgD2299 mutants bore many flowers in a reiterating pattern of shoots that all terminated with a single flower (Fig. 3B). Also intermediate forms, of which the phenotype varied over time, were seen (Fig. 3C), which indicates that the difference between the green and solitary flower evg mutants was not purely genetic, but also dependent on environmental factors.

Therefore, we selected green evg mutants from the hybrid W138/W115 background and stably transformed those with a 35S:DOT construct, or, as a control, with the empty vector (Rebocho et al., 2008; Sourer et al., 2008). 35S:DOT evg plants flowered very early and formed single flower inflorescences, comparable to 35S:DOT in a wild type background (Fig. 3D) (Sourer et al., 2008). Most of the flowers had a 35S:DOT appearance with six petals instead of five (wild type). The flowers with extreme numbers of floral organs, which regularly appear in untransformed evg plants (Fig. 1F), were not seen in 35S:DOT evg. In evg plants that were transformed with the empty vector, we did not observe any flowers at all. Together, these results show that the loss of floral meristem identity in evg mutants is due to failure to activate DOT expression.

EVG mRNA is expressed in the sympodial meristem at a relatively early developmental stage of this meristem. The observation that DOT expression overlaps with ALF expression (Sourer et al., 2008), while EVG does not, suggests that EVG and DOT are expressed in spatially separated domains. We confirmed this by double label in situ hybridizations and confirmed that EVG and DOT expression are also separated in time (Rebocho et al., 2008). That is, in the inflorescence domes that expressed EVG we could not detect DOT mRNA and vice versa. This suggests that EVG promotes DOT expression indirectly.

The fasciated stems of evg mutants (Fig. 1D, arrow) indicate that the role of EVG is not only to activate DOT expression, but also to separate the apical and sympodial meristems during inflorescence growth. To attempt to separate the roles of EVG in floral meristem identity and sympodial meristem outgrowth, we generated double mutants with extrapetals (exp), which is involved in maintenance of the sympodial meristem (Chapter 4). In exp the cymose inflorescence is reduced to a solitary flower (Fig. 3G) and alf exp double mutants generate an unbranched stem bearing bract- or
sepal-like-organs and lack normal flowers (Chapter 4). Surprisingly, exp evg mutants generated solitary flowers and were undistinguishable from exp single mutants (Fig. 3H), indicating that exp fully suppressed the effect of evg on floral meristem identity and DOT expression. To test whether this effect is specific for exp, we examined another solitary flower mutant, hermit (her, Fig. 3I)(Castel, 2009), and found that evg her double mutants generated solitary flowers, just like single her mutants (Fig. 3J). Thus, EVG is, unlike ALF, not required for floral identity in a solitary flower genetic background (exp or her). The observation that under certain conditions evg mutants are very well capable of producing flowers, combined with the fact that EVG is expressed in the sympodial meristem, indicates that EVG is not a floral meristem identity gene that is comparable to ALF or DOT, but that EVG is probably indirectly involved in the specification floral meristem identity, for example by repressing a mobile repressor of DOT in the sympodial meristem (see Discussion) (Rebocho et al., 2008).

Genome wide analysis of alf, dot and evg transcriptomes

We continued our analysis of alf, dot and evg by comparison of their transcriptome with cDNA-AFLP. As the DOT cDNA contains sites for both restriction enzymes that were used for the AFLP, EcoRI and MseI, we first verified the sensitivity of the method by confirming the differential expression of DOT. The regular cDNA-AFLP primer set contains two additional (selective) nucleotides 3’ of those complementary to the AFLP adaptors. These nucleotides increase the specificity of the primers and reduce the number of AFLP fragments amplified with a given primer combination. However, using this primer set and a radioactively labeled DOT cDNA marker, we found that a fragment of another, highly expressed gene ran at the expected height of the DOT fragment.

We therefore performed the reaction with a primer set containing three additional nucleotides per oligo that was expected to amplify the DOT fragment. Figure 4A shows that with this primer set we were able to visualize the differential expression of DOT. Using these primers, however, also led to the amplification of many seemingly randomly dispersed fragments on the gel (Fig. 4A). It seems that the advantage of increased sensitivity by the reduction of the number of bands is accompanied by a loss of binding specificity of the primers. We therefore performed the genome-wide cDNA-AFLP analysis with the regular set of primers containing two selective nucleotides.

Using this primer set we found only one fragment to be differentially expressed. This fragment was found in dot single mutants only (Fig. 4B), for which we named it BAND IN DOT (BAD). To avoid missing differentials due to masking of fragments by
Target genes of ALF, DOT and EVG

Figure 4. Identification and characterization of BAD.
(A) Differential expression of DOT (indicated by arrowhead) visible in a cDNA-AFLP gel. Also note the ghost bands that appear seemingly randomly. (B) cDNA-AFLP gel with 12 samples showing differential expression of BAD (red rectangle). (C) cDNA-AFLP gel with 6 samples in which the differential expression of BAD (red rectangle) was confirmed. (D) Genomic structure of BAD. Thick black line is the intron and rectangles are exons. The 5'- and 3'-untranslated regions are indicated in grey and the start en stop codons are indicated by open and closed circles respectively. (E) RT-PCR of BAD in different tissues. BAD is expressed in inflorescences and small flowers, and more specifically in stamens and carpels. In alf apices BAD is not expressed, and in dot apices in various levels. ACTIN was taken as a positive control. (F-H) No expression of BAD was seen in in situ hybridizations on wild type inflorescence apices. BAD hybridization on sympodial meristem and neighboring stage 4 (Smyth et al., 1990) floral meristem (F) and stage 3 and stage 5 floral meristem (G). (H) shows an in situ hybridization of EVG performed in the same experiment as (F) and (G). (I-K) High levels of ectopic expression of BAD does not lead to an aberrant phenotype. Phenotype of 35S:BAD (left) and non-transgenic sibling (right) (I). The arrow indicates the point at which the switch to flowering was made. Close up of the wild type inflorescence architecture of 35S:BAD (J). RT-PCR on leaves of two 35S:BAD and one non-transgenic sibling, showing high expression of bad in the plants containing the transgene (+), and no expression in the leaves of non-transgenic siblings (-) (K). The genotype in each lane in panels (A-C) is depicted by: m, homozygous transposon insertion mutant for gene depicted on the left; +, wild type for gene depicted on the left; b, bract; ca, carpel; flo, wild type floral buds; fm, floral meristem; inf, wild type inflorescence meristem; If, leaf; pe, petal; se, sepal; sm, sympodial meristem; st, stamen.
other bands, we switched to a slightly different strategy by using a more selective combination (+3/+2) of one primer extended with 3 nucleotides together with one having 2 selective nucleotides, which did not produce the random bands that appeared when using +3/+3 primers. Meanwhile we restricted the number of cDNA samples to be analyzed to only single mutants and one triple mutant, in order to keep the total number of reactions (that is the number of primers pairs times the number of RNA samples) within practical limits. Using this procedure we independently picked up the fragment of BAD (Fig. 4C) as the only differential, confirming the sensitivity and specificity of the cDNA-AFLP method. In total we used 224 primer combinations to display ~8000 cDNA-AFLP fragments and found, besides DOT, only BAD as being differentially expressed.

By PCR on W138 gDNA and somatic transposon insertion-mediated PCR (SOTI-PCR) (Rebocho et al., 2008) we isolated the complete protein coding sequence, intron sequence and 5’ and 3’ flanking regions of BAD (Fig. S1). BAD consists of two exons and one intron, and encodes a predicted protein of 213 amino acids (Fig. 4D).

BAD shows homology to proteins of unknown function from a wide range of organisms ranging from bacteria and archaea to plants and animals. The predicted protein contains a highly conserved region designated Domain of Unknown Function 567 (DUF567) in the Pfam database (Finn et al., 2010), which covers all but the extreme N-terminal end of the protein. The crystal structure of a mouse and Arabidopsis member of the same superfamily were visualized using X-Ray crystallography. The DUF567 domain was shown to be arranged as a 12-stranded, all anti-parallel, closed beta-barrel that surrounds the central alpha helix at the extreme carboxyl terminus of the protein, and which forms most of the hydrophobic core (Boggon et al., 1999; Levin et al., 2007). In Arabidopsis 29 proteins containing this domain have been identified.

BAD and other proteins containing the DUF567 domain are part of a large superfamily related to TUBBY from mouse (Mus musculus). Mutations in TUBBY lead to a tripartite phenotype comprising obesity, retinal degeneration, and hearing loss (Coleman and Eicher, 1990; Kleyn et al., 1996; Noben-Trauth et al., 1996). Structural analyses suggest that TUBBY-like proteins constitute a unique family of bipartite membrane-tethered transcription factors (Bateman et al., 2009; Boggon et al., 1999; Levin et al., 2007). What the role of such a gene could be in plants is unclear.

A gene like BAD that is expressed in dot mutants, but not in alf or evg, might be specifically expressed in the carpels, as these are regularly seen in dot mutants, but not in alf or evg mutants. Such a gene may be a far-downstream target of ALF or EVG, and need not be a (relatively) direct target. We therefore analyzed the BAD expression pattern by RT-PCR in different tissues, in order to determine whether
BAD was indeed carpel-specific. This revealed that BAD is expressed in wild type inflorescence apices, stamens and carpels, while its expression in inflorescence apices of single dot mutants was highly variable (Fig. 4E). These data neither exclude nor confirm that BAD is carpel-specific. However, the variable expression levels between dot samples are indicative that it might be the case: the number of carpels varies between dot plants, which would explain that some samples contain more (incipient) carpel tissue than others, and therefore more mRNA of a carpel-specific gene.

Next, we determined the expression pattern of BAD by in situ hybridization. However, with a dioxigenin-labeled antisense probe we never detected BAD mRNA in wild type inflorescence apices (Fig. 4F-G), while hybridization of an EVG antisense probe to sections of the same tissue gave a clear and specific signals (Fig. 4H). This indicates that BAD mRNA is expressed at very low levels.

Ubiquitous expression of BAD from the Cauliflower Mosaic Virus 35S Promoter (35S:BAD) in wild type petunia line W115 resulted in a wild type phenotype (Fig. 4I-J), even though high levels of expression were achieved (Fig. 4K). We hoped to obtain a transposon insertion mutant from the 3D insertion database by Vandenbussche et al. (Vandenbussche et al., 2008), but unfortunately no dTPH1 insertions in BAD were found. Based on these results we decided not to pursue the characterization of BAD any further.

**FBP MADS-box genes are differentially expressed in alf, dot and evg**

As the genome-wide cDNA-AFLP analysis did not uncover any genes besides DOT that seemed direct targets of ALF, DOT or EVG, we switched to a candidate gene approach. Therefore, we performed semi-quantitative RT-PCRs on the same ds-cDNA samples that were used for the cDNA-AFLP experiments to examine the expression of genes that we deemed likely targets of ALF, DOT and EVG (Fig. 5). These expected targets are the floral organ identity genes, a set of MADS-box genes in petunia known as the FLORAL BINDING PROTEINS (FBPs) (Angenent et al., 1992; Krizek and Fletcher, 2005).

During petunia development the E-type/SEP-like gene FBP4 is first expressed in bracts and the E-type gene FBP23 is first expressed in sepals (Ferrario et al., 2003; Immink et al., 2003). FBP4 and FBP23 expression thus mark subsequent steps in floral development.
In *evg* all floral organ identity genes that were analyzed are down regulated when compared to *alf* and *dot*, except for *FBP4* (Ferrario et al., 2003). Expression of this bract marker (Immink et al., 2003) indicates that *evg* mutants do make the switch to flowering, as bracts are specialized leaves that sub tend the flower and the lateral sympodial meristem in wild type petunia. However, they are arrested at an earlier stage of flower development than *alf*, *dot* and *alf dot*, as indicated by the reduced expression of several other *FBP* genes, such as *FBP23*. The E-type gene and sepal marker *FBP23* (Immink et al., 2003) is (more) strongly down-regulated in *evg* compared to in *alf*, *dot* or *alf dot*, similar to the regulation of *DOT*. This indicates that *alf* and *dot* (occasionally) progress to the production of (green) flowers with sepals that express the sepal marker *FBP23*. Furthermore, *dot* mutants express several more *FBPs* that are (more strongly) down regulated in *alf* and *alf dot*. The expression of C-, D- and E-type genes is necessary for the specification of the organs in the innermost whorl (carpels and ovules). Hence their relatively strong expression in *dot* is not surprising, given that carpels are regularly found in *dot*. Taken together, these RT-PCR data show that progressive loss of *DOT*, *ALF* and *EVG* is correlated with the expression of less floral organ identity MADS-box genes.

**Post-translational activation of ALF, DOT and EVG**

As an alternative to the cDNA-AFLP analysis of *alf*, *dot* and *evg* loss of function mutants we followed a gain-of-function approach for which we used translational fusions of ALF, DOT or EVG with the ligand-binding domain of the glucocorticoid receptor (GR). GR fusion proteins of transcription factors are inactive because they are bound to cytoplasmic heat shock proteins and cannot travel into the nucleus.

![Figure 5. Analysis of expression levels of FBP genes in mutant apices. RT-PCR on double stranded cDNA of single, double and triple mutants of alf, dot and evg for known FBP MADS-box genes. -, no-template negative control.](image_url)
When dexamethasone (DEX) is applied, the GR fusion protein is released and can enter the nucleus to activate the transcription of target genes (Picard et al., 1988). When in addition to DEX also the translation inhibitor cyclohexamide (CYC) is applied, the synthesis of proteins encoded by direct target genes is prevented. Therefore only direct targets of the GR fusion are expected to be activated upon DEX induction in the presence of CYC.

We constructed chimeric genes in which the 35S promoter drives the expression of a fusion of GR to the N-terminus of ALF (GR-ALF) or to the C-termini of DOT or EVG (DOT-GR and EVG-GR). GR was fused to the N-terminus of ALF because previous work suggested that a fusion of GR to the C-terminus of ALF (ALF-GR) was inactive. That is, when DEX was applied to ALF-GR alf plants by watering, or by dipping inflorescence apices into a DEX solution, no phenotypical alterations were observed (Alexandra Rebocho, unpublished results). The ALF-GR fusion protein was therefore considered to be inactive, possibly because the protein was unstable, because proper folding of the ALF protein was impaired by GR, or because the tertiary structure of the chimeric protein prohibited the induction by DEX. We hoped that an N-terminal fusion of GR to ALF would not suffer from these problems. Each transgene was stably transformed into petunia line W115.

Figure 6. GR-ALF and ALF-GR proteins are functional. (A) RT-PCR on leaves of primary GR:ALF transformants. High GR:ALF expression was observed in four ALF<sup>+</sup>/m and the alf<sup>m/m</sup> plants. ACTIN was taken as a constitutively expressed positive control. (B) In GR:ALF alf watered with mock solution the alf phenotype is maintained. (C) GR:ALF alf watered with 10µM DEX for four weeks. Note that the control (B) repetitively branches, whereas the DEX treated plant forms a cymose structure with green structures bearing supernumerary whorls of green organs (arrows). (D-F) Wild type (D) 35S:DOT (E) and 35S:ALF 35S:DOT seedling (F). (G) GR:ALF 35S:DOT seedlings grown on MS DEX. (H) ALF-GR 35S:DOT seedling grown on MS DEX.
As \textit{35S:ALF} plants are wild type (Souer et al., 2008), we could not assess whether GR-ALF was functional by DEX induction of GR-ALF in wild type. We therefore transformed GR-ALF to \textit{ALF}^{+/m} and to \textit{alf}^{m/m} plants and obtained eight transformants, of which one in \textit{alf} background. RT-PCR analysis showed that the transgene was expressed at high levels in four of the GR-ALF \textit{ALF}^{+/m} transformants, and in the GR-ALF \textit{alf}^{m/m} plant (Fig. 6A). The GR-ALF \textit{alf}^{m/m} plant had an \textit{alf} phenotype (Fig. 6B) and GR-ALF \textit{ALF}^{+/m} appeared wild type. Several cuttings of GR-ALF \textit{alf}^{m/m} were watered for four weeks with water containing 10 \(\mu\)M DEX or with water alone. Although induction was not able to fully complement the \textit{alf} phenotype, in many branches one of the two inflorescence shoots was replaced by a green structure bearing supernumerary whorls of green organs that resembled sepals or bracts (Fig. 6C). As these shoot-like structures do not branch, which is a typical character defining a sympodial inflorescence shoot, this suggests that DEX treatment of GR-ALF \textit{alf} does reduce the sympodial identity and increases floral identity, albeit very partially.

To further characterize the activity of GR-ALF, we used the fact that petunia seedlings constitutively expressing both \textit{ALF} and \textit{DOT} are arrested. These double overexpressors have closed cotyledons (Fig. 6F) and ectopically express a whole array of floral organ identity MADS-box genes (Souer et al., 2008). We crossed GR-ALF plants to \textit{35S:DOT} and germinated the seeds on Musharige and Skoog agar plates containing 25\(\mu\)M DEX (MS DEX) or mock. Of the 32 seedlings grown on MS DEX 10 showed a phenotype that was highly similar to that of \textit{35S:ALF 35S:DOT} seedlings (Fig. 6G) and 12 had a visible \textit{35S:DOT} phenotype (seedlings with curly leaves, Fig. 6E). All 42 seedlings on the mock plate had a wild type or \textit{35S:DOT} phenotype, and growth-arrested seedlings were not seen.

PCR analysis showed that the arrested seedlings with closed cotyledons contained both transgenes, whereas the other seedlings contained either GR-ALF or \textit{35S:DOT} alone or no construct at all. Out of 38 genotyped seedlings on the mock plates 10 contained both GR-ALF and \textit{35S:DOT}, but these had a wild type phenotype in the absence of induction (not shown). These data show that the GR-ALF fusion protein is functional upon induction, but that GR-ALF is inactive in the absence DEX.

As the above experiments with GR-ALF indicated that this protein is active in seedlings, it follows that the failure of DEX to induce phenotypic changes in mature flowering plants may be due to a poor penetrance of the DEX into developing meristems, rather than inactivity of the protein. This prompted us to re-examine whether the C-terminal fusion of GR to ALF was indeed inactive (as inferred from assays with DEX treatment of mature plants, see above) using the seedling assay. Therefore, we germinated the available seeds of a cross of \textit{ALF-GR} and \textit{35S:DOT} on MS DEX or MS and observed that also \textit{ALF-GR 35S:DOT} seedlings had the closed cotyledons
phenotype when germinated on MS DEX (Fig. 6H), but not when germinated on MS alone. This suggests that also ALF-GR is functional. We consider it likely that DEX treatment of mature flowering ALF-GR alf plants caused a phenotype that was as subtle as that of induction of mature GR-ALF alf (Fig. 6C) and was therefore not recognized at the time.

Figure 7. DOT-GR and EVG-GR are functional
(A) Phenotype of DOT-GR seedling grown on MS DEX. Note the characteristics of 35S:DOT in DOT-GR (curled leaves indicated by arrows), which are absent from wild type plants grown on MS DEX (cf. Fig. 6D and 7F). (B) RT-PCR analysis of DOT-GR seedlings grown on MS DEX. Wild type and EVG-GR seedlings and a no-template sample were used as controls. Note the up regulation in DOT-GR of B- and E-type MADS-box genes FBP1, GP and FBP9. ACTIN was used as a ubiquitously expressed positive control. -, negative no-template control (C) Mature DOT-GR plants watered with a 10µM DEX solution for four weeks. Note the extremely short internode between f1 and f2 and the curly bracts (arrow). f1, f2, f3, flowers numbered in order of development. (D-E) Cuttings of DOT-GR dot watered for four weeks with 10µM DEX. Note how the zig-zag cymose inflorescence structure returns in DOT-GR dot watered with DEX (D). The ‘green flowers’ often appear in pairs (arrow). The green flowers contain several whorls of sepals, no petals and stamens, and often end in a carpel (E). (F) EVG-GR (right) and wild type (left) seedling germinated on MS DEX. Internodes in EVG-GR are elongated compared to wild type and leaves are narrow and curled, similar to the phenotype of 35S:EVG (Rebocho et al., 2008). (G) EVG-GR seedling grown on MS DEX showing an exceptionally strong 35S:EVG phenotype.
In order to determine whether DOT-GR was functional we germinated DOT-GR seeds on MS DEX. 35S:DOT plants form single flower inflorescences and flower early, and young 35S:DOT seedlings are discerned from wild type by curly petaloid leaves that express high levels of B- and E-type floral organ identity genes (Souer et al., 2008). We observed the same phenotype in DOT-GR seedlings on MS DEX (Fig. 7A). RT-PCR analysis showed that they, like 35S:DOT seedlings, express B- and E-type MADS-box genes, whereas wild type seedlings germinated on MS DEX do not (Fig. 7B). In the absence of induction DOT-GR seedlings appear wild type.

When cuttings of mature DOT-GR plants in wild type background were watered for four weeks with 10 µM DEX, the newly emerged leaves were curly, comparable to the leaves of 35S:DOT (Souer et al., 2008). Although the cymose inflorescence structure of these plants was maintained, the internodes were of variable length and were often extremely compressed, as if the production of flowers was favored over the elongation of internodes (Fig. 7C). These effects were not seen in plants watered with a mock solution.

Next, we crossed the DOT-GR gene into dot mutants to examine whether DOT-GR could rescue floral meristem identity and the cymose inflorescence architecture. As expected cuttings from DOT-GR dot plants that were watered with mock kept the dot phenotype (data not shown), whereas DOT-GR dot cuttings watered with DEX formed curly leaves and determinate structures in the axils of the lower bracts, where flowers would form in wild type (Fig. 7D). These ‘green flowers’, which consisted of whorls of green, sepal-like organs with a central carpel (Fig. 7E), were surprisingly often formed in pairs of two (Fig. 7D, arrow). In wild type petunia, an axillary shoot is formed in the axil of the bract that subtends the flower. In the DEX-treated DOT-GR dot plants this axillary shoot was absent, and we therefore assume that in DEX treated DOT-GR dot plants this axillary shoot was converted into a second green, single flower.

We also tested the functionality of EVG-GR by the simple, but effective method of germinating seeds of primary transformants on DEX MS. 35S:EVG plants remain small and have thin stems and narrow leaves, a phenotype associated with the production of an excess amount of auxin (Rebocho et al., 2008). When grown on MS DEX EVG-GR seedlings developed the 35S:EVG phenotype (Fig. 7F), in some lines a phenotype stronger than ever observed in 35S:EVG was seen (Fig. 7G). EVG-GR plants grown on MS appeared wild type, indicating that EVG-GR is inactive in the absence of induction. These data show that EVG-GR is functional when induced with DEX.
Target genes of ALF, DOT and EVG

cDNA-AFLP of DEX-treated DOT-GR and EVG-GR seedlings

To identify target genes of DOT and EVG, we compared the expression profiles of induced and non-induced DOT-GR and EVG-GR seedlings by cDNA-AFLP. DOT-GR and EVG-GR seedlings were grown for 23 days on MS plates and were induced by emerging them in tubes containing water with DEX, CYC, DEX and CYC or a mock solution. This method of induction previously showed successful in experiments with Arabidopsis LFY-GR seedlings by Wagner et al. (1999) and floral buds of petunia (Spelt et al., 2000).

We treated two batches of DOT-GR seedlings for four hours each and two batches of EVG-GR seedlings, one for two hours and the other for four hours. We expected that in EVG-GR target genes would be up or down regulated quickly, because EVG is a homeodomain transcription factor that can directly bind to the promoters of its targets after induction. DOT, in contrast, is an F-box protein that will probably indirectly influence transcription of target genes, and DOT-GR may therefore influence transcription of these targets more slowly. A control group of non-transgenic seedlings was treated for four hours. For the cDNA-AFLP analyses we mixed the mRNAs of the two batches of the same GR genotype in a 50/50 ratio, hoping that if the induction of one of the two was less successful, target genes would be found nevertheless. We aimed to use a minimal number of samples (see Table S1) by using the two genotypes as mutual controls instead of comparing to non-transgenic seedlings.

Using 208 primers combinations, we analyzed the expression of ~7500 mRNA fragments, but found none that had an expression pattern that clearly indicated induction or repression by the fusion proteins, such as a strong band in either a DEX-treated sample alone or in both the DEX and in the DEX CYC treated sample of one genotype. Nonetheless we isolated 80 gene fragments whose expression seemed to be altered slightly by DEX treatment, reamplified and sequenced them. However, when we reassessed the expression levels by RT-PCR on the original panels of induced plants, we found no evidence at all that they were regulated by DEX treatment.

Possibly, the treatment of seedlings by submerging them in DEX-containing water did not work as expected. In Arabidopsis this method of induction did prove successful (Wagner et al., 1999), but petunia has a different cuticle and different trichomes than Arabidopsis and might therefore repel water more strongly, causing insufficient penetration of DEX into the tissue. Another possibility is that the number of transgenic seedlings or the average expression levels of DOT-GR or EVG-GR were too
low in the segregating families that we used for this experiment. Furthermore, the induction of DOT-GR targets may have failed because DOT(-GR) is dependent on ALF for its action, and endogenous ALF is only expressed in a limited number of cells in vegetative petunia (Souer et al., 2008; Souer et al., 1998). We therefore decided to search for targets of DOT by a different approach: by simultaneous induction of GR-ALF and DOT-GR in individually PCR-selected seedlings.

Simultaneous induction of GR-ALF and DOT-GR

Simultaneous ectopic expression of ALF and DOT in petunia seedlings causes expression of a wide array of ABCDE-type organ-identity genes, which are usually not expressed until the reproductive phase (Souer et al., 2008). To determine whether the FBP genes are early, or even direct targets of ALF and DOT, we crossed GR-ALF to DOT-GR. By germinating the seeds on MS DEX we determined that simultaneous induction of GR-ALF and DOT-GR was effective: the double GR seedlings terminated, like 35S:ALF 35S:DOT double overexpressors, but the phenotype deviated slightly. That is, instead of closed cotyledon phenotype that is typical for 35S:ALF 35S:DOT seedlings (Fig. 6F), the DOT-GR GR-ALF seedlings terminated with open cotyledons (6 out of 24 seedlings, Fig. 8A). When seedlings of the same crossing were grown on mock plates, only wild type seedlings were observed (24 seedlings).

Figure 8. DOT-GR directly regulates FBP9 and FBP26.
(A) Wild type (left) and GR-ALF DOT-GR (right) seedlings grown on MS DEX. Note that the GR-ALF DOT-GR seedling opened its cotyledons but is arrested in growth and did not form any true leaves. (B) RT-PCR analysis of individually PCR-genotyped seedlings, treated for six hours with DEX (D), CYC (C) or both (DC) or, as a control, with a mock solution (M). WT, wild type.
Target genes of ALF, DOT and EVG

We reasoned that analyzing target gene expression in a homogenous population would be more sensitive than in a mixed population segregating for the presence of two transgenes of which only 25% has the desired genotype. Therefore, we germinated seed of the cross GR-ALF x DOT-GR on MS and analyzed individual seedlings by PCR for the presence of the transgenes. In this way we were able to induce each of the four genotypes (wild type, GR-ALF, DOT-GR and GR-ALF DOT-GR) separately, by immersion for 6 hours in either mock solution, DEX, CYC or both DEX and CYC.

We subsequently assessed the expression of a range of *FBP* genes by RT-PCR. Most of the *FBP* genes that we tested were not expressed at levels detectable by RT-PCR. However, we observed that the expression of *SEPALLATA*-like gene *FBP9* (Immink et al., 2002) is activated when DOT-GR or GR-ALF DOT-GR seedlings were treated with DEX (Fig. 8B). This up regulation also takes place in the presence of translation inhibitor CYC, which indicates that *FBP9* is directly regulated by DOT, without the synthesis of an intermediate regulator. Up regulation is enhanced in double transgenic GR-ALF DOT-GR seedlings compared to single transgenic DOT-GR seedlings. This is probably because ALF is in DOT-GR expressed from the endogene only, hence in a limited number of cells, whereas in GR-ALF DOT-GR it is expressed ubiquitously.

Interestingly, also *FBP26*, which is a homolog of the A-type gene *FRUITFUL* from Arabidopsis (see Chapter 6 and Immink et al. (1999)) is up regulated in the double GR-ALF DOT-GR seedlings after six hours of DEX or DEX CYC treatment (Fig. 8B). The activation of *FBP26* upon combined DEX CYC induction is somewhat difficult to assess, because *FBP26* is already up regulated by treatment with CYC alone. Such a differential expression upon CYC treatment was seen for many genes in direct RT-PCR assays (e.g. *ACTIN*, Fig. 8B) and cDNA-AFLP analyses (data not shown) and is usually attributed to the decay of an already existing inhibitor or activator of the up or down regulated gene (see e.g. (Picard et al., 1988)). These regulators are not replaced in the presence of translation inhibition by CYC. However, in GR-ALF DOT-GR seedlings, *FBP26* is up regulated more strongly by DEX CYC treatment than by CYC alone, indicating that also *FBP26* is a direct target of ALF and DOT. These data provide independent evidence that DOT directly activates ALF target genes, which was suggested by Souer et al. (2008).
Discussion

This chapter describes the search for targets of the petunia meristem identity genes *ALF*, *DOT* and *EVG*. We employed two strategies to identify their targets, a loss of function approach based on the analysis of mutants, and a gain of function approach based on ectopic post-translational induction of fusion proteins. A priori, we assumed that *ALF*, *DOT* and *EVG* specify floral meristem identity by distinct mechanisms and target genes, because they encode for essentially different proteins that are expressed in different patterns. However, detailed characterization of the mutants, which is documented here and elsewhere (Castel, 2009; Rebocho et al., 2008; Souer et al., 2008; Souer et al., 1998), showed that this is not the case. Our data show that the *ALF*, *DOT* and *EVG* pathways converge earlier than expected, and are all three needed to generate an activated form of *ALF* that specifies floral meristem identity and drives transcription of downstream genes, such as *FBP9* and *FBP26*.

Genetic data suggested that *ALF* and *DOT* are interdependent for activity and together are sufficient to activate B- and E-type organ identity genes, and in combination with the WUS-homolog TERMINATOR also C- and D-type genes (Souer et al., 2008). Since *ALF* and *DOT* directly interact it is believed that the role of *DOT* is to activate the transcription activation domain of *ALF* to trigger transcription of target genes. The results presented here lend further support to this model. From the finding that the transcriptome of *alf* and *dot* mutants are (nearly) identical it follows that *DOT* is required for the activation of (nearly) all *ALF* target genes. Furthermore, the model predicts that the activation of *ALF* target genes by *DOT* should be direct (Souer et al., 2008), which is indeed what we found for the activation of *FBP9* and *FBP26*.

At this stage we cannot explain why the *alf* and *dot* phenotype are, though very similar, not fully identical. Although the *dot* phenotype is much stronger than that of homologous mutants in *Arabidopsis (ufo)* or *Antirrhinum (fimbriata)* it is still slightly weaker than the *alf* phenotype, which is evident from the relatively frequent formation of carpels in *dot*. One possibility is that the function of *DOT* is slightly redundant with that of other genes or pathways. Another possibility, not excluding the previous one, is that a small number of *ALF* target genes are not at all or less dependent on *DOT*. The observation that a weak *dot* allele only compromises the identity of petals, but not the identity of the floral meristem or other floral organs indicates that the activation of B-type organ identity genes requires higher levels of *DOT* activity than other *ALF* targets, such as C-type genes (Souer et al., 2008). Furthermore, in (double) mutant background with either *veggie* (Roobeek, 2011), *hermit* (Castel, 2009) or *extrapetals* (Chapter 4), *alf* appears to block the onset of flowering completely, whereas *dot* does not, suggesting that at least some targets of *ALF* may be completely independent of *DOT*. These unknown *ALF* targets should
be active during the switch to flowering, but may be inactive during inflorescence development and therefore may have escaped detection in our mRNA profiling of inflorescence apices.

The observation that EVG is exclusively expressed in the emerging sympodial meristem well before ALF or DOT become active there suggested that EVG promotes floral identity via a pathway that is distinct from the ALF/DOT pathway. However, our finding that the absence of flowers in evg mutants was due to down regulation of DOT was highly surprising, and also explains why we found only a few genes that were differentially expressed between alf, dot and evg. However, the mechanism by which EVG promotes DOT expression seems rather complex and is unlikely to involve a simple transcriptional cascade. A model for the regulation of DOT expression needs to account for two apparently paradoxical observations. First, expression of DOT in the floral meristem is dependent on expression of EVG in the sympodial meristem. Second, mutations that disrupt the initiation or identity of the sympodial meristem fully suppress this EVG-dependency of DOT.

Figure 9. Model explaining the phenotype of evg in different backgrounds. During development floral identity (indicated in red) is specified by ALF and DOT. Initiation and identity of the sympodial meristem (blue zone) is controlled by EXP and HER. The model assumes that an unknown mobile factor, designated ‘X’, synthesized in the sympodial meristem (anlagen) inhibits DOT expression in the sympodial meristem and in the neighbouring floral meristem, and thereby specification of floral identity. EVG disrupts the inhibitory effect of X on DOT expression in the floral meristem, possibly indirectly by promoting proliferation of the lateral sympodial meristem and separation from the apical floral meristem. In exp and her mutants development of the sympodial meristem is compromised and X is not made; hence EVG is no longer required for DOT expression. This Figure is adapted from (Rebocho et al., 2008).
To explain these observations, we postulate that the sympodial meristem produces a non-flowering signal (indicated as ‘X’ in Fig. 9), which negatively regulates DOT expression. If the action of X is not cell-autonomous, X would also inhibit DOT expression in the cells that will form the floral meristem as long as they do not separate from the emerging sympodial meristem (Fig. 9). Because EVG promotes the proliferation of the sympodial meristem and thereby its physical separation from the floral meristem, this provides an explanation for how EVG may lift the inhibitory effect of X on the expression of DOT. This would imply that the absence of flowers in evg mutants in the W138 background is an indirect effect of the fusion of floral and sympodial meristems and the continued inhibition of DOT expression in both the floral and sympodial zones. An alternative explanation might be that EVG represses X or activates DOT in a cell-autonomous way. Such a scenario requires a mechanism that can account for the time span of ~2 days between EVG and DOT expression in the same meristem dome.

The hypothetical mobile factors that inhibit (‘X’) or promote floral identity are not known. To account for non cell-autonomous effects, X need not necessarily be a classical signalling molecule, but could also be a transcription factor, as several transcription factors are known to move between cells over short distances, such as LFY, the maize homeodomain protein KNOTTED1 and the Antirrhinum MADS-box protein DEFICIENS (Kim et al., 2002; Lucas et al., 1995; Perbal et al., 1996; Sessions et al., 2000; Wu et al., 2003). Given that exp and her mutants produce, like 35S:DOT, solitary flowers it is possible that EXP or HER encode X or (indirectly) promote the synthesis of X. In either way, mutation of EXP or HER would be expected to abolish the expression of X and fully suppress the requirement of EVG for expression of DOT and the formation of flowers (Fig. 9). The finding that evg mutants display a solitary flower phenotype in a mixed W115/W138 genetic background supports the view that EVG promotes floral identity indirectly. The simplest explanation would be that the evg phenotype gets stronger in this background, -to become essentially similar to the exp and her phenotype- resulting in a (nearly) complete block of sympodial meristem development and formation of ‘X’ (Fig. 9).

Shortly after the publication of EVG, a paper by Lippmann et al. appeared that described the Solanum lycopersicum (tomato) orthologs of DOT and EVG, ANANTHA (AN) and COMPOUND INFLORESCENCE (S), respectively (Lippman et al., 2008). Tomato is, like petunia, a member of the Solanaceae family and a close relative of petunia. AN is very comparable to DOT with respect to the encoded protein, the expression pattern and the phenotype of the mutant. Also S and EVG show many similarities. The phenotype of s was described for several alleles and it varies markedly among these alleles: sometimes the mutation results in highly branched 'panicle-like' tomato inflorescences with
supernumerary flowers, whereas other alleles more resembled an. According to Lippman et al. this variation was caused by different genetic backgrounds and not by the different mutant alleles (Lippman et al., 2008). Lippmann et al. postulated that the expressivity of the s allele was determined by differences in growth conditions and uncharacterized ‘modifier loci’ that differ between tomato lines. It appears that in petunia evg mutants a very similar mechanism is at work.

The expression profiles of alf, dot, evg and their double and triple mutants were found to be highly similar, but the expression levels of several floral organ identity MADS-box genes showed to be different between alf, dot and evg (Fig. 5). These FBP RT-PCR data confirmed the idea that evg is epistatic over alf and dot, as indicated by the fact that the phenotype of alf evg, dot evg and the triple mutant is indistinguishable from evg. The RT-PCR data indicate that floral development in evg (at least in the fully flowerless apices in W138 background that we used in this study) is arrested earlier than in alf, as the sepal marker FBP23 and C-type gene FBP14 are expressed in alf, and not in evg. alf is again arrested slightly earlier than dot, as indicated by the complete block of carpel formation and reduced expression of C-, D- and E-type organ identity genes (resp. FBP6, FBP11 and FBP9). These data indicate that EVG, ALF and DOT are active in subsequent stages of petunia floral development, which corresponds to the order in which they are expressed in the sympodial meristem.

In our experiments with the GR fusion proteins, we did not assess the effectiveness of CYC translation blockage. This means that we cannot be sure if FBP9 and FBP26 are indeed direct targets of (ALF and) DOT. However, CYC treatment was performed similarly as in other experiments in which it was proven to block translation completely (Wagner et al., 1999), and the differential regulation of many genes upon CYC treatment indicates that CYC was also active in our experiments. Moreover, the relatively brief induction period of six hours used in the experiments with GR-ALF DOT-GR seedlings makes it unlikely that multiple tiers of downstream genes were activated.

The induction experiments with GR fusion proteins showed that FBP26 expression is up regulated by simultaneous induction of GR-ALF and DOT-GR. As DOT is dependent on ALF for its function (Souter et al., 2008), we expect that differential expression of any gene via induction of DOT-GR requires co-expression of ALF. In DOT-GR such co-expression is confined to young leaf primordia, where endogenous ALF is expressed, while in GR-ALF DOT-GR it occurs in all cells. That both GR-ALF and DOT-GR are required for induction of FBP26 suggests the involvement of one or more additional (co-)activators that are not expressed in leaf primordia, similar to the activation of C- and D-genes in 35S:ALF 35S:DOT seedlings (Souter et al., 2008).
The direct activation of \textit{FBP26} is interesting, because the \textit{Arabidopsis} A-type gene \textit{APETALA1} (\textit{AP1}), which groups in the subclade neighboring \textit{FRUITFUL}/\textit{FBP26}, is a direct target of \textit{LFY-GR} (Wagner et al., 1999). \textit{AP1} is a major determinant of floral identity in \textit{Arabidopsis}, and a large part of the early flowering effect of \textit{35S:LFY} is achieved through up regulation of \textit{AP1} (Weigel and Nilsson, 1995). Whereas in our experiments in petunia \textit{DOT} and \textit{ALF} are required together to activate \textit{FBP26}, activation of \textit{LFY} alone is sufficient for \textit{AP1} up regulation in \textit{Arabidopsis}.

\textbf{Acknowledgements}

We could not have done this research without the excellent plant care of Pieter Hoogeveen, Daisy Kloos and Martina Meesters. Matthijs van Lint did a great job in verifying possible \textit{DOT} and \textit{EVG} targets. This work was supported by a grant to R.K. from the Netherlands Organisation for Scientific Research (NWO).
Materials and methods

Plant material
The transposon insertion alleles alf<sup>W2167</sup>, dot<sup>A2232</sup> and the stable recessive allele evg<sup>D2299</sup> have been described in detail elsewhere (Rebocho et al., 2008; Souer et al., 2008; Souer et al., 1998). The single, double and triple mutants were generated in W138 background by self fertilization of triple heterozygous ALF<sup>W2167/+</sup>; DOT<sup>A2232/+</sup>; EVG<sup>D2299/+</sup> plants. Mutants were scored by the flowerless and sometimes fasciated (evg) phenotype, and subsequently by PCR using gene-specific primers flanking the transposon insertion sites (Table S2).

All plants were grown in a greenhouse. Care was taken that for comparisons of phenotypes plants were grown side by side to exclude that any phenotypic differences were caused by variations in greenhouse conditions.

RNA analysis and cDNA-AFLP
For cDNA-AFLP PolyA<sup>+</sup> RNA was extracted from the top 2-3 mm of alf, dot, evg single, double and triple mutant inflorescence apices or from whole (DEX-induced) seedlings using the TRizol reagent (Invitrogen) and a polyAtract mRNA system III (Promega). The PolyA<sup>+</sup> RNA was converted to cDNA with Superscript reverse transcriptase (Invitrogen) and made double stranded with RNase H and DNA polymerase I (Promega), all according to the manufacturers’ specifications. mRNA abundance was determined by RT-PCR as described (Quattrocchio et al., 2006), using two gene-specific primers (Table S2) and for many additional mRNA fragments by cDNA-AFLP.

cDNA-AFLP analysis of mRNA isolated from apices or seedlings was performed essentially as described (Bachem et al., 1996) using the restriction enzymes Msel and EcoRI (apices) or Msel and HindIII (seedlings) and corresponding adapters and primers.

Quantitative RT-PCR analysis on RNA samples other than the double stranded cDNA samples used for cDNA-AFLP was performed as described previously (Quattrocchio et al., 2006). In brief, total RNA was extracted using TRizol (Invitrogen) and first strand cDNA was made with Superscript reverse transcriptase (Invitrogen) and first strand cDNA was made with Superscript reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. RT-PCR on first strand cDNA was performed with a reduced number of cycles to prevent saturation, using two gene-specific primers (Table S2). The PCR products were run on agarose gels (Sigma), blotted onto Hybond-N<sup>+</sup> membranes (Amersham) and hybridized with <sup>32</sup>P-labeled gene-specific probes. Hybridization signals were detected by a Phosphorimager (GE Healthcare). We used ACTIN as a constitutively expressed positive control.

Construction of transgenes and plant transformation
To generate the ALF-GR, DOT-GR and EVG-GR construct the cDNAs were amplified from the 35S:ALF, 35S:DOT (Souer et al., 2008) or 35S:EVG vector (Rebocho et al., 2008) respectively with Phusion High-Fidelity DNA Polymerase (Finnzymes) using primers flo11 and flo1; puf29 and puf30; and evg11 and evg24 The PCR products were ligated into the XbaI/BamHI sites of pGreen5K (Hellens et al., 2000).

GR-ALF was constructed by amplifying the ALF cDNA from the 35S:ALF vector with Phusion High-Fidelity DNA Polymerase, using primers flo43 and flo9 and ligating the digested PCR product into the XbaI/BamHI site of pGreen1K (Hellens et al., 2000). Subsequently, the sequence encoding the GR receptor
domain was amplified from pGreen5K using Phusion and primers GR ATG F and GR R2 and ligated into the Xbal site of the vector obtained in the previous step.

35S:BAD was constructed by ligating a Phusion PCR product made with primers 02_02F4 and 02_02R5 on W138 inflorescence cDNA into the HindIII site of pGreen7K (Hellens et al., 2000).

All constructs were re-sequenced before introduction into plants.

The transgenes were introduced into heterozygous F1 hybrids of the lines W138-derived lines W229 (alf<sup>W2167</sup>) or W232 (dot<sup>A2232</sup>) and the unrelated line W115 (ALF<sup>+</sup> DOT<sup>+</sup> EVG<sup>+</sup>) using Agrobacterium tumefaciens (strain AGL0) mediated leaf disc transformation (Horsch et al., 1985). For the generation of 35S:DOT evg plants we selected F2 progeny from the cross W264 (evg<sup>D2299</sup>) x W115 (EVC<sup>+</sup>) with the ‘green’ evg phenotype and transformed those by leaf disk transformation using the 35S:DOT construct described elsewhere (Souer et al., 2008). The sequences of the primers used are listed in Table S2.

**Induction of GR fusion proteins**

For continuous induction of GR fusion proteins sterilized seeds were germinated on 1x Musharige and Skoog (MS, Duchefa) plates containing 10 µM dexamethasone (DEX, Sigma) (MS DEX) or control plates (Mock, M) containing 0.1% ethanol.

Transient induction of seedlings was performed by submerging 23-day old seedlings germinated on 1x MS in 50 ml tubes with demineralized water containing 25 µM DEX (D) or 25 µM DEX and 10 µM cycloheximide (CYC, DC) (Sigma) under gentle shaking. Control seedlings were submerged in demineralized water containing 0.1% ethanol (M) or 0.1% ethanol and 10 µM CYC (C). Two independent induction experiments were performed for each genotype: twice 4 hours for DOT-GR seedlings and once 2 hours and once 4 hours for EVG-GR. Non-transgenic control seedlings were treated for 4 hours. After induction the tissue was flash frozen and stored at -80°C until further use.

GR fusion proteins in mature plants were induced by prolonged daily watering (three to four weeks) with water containing 10 µM DEX. Control plants were treated with a mock solution (water containing 0.1% ethanol).

**In situ hybridization experiments**

In situ hybridization was performed as described by Souer et al. (Souer et al., 1998). Dioxigenin-labeled BAD or EVG antisense RNA probes spanning the full coding region were in vitro synthesized using T7 polymerase and dioxigenin labeling kits (Roche) according to the manufacturer’s specifications. Post-hybridization treatment included a RNaseA treatment, which eliminates non-specifically bound probe as well as cross-hybridization to related mRNAs (Rebocho et al., 2008).

**Isolation of BAD**

BAD was found as a fragment only expressed in dot single mutants on a cDNA-AFLP gel. The band was isolated, reamplified and sequenced with the corresponding cDNA-AFLP primers. The full length BAD protein coding sequence and the gDNA sequence was obtained by somatic transposon insertion-mediated PCR (SOTI-PCR, see Rebocho, 2008).
Genbank accession numbers
ALF, AF030171 ; DOT, EU352681; EVG, EF187281; FBP1/PhGLO, M91190/AY532265; FBP2, M91666; FBP4, AF335234; FBP5, AF335235; FBP6, X68675; FBP7, X81651; FBP9, AF335236; FBP11, X81852; FBP14 (pMADS3), X72912; FBP23, AF335241; FBP26, AF176783; GP/PhDEF, AY370519/DQ539416; BAD, to be submitted
Supporting data

CTGGSYTTAC GCASMTAKTG AAGMATTTT TTAGGGAACATA ATACAACACGT GCCTAAGCTG TTTCGAAAATT 0070
TGCAAGTTATG AATTAACCTT ATCTAAAGGA ATATTACTT CTATTGAGGA GAAATATTATA TATTCAACGTC 0140
GAGATAAGCC TCCAAATAGCC ATATGACCTC CAGATATTTAA AATATCAAAT GTGGAAGACTG CACACAGCAAT 0210
TCAAATGATTT AAAATATCTA AAGAAAGAAG TAAACACATT ATTTAACTCCC TCCTCCACAG TCACAATTTC 0280
ACTCTTTCTCG ATTTGAATAT TTCTTCTACTCT TTCTCTACTCT TTCTCATTTC 0350
AAAAGTTGATG TAATGGAAGT GAGTGAGCTA TTCAATTGGA ATTAATGGAA GTAAGTACTAG TATTTGGTTG 0420
AGGAGAGGGA TATGTGCAAA AGGTATTGGG TTGGAAAAGG ACACAAAGCT CAGATAGTAA AAAAGTTCAT 0490
TGTTCTATTC TGTTGATTGGC TTCTATCTGC ATATCCCTGC TGTTGAAATT GTCTTCTGAG TGTTGATCAT 0560
CTGGCCGGAT TTTACCAAAC ATGAGGACTG GTTCCTCACTG AATTCTGCGC GCAATCTTCA GTCTCACACCT 0630
CACCCGAGG TTTGGTATTC TCTAGTTTAT GTTCTCATCT TAATTCCTAGT TTCTGCTTGT AGCTTACAAG 0700
TGTTTGACCA TTAGTGCCAT TATCAAAATT GCTAAATGATA ATATCCCTTT CTAAATATTC TTTAGTGTTG 0770
AAGGAAACTT TAAAGAAGGA ATGTTAATTA CGTTTCTGTC CAAAGATATT TGATTTCTCTT 0840
TCCCCGATT GTAAGGATAA AAAAAACTAA AAGGATGAAA AGTATAAGAC ACCTCACATA TCAAAACACC 0910
TAAAGCCTGTC AAGTGGTAAAA GAGCAAACCA AATAATGGAAG TTATGAGGAT GCTCAATGG TCACACAACC 0980
ATTCTTGTGC AAAAAACAAA AAAAAAAT ACAAAGCTTT TCACATTTCT CAATACGATA TTGTAGTAGT 1050
TGAACCAACTC AATTTGGAAT ATGGAGGTGG TATAAAGTAA CAACCATGTT GAGCATAAAT ATATCCTCTT 1120
CTGGAGAAGT CCTAAACTCTT CTATATCTTT CTAGATTACA ATAACCTTTGAG ATGTCCCAAAC CACATACTGA 1190
TGATAGAGTTA TATAGGAATTA AGATAAATTT AGTCTGACTCT TCTTCTGGAG TTTGTATATAA TTGTATCTCT 1260
AATTTTTTATC AAAACATCTT AGAGCCAAAG CTTCTACATG AGGTGGAGAG GTTCTCTGAG AGAGAAAAAA 1330
GAAGGGCAAG GACCAACTTC CAGCTATTCA AAGTCATCAA TAATAGGAC ACAACGACAA GTTGCGAGAG 1400
TCTACAGTGA TTTTACCTCA GATATCAAAC TCAAGGCTGCT ATACTCGAGC AGAAGTGTCA CCAATATCGT 1470
TAATCCTAAA ACAGATCAGA TAAACCACAA AGAAGTGTGA GCTGAAACTA AAAGAAAAT GAAAGCCCAG 1540
ACAAATGTATTA TATTGGCTAA GAGATTTTTTC TTCTCTGCTGC TTAAGGCTGG TATTAGTGGG GCTTCATGAA 1610
TGCTTTGATT ACTCTTCCTTT GATCAAGTGG AGGGAGATGA TTTTATATTTT AAAAAAAAAA GTGTAGTACA 1680
TATCTGAGC ATTTAAACCTTG TGGAGGAAGA AGTTTACCTTG TCTTGGTTGT TTAAAGGAAA ATTTTAGGCTT 1750
TTTTCCTCCCT AATTAGGTCAT TATGTATGTTG TTAGATCAT TAACACATG GAATGTTCAAAC 1820
TATATGGGAT TTGTCATTTT TATCTACTTT GCTTTGGATATT CTGGCGAAAA ATTATAGAAC TATTTAACAG 1890
TAAACACATCT GCTTTGATTC CTAATATGTTA TAAATAGGTA TTTGATATCT ATACCTCAAAT ATACGCTTGA 1960
CATAGGGGAA TTTTTATCAA ATTTTTCAAA GGCGCAACTG GAGGTTATAC CATTTTCTGA GGAGCAAAAA 2030
GGTTAATTTA CCGGGTACTG ATGCTTGTACG GTCTGACGATT GAATTTAATTT 2100
TCTTGCCTTC ATTTTCTTTGG CTATATGTCCT TTTCTTACACC AAGAAGTAAAT AGTGGTATGAT GAAATAGGAA 2170
GTGTACCTGG ACAGCGCTGC ACCATATTGG AGGAAGTCGG ATATCATGATG TTTGACACTG 2240
TTTTAGTTTG TACCTGGTTTA TCTTTTACCC AAGAAGTGAAT TTGGTATTTT CATAGAAAC AACTTTTGAAT 2310
TTTGAGATTG CCCATACATC TGGAGGAAGA AGTGGTATGAT TTTGATATCT 2390
TGGAATACATT TGAAGGGTAA CCAACAGACT GTGAAATTAAT TTAGATGAAG ATTTATATTTA TTGGACTGTTG 2450
TTCAGTTAATT ACTTGGGAATC TGTTTATATT CTAATACATG TGTTGACCTGC TTACGTACAA TAAGATATTT 2520
CAGTTAGTATT ATGTTACATT TTAACAGTTG AGCTTGTTTCT ATACCTTTCA GAATTAAGAG ATATGGAATT 2590
TGTTGATCTTC 2600

Figure S1. BAD gDNA sequence.
Translation start and stop codon are indicated by a box, coding regions are underlined and non-coding regions are depicted in italics.
Table S1. Overview of samples used in cDNA-AFLP analysis of GR-seedlings. Treatments: M, mock; D, 10\(\mu\)M DEX; C, 10\(\mu\)M CYC; DC, 10\(\mu\)M DEX and 10\(\mu\)M CYC.

<table>
<thead>
<tr>
<th>Lane</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transgene</td>
<td>DOT-GR</td>
<td>EYG-GR</td>
<td>DOT-GR</td>
<td>EYG-GR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>M</td>
<td>D</td>
<td>M</td>
<td>D</td>
<td>C</td>
<td>DC</td>
<td>C</td>
<td>DC</td>
</tr>
<tr>
<td>Duration (h)</td>
<td>4</td>
<td>4</td>
<td>2/4</td>
<td>2/4</td>
<td>4</td>
<td>4</td>
<td>2/4</td>
<td>2/4</td>
</tr>
</tbody>
</table>
### Table S2. Primers used for RT-PCR and construction of transgenes.

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Primer ID</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>gacatg</td>
<td>BAD-ATG U5 primers</td>
<td>2850</td>
<td>TCCGACATCTGAGGAGATGTTG</td>
</tr>
<tr>
<td>gc1118</td>
<td>BAD-ATG U5 primers</td>
<td>2812</td>
<td>TCCGACATCTGAGGAGATGTTG</td>
</tr>
<tr>
<td>se1063</td>
<td>BL-PCE ACT primers</td>
<td>2802</td>
<td>AGATATCGAATCATATATCC</td>
</tr>
<tr>
<td>se602</td>
<td>RT-PCR ACT primers</td>
<td>2803</td>
<td>AGATATCGAATCATATATCC</td>
</tr>
<tr>
<td>se1135</td>
<td>RT-PCR ACT primers</td>
<td>2803</td>
<td>AGATATCGAATCATATATCC</td>
</tr>
<tr>
<td>se1135R</td>
<td>RT-PCR ACT primers</td>
<td>2803</td>
<td>AGATATCGAATCATATATCC</td>
</tr>
<tr>
<td>AcgIR</td>
<td>DDC 5'cloning R primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
<tr>
<td>AcgIF</td>
<td>DDC 5'cloning F primers</td>
<td>2841</td>
<td>CGGATTAGGATGATGAGAGAG</td>
</tr>
</tbody>
</table>
References


