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The pollen-specific *DEFH125* promoter from *Antirrhinum* is bound in vivo by the MADS-box proteins DEFICIENS and GLOBOSA

Abstract The *Antirrhinum* DEFH125 MADS-box protein is expressed in maturing pollen and thus likely participates in the regulation of pollen development. Here, we describe the characterization of a 2.5 kbp promoter fragment conferring pollen-specific GUS expression in *Antirrhinum*, as well as in the distantly related species *Arabidopsis*. Taking advantage of the higher sensitivity of the diphtheria toxin A-chain (*DTA*) reporter gene assay, onset of *DEFH125* promoter activity could be defined to start at the late unicellular microspore stage. Stamen development in *Antirrhinum* is governed by the class B MADS-box genes *DEFICIENS* (*DEF*) and *GLOBOSA (GLO)*. The respective proteins form a heterodimer and are expressed throughout stamens, except for microspores. Complementary expression patterns of *DEFH125* and *DEF/GLO* during later stamen development tempted us to investigate whether the DEF/GLO heterodimer might bind the *DEFH125* promoter and could thus be involved in repressing the *DEFH125* expression. The ChIP technique was applied to investigate protein/DNA interactions occurring in vivo. We report the identification of a 200 bp *DEFH125* promoter fragment that is in vivo bound by DEF and GLO proteins. This fragment contains a CArG-box motif, known to mediate DNA binding of MADS-box proteins. Implications for a likely function of *DEF* and *GLO* in the transcriptional control of *DEFH125* are discussed.

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Introduction

Stamens are the male reproductive organs of flowering plants. In *Antirrhinum*, *Arabidopsis* and other model species, stamen organogenesis is controlled by the overlapping activity of homeotic class B and C MADS-box transcription factors (Schwarz-Sommer et al. 1990; Coen and Meyerowitz 1991). The *Antirrhinum* class B genes *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*) regulate stamen and petal organogenesis (Schwarz-Sommer et al. 1992; Tröbner et al. 1992). Lack of either one of the class B function genes in *Antirrhinum* causes a conversion of stamens into carpels and petals into sepals (Sommer et al. 1990; Tröbner et al. 1992). Analysis of the DEF and GLO protein expression pattern showed that the expression is restricted to the second and third whorl organs and starts at early floral stages (Zachgo et al. 2000). Analysis of the subcellular protein localization detected a strong expression in the nuclei, being in accordance with their function as transcriptional activators and repressors throughout petal and stamen development (Zachgo et al. 1995; Bey et al. 2004). DEF and GLO proteins were shown to form a heterodimer that can bind to short regulatory DNA fragments, so-called conserved CArG-box motifs (Tröbner et al. 1992). After initiation of petal and stamen primordia, DEF and GLO expression is maintained by an autoregulatory loop, likely by directly binding CArG-box motifs in their own promoters as shown by in vitro binding studies (Zachgo et al. 1995). Recently, it has been shown for *Antirrhinum* and

Arabidopsis that additional MADS-box proteins can interact with the class B heterodimer (Egea-Cortines et al. 1999; Honma and Goto 2001). The combinatorial activity of class B/C genes in the control of stamen identity is thus likely exerted by the formation of multimeric MADS-box complexes (Honma and Goto 2001; Theißen and Saedler 2001).

However, little is known about the processes controlling the formation of various tissues and cell types within the stamens during further development. Differentiation of stamen primordia starts with the formation of a stalked basis, the filament, and convex protrusions on the top that give rise to the pollen sacs. Within the anther, diploid male sporogenous cells differentiate into pollen mother cells. In *Arabidopsis*, this process is under the control of the putative transcription factor *NOZZLE/SPOROCYTELESS*, as lack of function mutants fail to form pollen mother cells (Schiefthaler et al. 1999; Yang et al. 1999). The pollen mother cells undergo meiosis leading to the formation of a tetrad of cells; held together by a thick callose wall. The tapetum, a nutritive sporophytic cell layer lining the locules containing the developing microspores, releases an enzyme mixture called callase that separates the haploid microspores (Scott et al. 2004). During further microgametogenesis, microspores enlarge and undergo an asymmetric division, generating two cells with very distinct fates (McCormick 2004). The large vegetative cell accumulates dense cytoplasm containing lipids, proteins and carbohydrates to provide storage compounds for the period of pollen tube growth. The small generative cell contains highly condensed chromatin and undergoes another mitosis producing the two sperm cells.

Other transcription factors known to regulate gametophytic development like the *MALE STERILE* and *ABORTED MICROSPORE1* gene seem to affect gametophytic development rather indirectly by regulating tapetal gene expression in *Arabidopsis* (Wilson et al. 2001; Sorensen et al. 2003). Overall, recent *Arabidopsis* microarray analysis revealed an under-representation of transcription factors during pollen development (Honys and Twell 2003, 2004; Pina et al. 2005).

Besides the well-characterized pollen-specific promoters from the tomato genes *LAT52* and *LAT59* (Bate and Twell 1998), there is still the need for other characterized pollen-specific promoters that confer different expression strengths or different expression windows (McCormick 2004). The protein expression of the *Antirrhinum* MADS-box transcription factor *DEFH125* is restricted to pollen in unfertilized plants. Upon fertilization, DEFH125 protein was also detected in the transmitting tract (Zachgo et al. 1997). Here, we report on the characterization of the pollen-specific *DEFH125* promoter in *Antirrhinum* and *Arabidopsis* using reporter genes of different sensitivities. Furthermore, chromatin immunoprecipitation (ChIP) experiments demonstrate an in vivo interaction of the DEF/GLO proteins at the *DEFH125* promoter.

Materials and methods

DEFH125 promoter analysis

A genomic *Antirrhinum* phage library was screened with a *DEFH125* cDNA probe as described by Zachgo et al. (1997). A 7 kbp phage insert was isolated and sequenced containing 2.5 kbp upstream of the putative *DEFH125* start codon. For GUS expression analysis, the promoter fragment was cloned into the pGPTV-hpt vector (Becker et al. 1992). For the *DEFH125::DTA* cell ablation construct, the *GUS* cDNA was removed from the *DEFH125::GUS* vector and replaced by *DTA* that was produced by PRC using *DTA* primers introducing additional restriction sites (5′ CCAC*GAGCTC*GCCTT CACAAAGATCGCCTG, SacI; 3′ CAGC*TCTAGA*GC CATGGATCCTGATGATG, XbaI) for suitable cloning into the binary vector. DTA gene was obtained from I. Maxwell, University of Colorado Health Sciences Center, Denver, CO, USA. Constructs were transformed into *Antirrhinum* (ecotype 165E, MPIZ, Cologne) and *Arabidopsis* (Columbia, Nottingham Stock Center), following protocols from Heidmann et al. (1998) and Clough and Bent (1998), respectively. After hygromycin selection, transgenic T1 plants were analyzed.

Expression studies

GUS staining, protein immunolocalization and DAPI staining were conducted as described by Heidmann et al. (1998) and Zachgo et al. (1995). For detailed GUS analysis, transgenic plants were fixed in FAA (5 ml glacial acetic acid, 10 ml 37% formaldehyde and 85 ml 70% ethanol), embedded in paraffin and sectioned. Sections from the anthers harboring the *DEFH125::DTA* construct were stained with 0.1% Naphthol Blue Black (Aldrich Chemical Company, Inc.). Images were captured using the digital camera KY-F70B (JVC). For pollen tube GUS staining, mature pollen were dropped on the surface of germination medium (1 mM CaCl$_2$, 1 mM MgSO$_4$/H$_2$O, 1 mM KNO$_3$, 0.01% H$_3$BO$_3$ and 10% sucrose) and incubated for 6 h before adding the staining solution. *Arabidopsis* pollen germination was conducted as described by Chen and McCormick (1996).

Chromatin immunoprecipitation experiments

Plant fixation and processing were largely performed as described by Müller (2003). The material was fixed with 1% paraformaldehyde in 0.1 M Na-phosphate buffer, pH 7.4, for 20 min under vacuum and for 100 min on a rotating wheel at 4°C. Fixation was stopped with 0.125 M glycine. Samples were washed three times 10 min with washing buffer (0.1 M Na-phosphate buffer, pH 7.4, 0.125 M glycine).

Fixed samples were ground in liquid nitrogen. To the sample powder, 30 ml of buffer A (10 mM Hepes/KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.1% NP-40, 10% glycerol) including 200 μl proteinase inhibitor (Sigma P-9599) was added. The slurry was filtered successively through 300, 75, 20 and 10 μm nylon membranes and nuclei were collected by 10 min centrifugation at 500g. The nuclei pellet was washed five times with 10 ml buffer A, then transferred into a 2 ml eppendorf tube. Nuclei were resuspended in 450 μl sonication buffer (10 mM Hepes/KOH, pH 7.9, 100 mM NaCl, 1 mM EDTA, pH 8.0, 1 mM EGTA, pH 8.0, 0.1% Triton X-100, 0.1% sodium deoxycholate, 0.125 M glycine). Chromatin was solubilized by sonication using a Hielscher UP50H sonicator (Dr. Hielscher GmbH, Teltow, Germany) with an amplitude setting of 60% and a cycle control of 0.65 for 20 times, each 10 s. Immunoprecipitation was carried out as described by Turck et al. (2004) and the immunoprecipitated DNA was concentrated using MinElute columns (Qiagen). Semi-quantitative polymerase chain reaction (SQ-PCR) amplifications (56°C annealing temperature with 45 s extension for 33 cycles) were performed using one-tenth of the volume from the immunoprecipitated DNA as template and Ampliqon Taq polymerase (Ampliqon, Roedovre, Denmark). PCR bands were quantified using a phosphoimager scanner (Molecular Dynamics) and Image Quant (Molecular Dynamics).

The following oligonucleotide sequences were used:

Region A	fwd	CGTACCCCCTCATGTTAGTAAAGATTTGC
	rev	GCTCTTTTAAAGCCACATAAACCGTAATACC
Region B	fwd	GAAAGGCACACCATCTACTTAACTAATC
	rev	GTTTGGGATAATTACAGCTACACCCACTG
Region C	fwd	ATGCTTAGAACTTCCTCGATTCATCTC
	rev	GAGATATTGACACGTGGGAAATCAACTC
Region D	fwd	CCTCAGTGGGTGTAGCTGTAATTATCCC
	rev	CTTGTGTTAGATCTCGAAACCACTTAGGTC
Region E	fwd	CAATTAAGGGCTAATTGACTTTGATATACCG
	rev	CAACTTCTGCATCACAAAGAATAGCAAGC

Statistical analysis

In order to assess whether the variance between two sets of data was due to sampling bias or statistically relevant, a Student's t test was performed with the wild-type, mutant and input data. The t test compares the actual difference between two means in relation to the variation in the data. MS Excel (MicroSoft, USA) was used to compute the t value. The significance threshold of the t value commonly used is 0.05. For values below 0.05, there is a 95% probability of a significant difference of the two investigated data sets (Miller 1986). For example, values below 0.05 were obtained when ratios from the regions C/A or D/A from wild-type and input data were compared, showing that the wild-type enrichment was not simply due to sampling bias. Values above 0.05 were obtained for mutant and input data demonstrating that no region of the

DEFH125 promoter was specifically enriched under these conditions.

Results and discussion

Isolation of the *DEFH125* promoter

Recent expression analysis of the *Antirrhinum* MADS-box protein DEFH125 revealed a pollen-specific expression. We isolated and sequenced a 2.5 kbp fragment upstream of the putative *DEFH125* ATG start codon in order to characterize its capacity to confer pollen-specific expression (DQ323884). We searched the promoter for the presence of CArG-box motifs, the conserved consensus sequence $CC(A/T)_6GG$, known to mediate DNA binding of MADS-box proteins (Schwarz-Sommer et al. 1992). Three CArG-motifs were identified, allowing a change of one of the CC or GG nucleotides into an A/T (Fig. 1).

DEFH125 promoter drives GUS expression in pollen and pollen tubes

To investigate the *DEFH125* promoter activity, the 2.5 kbp promoter fragment was fused to the *Escherichia coli uid*A gene, which encodes the β-glucuronidase (GUS) enzyme. *Antirrhinum majus* wild-type plants were transformed with *Agrobacterium* harboring the *DEFH125::GUS* construct. Three independent transgenic plants (line 30, 47 and 102) were obtained. All lines revealed pollen-specific reporter gene expression and a wild-type-like phenotype. Line 30 and 102 showed GUS staining of ~50% of the pollen, indicating that most likely a single transgene integrated into the genome. For further analysis, line 47 was selected, as here, all pollen were stained, probably due to at least two independent integration events. After selfing of the plants, progeny of line 47 was analyzed by staining 2 to 3-week seedlings and flower buds at different stages. GUS color was detected in anthers of older flower buds that reached a length of 7 mm (Fig. 2a). No GUS staining was observed in sepals, petals and carpels before and after fertilization (Fig. 2a) and vegetative parts of the plant (data not shown). To precisely determine the temporal and spatial GUS expression conferred by the *DEFH125* promoter, anthers from an early unicellular stage (5 mm bud length) and an older, bicellular stage (7 mm bud length) were stained. After fixation and embedding, sections were observed under the microscope. GUS color was not detectable in uninucleate microspores (Fig. 2b). The onset of GUS staining appeared in an early bicellular stage when the first pollen mitosis was just accomplished and produced a vegetative and a generative cell (Fig. 2c, inset). As pollen development proceeded further, GUS color became stronger in the cytoplasm of the vegetative cell and was also detectable in pollen tubes that were germinated in vitro (Fig. 2d).
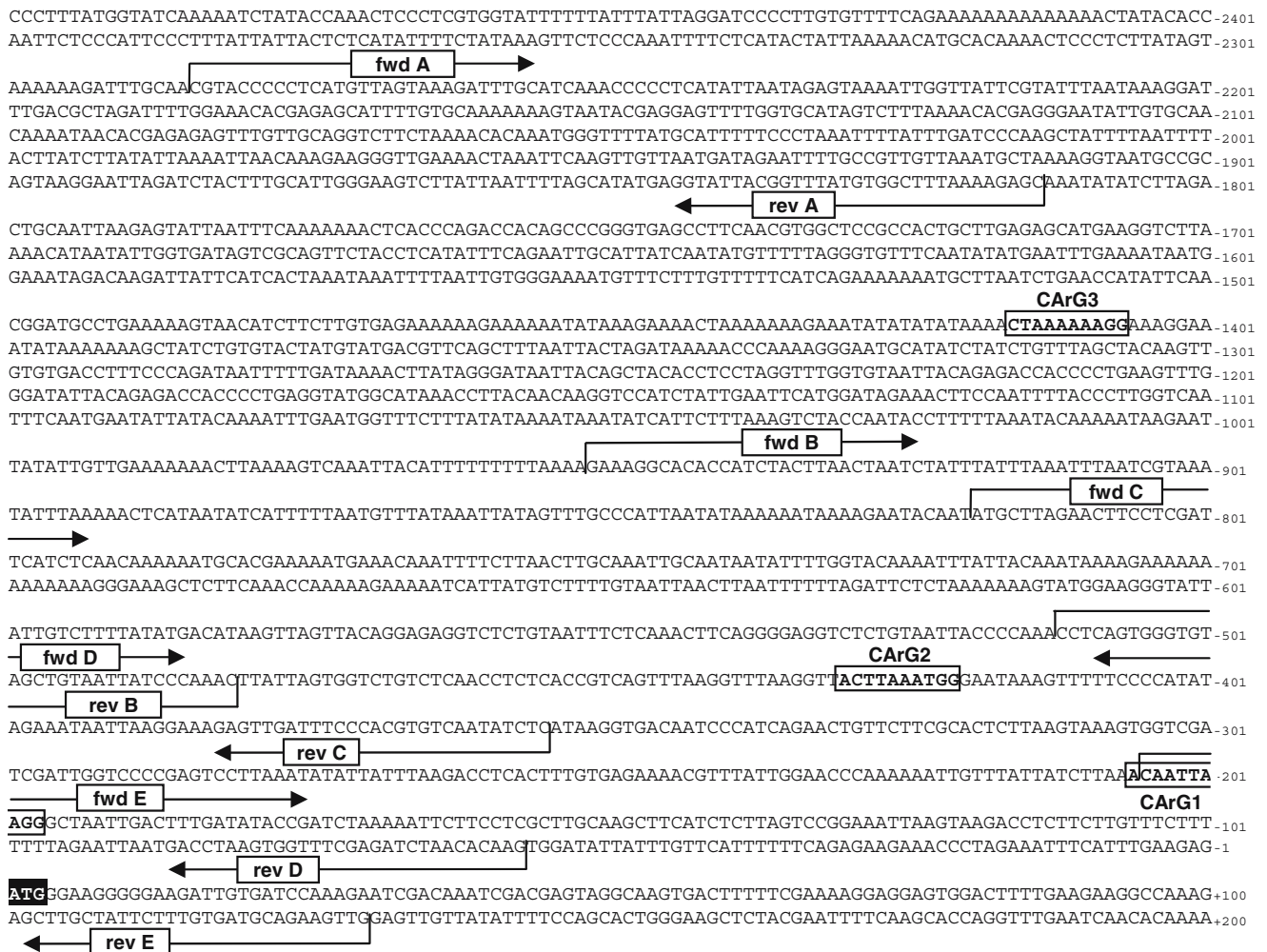
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CCCTTTATGGTATCAAAAATCTATACCAAACTCCCTCGTGGTATTTTTTATTTATTAGGATCCCCTTGTGTTTTCAGAAAAAAAAAAAAAACTATACACC-2401
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AAAAAAGATTTGCAACGTACCCCCTCATGTTAGTAAAGATTTGCATCAAACCCCCTCATATTAATAGAGTAAAATTGGTTATTCGTATTTAATAAAGGAT-2201
               ┌─────┤ fwd A ├────────►
TTGACGCTAGATTTTGGAAACACGAGAGCATTTTGTGTCAAAAAAAGTAATACGAGGAGTTTTGGTGCATAGTCTTTAAAACACGAGGGAATATTGTGCAA-2101
CAAAATAACACGAGAGAGTTTGTTGCAGGTCTTCTAAAACACAAATGGGTTTTATGCATTTTTCCCTAAAATTTATTTGATCCCAAGCTATTTTAATTTT-2001
ACTTATCTTATATTAAAATTAACAAAGAAGGGTTGAAAACTAAATTCAAGTTGTTAATGATAGAATTTTGCCGTTGTTAAATGCTAAAAGGTAATGCCGC-1901
AGTAAGGAATTAGATCTACTTTGCATTGGGAAGTCTTATTAATTTTTAGCATATGAGGTATTACGGTTTATGTGGCTTTAAAAGAGCAAATATATCTTAGA-1801
                                               ◄────────┤ rev A ├────────
CTGCAATTAAGAGTATTAATTTTCAAAAAAACTCACCCAGACCACAGCCCGGGTGAGCCTTCAACGTGGCTCCGCCACTGCTTGAGAGCATGAAGGTCTTA-1701
AAACATAATATTGGTGATAGTCGCAGTTCTACCTCATATTTCAGAATTGCATTATCAATATGTTTTTAGGGTGTTTCAATATATGAATTTGAAAATAATG-1601
GAAATAGACAAGATTATTCATCACTAAATAAATTTTAATTGTGGGAAAATGTTTCTTTGTTTTTCATCAGAAAAAAATGCTTAATCTGAACCATATTCAA-1501
                                                                           CArG3
CGGATGCCTGAAAAAGTAACATCTTCTTGTGAGAAAAAAGAAAAAATATAAAGAAAACTAAAAAAAGAAATATATATATAAA|CTAAAAAAGG|AAAGGAA-1401
ATATAAAAAAAGCTATCTGTGTACTATGTATGACGTTCAGCTTTAATTACTAGATAAAAACCCAAAAGGGAATGCATATCTATCTGTTTAGCTACAAGTT-1301
GTGTGACCTTTCCCAGATAATTTTTGATAAAACTTATAGGGATAATTACAGCTACACCTCCTAGGTTTGGTGTAATTACAGAGACCACCCCTGAAGTTTG-1201
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TTTCAATGAATATTATACAAAATTTGAATGGTTTCTTTATATAAAATAAATATCATTCTTTAAAGTCTACCAATACCTTTTTAAATACAAAAATAAGAAT-1001
                                          ┌─────┤ fwd B ├─────►
TATATTGTTGAAAAAAACTTAAAAGTCAAATTACATTTTTTTTTAAAAGAAAGGCACACCATCTACTTAACTAATCTATTTATTTAAATTTAATCGTAAA-901
                                                                            ┌─────┤ fwd C ├─────
TATTTAAAAACTCATAATATCATTTTTAATGTTTATAAATTATAGTTTGCCCATTAATATAAAAAATAAAAGAATACAATATGCTTAGAACTTCCTCGAT-801
────►
TCATCTCAACAAAAAATGCACGAAAAATGAAACAAATTTTCTTAACTTGCAAATTGCAATAATATTTTGGTACAAAATTTATTACAAATAAAAGAAAAAA-701
AAAAAAAGGGAAAGCTCTTCAAACCAAAAAGAAAAATCATTATGTCTTTTGTAATTAACTTAATTTTTTAGATTCTCTAAAAAAAGTATGGAAGGGTATT-601
                                                                                     ┌────
ATTGTCTTTTATATGACATAAGTTAGTTACAGGAGAGGTCTCTGTAATTTCTCAAACTTCAGGGGAGGTCTCTGTAATTACCCCAAACCTCAGTGGGTGT-501
                                                     CArG2
     ┌─────┤ fwd D ├─────►                                              ◄────────
AGCTGTAATTATCCCAAACTTATTAGTGGTCTGTCTCAACCTCTCACCGTCAGTTTAAGGTTTAAGGTT|ACTTAAATGG|GAATAAAGTTTTTCCCCATAT-401
   ───┤ rev B ├───┘
AGAAATAATTAAGGAAAGAGTTGATTTCCCACGTGTCAATATCTCATAAGGTGACAATCCCATCAGAACTGTTCTTCGCACTCTTAAGTAAAGTGGTCGA-301
                                                                                        ┌─|ACAATTA|-201
TCGATTGGTCCCCGAGTCCTTAAATATATTATTTAAGACCTCACTTTGTGAGAAACGTTTATTGGAACCCAAAAAATTGTTTATTATCTTA
          ◄────┤ rev C ├────
   ┌─────┤ fwd E ├─────►                                                                  CArG1
AGG|GCTAATTGACTTTGATATACCGATCTAAAAATTCTTCCTCGCTTGCAAGCTTCATCTCTTAGTCCGGAAATTAAGTAAGACCTCTTCTTGTTTCTTT-101
TTTAGAATTAATGACCTAAGTGGTTTCGAGATCTAACACAAGTGGATATTATTTGTTCATTTTTTCAGAGAAGAAACCCTAGAAATTTCATTTGAAGAG-1
                          ◄────┤ rev D ├────
|ATG|GGAAGGGGGAAGATTGTGATCCAAAGAATCGACAAATCGACGAGTAGGCAAGTGACTTTTTCGAAAAGGAGGAGTGGACTTTTGAAGAAGGCCAAAG+100
AGCTTGCTATTCTTTGTGATGCAGAAGTTGGAGTTGTTATATTTTCCAGCACTGGGAAGCTCTACGAATTTTCAAGCACCAGGTTTGAATCAACACAAAA+200
       ◄────┤ rev E ├────
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Fig. 1 Sequence and structure of the *DEFH125* promoter. The sequence of 2.5 kbp of the *DEFH125* promoter is reported together with a fragment of the *DEFH125* coding region. Forward (*fwd*) and reverse (*rev*) primers employed in the ChIP analysis are indicated. *Letters A–E* correspond to the five amplified regions (see Fig. 2). Positions of three CArG-boxes are shown (CArG1, 2 and 3). They were identified allowing one substitution from the CArG consensus $CC(A/T)_6GG$ in the GG or CC nucleotides. The putative ATG start codon is indicated by a *black box*

Upon self-pollination of line 47, pollen tubes growing through the style toward the ovules were also expressing GUS (data not shown). Taken together, our data show that *DEFH125* promoter confers a pollen-specific GUS expression in *Antirrhinum*, starting after the first mitotic division of the microspore had occurred. Thus, the isolated 2.5 kbp promoter fragment confers an expression identical to the endogenous DEFH125 protein expression pattern (Zachgo et al. 1997). Due to the small size of the generative cells, being embedded in the large vegetative cytoplasm, the GUS expression study did not allow to discriminate if the *DEFH125* promoter also drives expression in the generative cells.

To check whether the *DEFH125* promoter activity is also conserved in distantly related species, we transformed the *DEFH125::GUS* construct into *Arabidopsis thaliana*. Fifteen transgenic T1 lines were obtained and analyzed; all of them showed a GUS signal confined exclusively to anthers at late floral stages (Fig. 2e).

Analysis of anthers at different stages gave similar results as in *Antirrhinum*. No GUS color was detectable in microspores at the uninucleate microspore stage. However, at this stage, transgenic *Arabidopsis* plants showed a weak and transient GUS signal in the tapetum that was not detectable in *Antirrhinum* (compare Fig. 2b, f). As in *Antirrhinum*, onset of GUS staining was detectable from the early two cellular microspore stages onward (Fig. 2g and inset) and was observed later in matured pollen and in in vitro germinated pollen tubes (Fig. 2h).

Analysis of the *DEFH125* promoter revealed a conserved regulatory mechanism conferring pollen and pollen tube expression of *DEFH125* in two species belonging to the asterid and rosid clades. Besides the conservation of the pollen-specific expression, minor regulatory differences seem to have evolved that lead to a short, transient tapetum activity of the *DEFH125* promoter in *Arabidopsis*, not observed in *Antirrhinum*. The monocot MADS-box gene *ZmMADS2*,
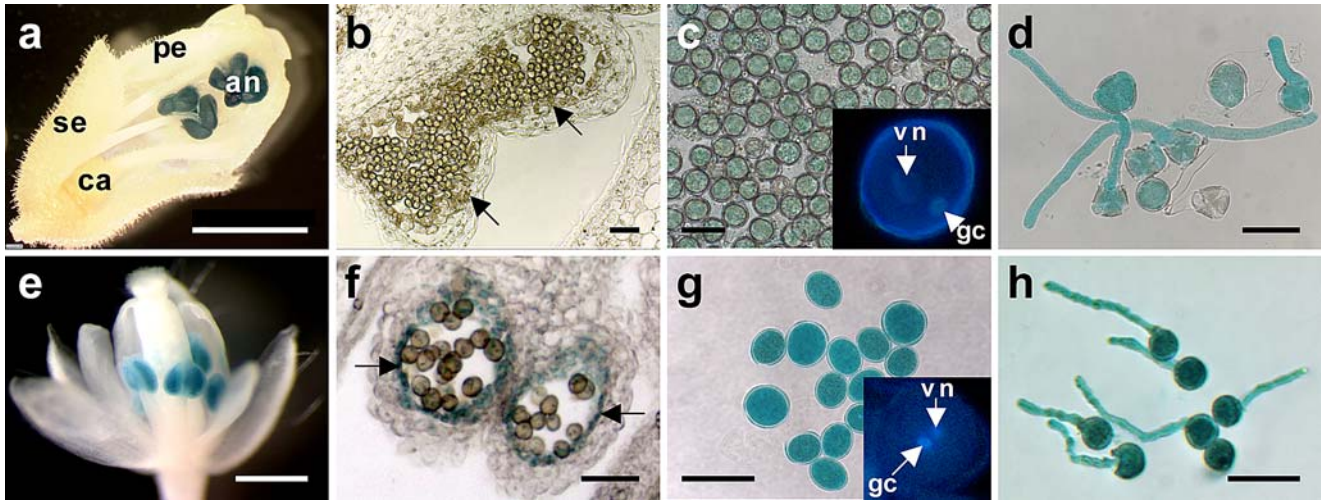
Fig. 2 *DEFH125::GUS* expression in *Antirrhinum majus* and *Arabidopsis thaliana*. Analysis of *DEFH125::GUS* expression in **a–d** *Antirrhinum* and **e–h** *Arabidopsis*. **a** An *Antirrhinum* flower with mature pollen (15 mm length) from the transgenic plant 47 shows only in anthers strong GUS staining. *se* sepal, *pe* petal, *an* anther, *ca* carpel. **b** Cross-section through a stained anther at the uninucleate stage (5–6 mm length) reveals the absence of GUS staining in uninucleate microspores and tapetum (indicated by *arrows*). **c** Cross-section through a stained pollen sac at a bicellular stage (10 mm length). Homogenous GUS staining is observed in all pollen. *Inset* shows a DAPI-stained pollen at the same stage to visualize the vegetative nucleus (*vn*) and generative cell (*gc*), indicated by *white arrows*. **d** Strong GUS staining of in vitro germinated pollen is visible in the cytoplasm of pollen and pollen tubes. **e** A mature flower bud (stage 12, according to Sanders et al. 1999) from a transgenic *Arabidopsis* line was stained, showing the GUS signal uniquely appearing in anthers. **f** Two pollen sacs from stained and sectioned anthers at the unicellular stage (stages 8–9). Whereas no staining was detectable in the microspores, a transient GUS expression was detectable in the tapetum (*arrows*). **g** A group of pollen at bicellular stage (stage 11) reveals a strong GUS signal. *Inset* shows a DAPI-stained pollen from the same stage with vegetative nucleus (*vn*) and generative cell (*gc*). **h** Transgenic, in vitro germinated pollen show strong GUS expression in cytoplasm and pollen tube. *Bars* **a** = 5 mm, **b–d** = 50 μm, **e** = 500 μm, **f–h** = 25 μm

a *DEFH125* homolog from *Zea mays*, is also expressed in pollen and pollen tube but has evolved an additional function. *ZmMADS2* expression is also transiently detectable in the endothecium and connective tissues of the anther (Schreiber et al. 2004). Functional analysis showed that this gene not only plays a role in pollen maturation but is also involved in anther dehiscence (Schreiber et al. 2004).

Determination of *DEFH125* promoter activity onset by DTA assay

The *DEFH125* promoter was used to drive expression of the bacterial *DTA* gene in *A. thaliana* to conduct a highly sensitive cell ablation assay. The DTA protein is highly toxic to eukaryotic cells and a few molecules are sufficient to inhibit protein translation leading to cell death (Yamaizumi et al. 1978). In plants, tissue-specific promoters driving *DTA* gene expression have been successfully applied to determine the onset of the respective promoter activities (Day et al. 1995; Twell 1995). We analyzed 16 transgenic *DEFH125::DTA* T1 lines. All lines showed partial or complete male sterile phenotypes, which was caused by abortion of pollen. Otherwise, transgenic plants displayed a wild-type-like phenotype (data not shown). To determine from which stage on pollen development was affected, serial sections of anthers from different transgenic lines were prepared.

Compared to wild type, microspore development in the *DTA* lines was only normal until the tetrad stage, after the meiotic divisions were completed (data not shown). The first detectable difference was found at the late uninucleate microspore stage. At this stage, wild-type microspores possess a big vacuole in the cytoplasm (Fig. 3a), which is broken down after the first mitosis (Yamamoto et al. 2003). However, this vacuole was not detectable in all microspores from the transgenic *DTA* lines (Fig. 3b). Microspores incapable of forming a vacuole never progressed further through development to conduct the first mitotic division that gives rise to the generative and vegetative cell. Instead, microspores lost their cytoplasm, collapsed and were visible as shrunken structures. Some *DTA* lines showed ~50% of the pollen being aborted (Fig. 3c). These lines likely contained only one T-DNA copy, thus 50% of the haploid pollen carrying no transgene did develop normally in the pollen sacs. The *DEFH125::GUS* analysis showed a transient *GUS* expression in the *Arabidopsis* tapetum at the unicellular stage (Fig. 2f). Infered from this observation, it could be possible that *DTA* under the control of the *DEFH125* promoter is also expressed in the tapetum. However, degeneration of the tapetum in these transgenic plants occurred at a similar stage as in wild-type anthers and did not hinder development of ~50% normal haploid pollen (data not shown). Other *DTA* lines, showing a complete ablation of all pollen, are presumed to contain more independent T-DNA copies (Fig. 3d).
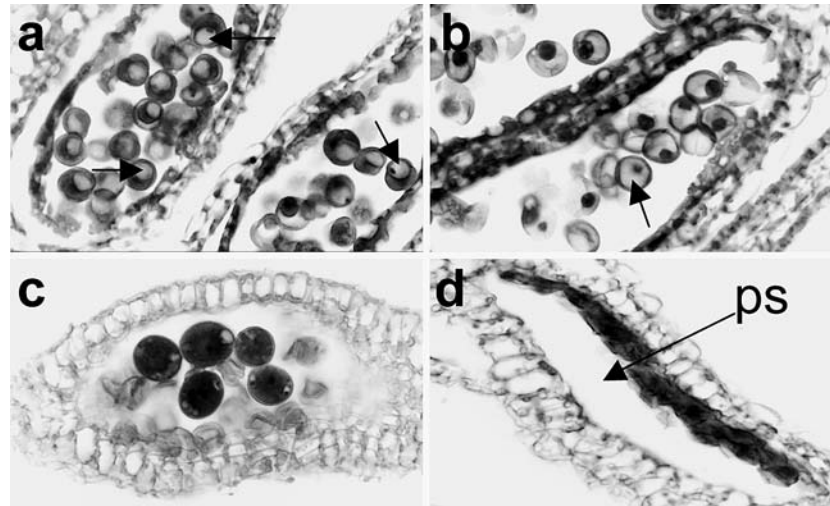
Fig. 3 Analysis of *DEFH125::DTA* in *Arabidopsis* plants. **a** Two pollen sacs from a sectioned wild-type *Arabidopsis* anther at a late uninucleate stage (stage 9), showing the presence of a large vacuole in microspores (*arrows*). **b** Pollen sacs from a *DTA* line at same stage as **a**. Most microspores in these pollen sacs are lacking an obvious vacuole (*arrow*). **c** Pollen sac from a *DTA* line at the bicellular stage (stage 11), showing about 50% dead pollen and 50% normally developed pollen. **d** Pollen sac from another *DTA* line at a comparable stage as in **c**; however, likely due to a high copy number insertion, pollen have not at all developed. Dead, aborted pollen are lining up along the anther wall. Tissue was stained with Naphthol Blue Black. *Bars* 25 μm

The *DTA* results demonstrate that microspores containing expressed *DTA* are aborted before they progress into the bicellular stage. Due to the higher sensitivity of the DTA assay, onset of *DEFH125* promoter activity could be defined to occur already at the unicellular stage, thus predating the onset of GUS expression detection observed at the bicellular stage. To summarize, the *DTA* data support that *DEFH125* promoter activity starts at the uninucleate stage in *Arabidopsis*, which may be similar in *Antirrhinum*. In pollen development, the first mitosis is an asymmetrical division and generates two daughter cells adopting different cell fates (Scott et al. 2004). As *DEFH125 (i)* expression starts before pollen mitosis I, the *DEFH125* MADS-box gene might be involved in this decisive process. Other MADS-box genes from *Arabidopsis*, like *AGL18* (Alvarez-Buylla et al. 2002) and *AGL21* (S. Xing and S. Zachgo, unpublished observation), are also expressed before and after pollen mitosis I, indicating the complexity and likely redundancy in the control of this process.

## DEF and GLO proteins are expressed throughout petals and stamens but absent from microspores

Expression analysis of the *Antirrhinum* class B DEF and GLO proteins governing petal and stamen organogenesis revealed that both are expressed uniformly throughout the petal and stamen primordia during early floral stages (Zachgo et al. 1995, 2000; Fig. 4a, b). In this study, we analyzed the expression pattern during later stages, after the differentiation of floral organs has started. Figure 4c, d shows immunolabeled sections through young flower buds with anthers containing young microsporocytes that will soon undergo meiosis, form tetrads and release haploid microspores. DEF and GLO proteins are still expressed throughout the petals. However, in young stamens, expression is restricted to the filaments, connective tissue and vasculature, and no signal could be detected in the microsporocytes (Fig. 4c, d). Although DEF/GLO are required for stamen organogenesis, a lack of expression in microsporocytes indicates that the formation of a vegetative and generative cell is likely under the control of other regulatory genes. Figure 4e, f shows an advanced floral stage, after accomplishment of meiosis and the first mitotic division, in which bicellular microspores were generated that are visible as almost mature pollen in the pollen sacs. DEF and GLO protein expression is still absent from the pollen and only detectable in the filament, connective tissue and vasculature. In older petals and filaments, expression is strongest in epidermal cell layers. Analysis of the *DEFH125* and *DEF/GLO* expression at older developmental stages thus revealed a complementary expression pattern in stamens. This observation raised the question, whether the DEF/GLO heterodimer could be involved in regulation of *DEFH125* by binding its promoter and acting as a repressor of the *DEFH125* transcription.

Establishment of the ChIP technique to analyze an in vivo interaction of the DEF/GLO heterodimer at the *DEFH125* promoter

The ChIP technique delivers the proof of in vivo protein/DNA interactions. The technique has been initially applied to study transcription factor/DNA interactions

Fig. 4 DEF and GLO protein expression in older floral stages. DEF protein (**a, c, d**) and GLO protein expression patterns (**b, d, f**) in wild-type *Antirrhinum* buds were detected on longitudinal sections using anti-DEF and anti-GLO sera. **a, b** Young stages with undifferentiated floral primordia. **c, d** Organ differentiation has started and expression is detectable in different petal and stamen tissues but no expression could be observed in the microsporocytes developing in the microsporangia. **e, f** Similarly, older flowers show no DEF/GLO expression in almost mature pollen. *se* sepal, *pe* petal, *st* stamen, *ca* carpel, *ps* pollen sac, *fi* filament. *Bars* 200 μm.
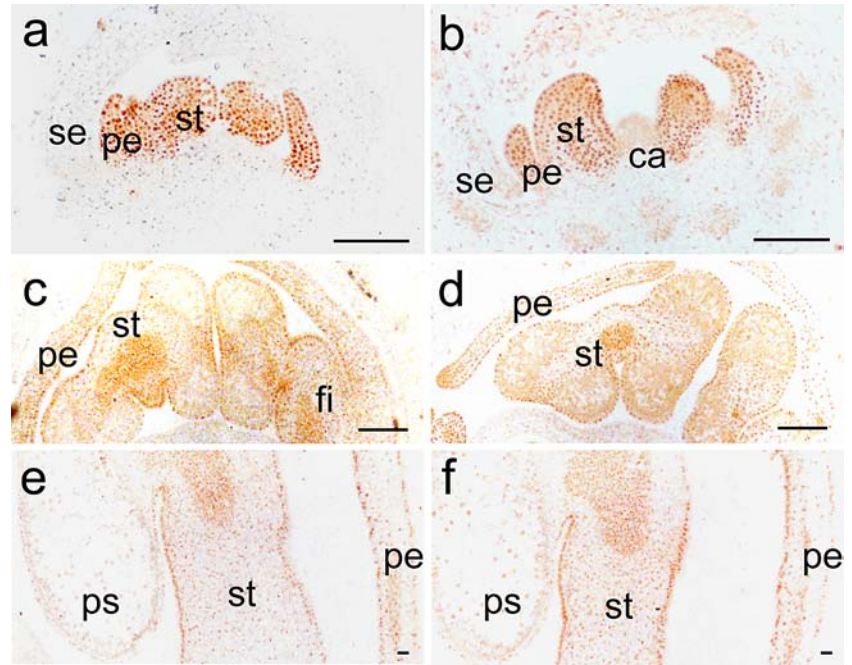
in *Drosophila* (Orlando et al. 1997). Recently, the technique has also been employed for plant protein/DNA studies (Turck et al. 2004; William et al. 2004; Gomez-Mena et al. 2005). We were intrigued by the observation that the DEF protein binds to a *DEFH125* promoter region of about 1 kbp (Müller 2003). Therefore, we investigated the binding of both, the DEF and GLO proteins, to analyze precisely their in vivo interaction with a 2.3 kbp long *DEFH125* promoter region by conducting an in-depth ChIP analysis. After fixation of *Antirrhinum* plant material, nuclei were extracted and DNA fragments of an average size of 300–400 bp were generated by random shearing. Immunoprecipitation was carried out with anti-DEF and anti-GLO sera that were successfully used in immunolocalization studies (Zachgo et al. 1995, 2000). The enrichment of specifically bound DNA sequences in the immunoprecipitated DNA pool was determined by SQ-PCRs, for which an example is given in Fig. 5b. To prove the reliability of the data and to allow a comparison between the different analyzed promoter regions, several control steps were included. To circumvent the intrinsic variability of the PCR, each ChIP experiment and consecutive PCRs were conducted at least six times. To discriminate between real in vivo protein/DNA interactions and amplification of non-specific background, and thus to demonstrate serum specificity, each experiment was conducted in parallel with the material from the *DEF* and *GLO* null-mutants, *def-gli* and *glo*, respectively. For determination of DEF/GLO DNA-binding sites in the *DEFH125* promoter, four partially overlapping promoter regions (B–E) were considered (Fig. 5a). Each fragment spans about 400 bp and together, they cover about 1,000 bp upstream of the putative start codon. Region A, located ∼2.3 kbp upstream of the ATG, was shown to be suitable as a background control. Within this interval, the overall chromatin structure is supposed to be still comparable and thus its impact on the shearing and immunoprecipitation procedure similar (Schwartz et al. 2005).

Fig. 5 Overview on the *DEFH125* ChIP experiments. **a** Investigated regions A–E in the *DEFH125* promoter, as well as position and sequence of the CArG1 and CArG2 motifs, are indicated. **b** Representative example of a SQ-PCR conducted on 'wild-type', 'mutant' and 'input' material. Immunoprecipitation was carried out with anti-DEF serum. Primers for amplification of regions A–E are reported in Fig. 1
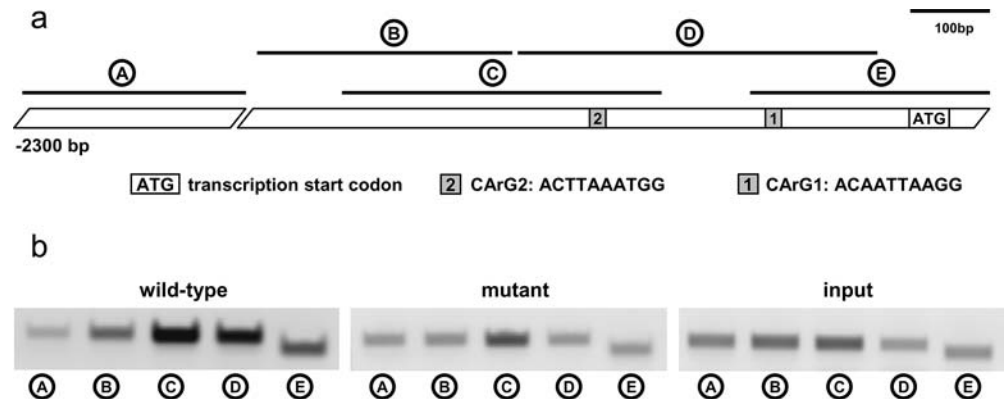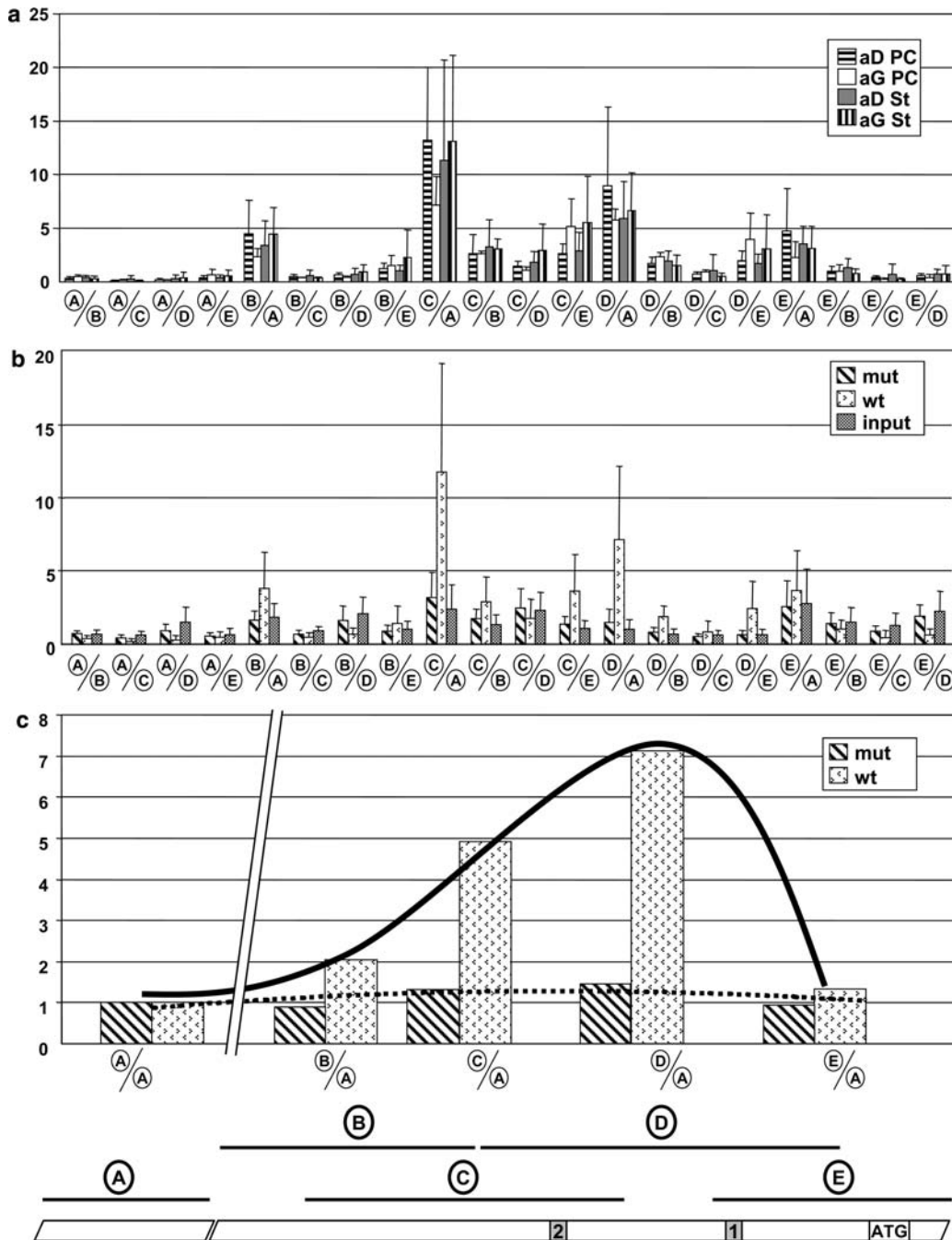
Fig. 6 Binding of DEF/GLO to the *DEFH125* promoter. **a** The chart illustrates the average values of all band intensity ratios of the ChIP experiments conducted on petals and stamens using anti-DEF (aD Pe, aD St) and anti-GLO serum (aG Pe, aG St), respectively. **b** 'Wild-type' (*wt*) columns correspond to average values of four sets of data: stamen and petal material treated with the two anti-sera, reported separately in Fig. 5a. Values corresponding to 'mutant' (*mut*) comprise results of experiments conducted with anti-DEF serum and anti-GLO serum on *def-gli* and *glo* mutant inflorescences, respectively. As no significant differences were noted between these two sets of data they were reported together (16 replicates in total). Input (*input*) values are the average of 21 independent SQ-PCRs. **c** Further on, only the set of values generated in relation to region A were considered and divided by the input values for normalization. A *bell-shaped curve* (top enrichment corresponding to region D) for experiments using wild-type material could be extrapolated. An *almost flat line* reflects the lack of enrichment in the mutant experiments

For normalization of primer strength, control SQ-PCRs were conducted in parallel with 'input' DNA (Fig. 5b). Input DNA originated from an aliquot taken after the sonication step, but before the immunoprecipitation, and was diluted to a concentration comparable to the immunoprecipitated DNA. To determine the relative fragment enrichment, PCR band intensities were quantified and ratio values were pairwise calculated within each experiment. Mean values of ratios and the corresponding standard deviations were calculated.

Furthermore, a statistical approach, the Student's *t* test (see Materials and methods), was conducted to assess whether differences between data sets from wild-type, mutant and input were due to a specific enrichment or caused by sampling bias.

DEF and GLO bind in vivo to the *DEFH125* promoter

The ChIP experiments were conducted in parallel with petal and stamen material harvested from ~1 cm long wild-type flowers and with inflorescences from *DEF* and *GLO* mutants using anti-DEF and anti-GLO sera. After quantification of the PCR repetitions, ratios between all five investigated regions in one experiment were calculated and are shown in Fig. 6a. The clear tendency of region D to be enriched with respect to the other regions was confirmed by further processing of the data (see also PCR gel pictures in Fig. 5b). Very similar ratio values were obtained in petals and stamens using both anti-DEF and anti-GLO sera, respectively (Fig. 6a). Therefore, these four data sets were treated together as one wild-type group for further normalization steps. Similarly, data collected from mutant tissues showed negligible variations (data not shown) and were thus treated as one set of data (Fig. 6b). Applying a Student's *t* test, the specificity of the observed enrichment of regions C and D in wild-type tissues was verified. On the contrary, calculated ratios from input and mutant data were low (Fig. 6b). Furthermore, region A showed the lowest enrichment in the wild-type experiments, demonstrating its applicability as a background control. Together, our data provide evidence that DEF and GLO proteins bind in vivo to the same area in the *DEFH125* promoter. It is therefore likely that they bind the DNA as a heterodimer, as has been shown by former in vitro binding studies (Tröbner et al. 1992; Zachgo et al. 1995).

To correct for the differences in primer strength, values depicted in Fig. 6b from wild type and mutant were divided by the corresponding input value. For better visualization of the normalized data, Fig. 6c shows the set of values determined in relation to the background control (region A). The curve extrapolated from the wild-type data approximates a Gaussian distribution, describing the relation between enrichment of a region and its distance from the binding site (Fig. 6c). For the mutant data set, an almost flat line could be projected. No region of the *DEFH125* promoter can be specifically immunoprecipitated in the absence of the investigated protein in the respective mutants, thus demonstrating the specificity of the serum. The peak of the curve is represented by region D and a slightly reduced enrichment was detected for region C. Enrichment values decrease for regions B and E and reach the lowest value for region A. The majority of the sheared fragments spans about 400 bp. However, as random sonication also generates lower amounts of longer fragments, these can still be precipitated and PCR amplified with primers not spanning the binding site and thus explain the slight enrichment observed for regions B and E.

Mapping of the DEF/GLO binding sites defines CArG2 as a likely interaction site

Given that the PCR primers were designed to amplify partially overlapping 400 bp long regions, the ChIP data allowed defining the DEF/GLO binding region more precisely. Regions C and D displayed the strongest enrichment, whereas values for region B dropped down to a level more similar to that observed for region A, the background control. This allows restricting the binding area for the DEF/GLO heterodimer to an area of ~200 bp in the *DEFH125* promoter, located 220 bp upstream of the putative start codon. This area contains the CArG2 motif (ACTTAAATGG) showing one single mismatch from the conserved CArG consensus motif $CC(A/T_6)GG$. Besides recognizing the CArG1 motif in the *DEF* promoter that fully matches the CArG-box consensus sequence (CCTTTTTAGG), slight deviations from the consensus were also shown to be recognized by DEF/GLO and AP3/PI heterodimers (Tröbner et al. 1992; Tilly et al. 1998; Hill et al. 1998). The ChIP data demonstrate specificity of the interaction in region D comprising CArG2, as other tested CArG-boxes were not promiscuously bound by the DEF/GLO heterodimer. One additional motif (CArG3, see Fig. 1) was identified upstream of CArG2 showing similar deviations from the conserved CArG-box consensus. However, in spite of sequence similarity, no specific enrichment could be observed in ChIP experiments (data not shown). This observation further strengthens the interaction specificity that might be conferred by additional cofactors and/or a specific chromatin structure to bind to the region containing CArG2.

Conclusion

In this study, we characterized the *DEFH125* promoter by different reporter gene studies and demonstrate its usability for pollen-specific studies in *Antirrhinum* and *Arabidopsis*. Furthermore, being intrigued by the complementary *DEFH125* and *DEF/GLO* expression pattern in stamens, we conducted a thorough ChIP binding analysis showing that the *DEFH125* promoter is bound in vivo by DEF/GLO. We demonstrate that the ChIP technique can be exploited to map the DEF/GLO binding site down to a region of about 200 bp in the *DEFH125* promoter. The whole investigated promoter fragment comprises several CArG-boxes, short DNA-binding motifs for MADS-box proteins. However, only one motif within the 200 bp region, CArG2, seems to be directly recognized by the heterodimer, whereas other similar CArG-box motifs did not contribute to a specific in vivo interaction. So far, maintenance and up-regulation of later class B and also class

C MADS-box gene expression by an autoregulatory loop has been interfered from genetic and biochemical studies conducted in *Antirrhinum* and *Arabidopsis* (Zachgo et al. 1995; Jack et al. 1994; Tilly et al. 1998; Gomez-Mena et al. 2005). Our in vivo ChIP experiments and expression studies suggest that the class B MADS-box proteins DEF and GLO could also be involved in the direct repression of the expression of another MADS-box gene, namely *DEFH125*, and could thereby contribute to the formation of mature male gametophytes.

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