The maize (Zea mays) late pollen gene ZmMADS2 belongs to the MIKC type of MADS box transcription factor genes. Here, we report that ZmMADS2, which forms a homodimer in yeast (Saccharomyces cerevisiae), is required for anther dehiscence and pollen maturation. Development of anthers and pollen was arrested at 1 d before dehiscence in transgenic plants expressing the ZmMADS2-cDNA in antisense orientation. Temporal and spatial expression analyses showed high amounts of ZmMADS2 transcripts in endothecium and connective tissues of the anther at 1 d before dehiscence and in mature pollen after dehiscence. Transient transformation of maize and tobacco (Nicotiana tabacum) pollen with the luciferase reporter gene under the control of different ZmMADS2 promoter deletion constructs demonstrated the functionality and tissue specificity of the promoter. Transgenic maize plants expressing a ZmMADS2-green fluorescent protein fusion protein under control of the ZmMADS2 promoter were used to monitor protein localization during anther maturation and pollen tube growth. High amounts of the fusion protein accumulate in degenerating nuclei of endothecial and connective cells of the anther. A possible function of ZmMADS2 during anther dehiscence and pollen maturation and during pollen tube growth is discussed.
with anther burst and release of mature pollen (Goldberg et al., 1993).

Several mutants have been described affecting both anther and pollen maturation in corn and, thus, result in male sterility (e.g. Beadle, 1932; Cheng et al., 1979; Albertsen and Phillips, 1981; Neuffer et al., 1997). For example, Cheng et al. (1979) demonstrated that development of tapetal cells is affected at the young microspore stage in $ms10$, whereas microspores show cytoplasmic degeneration and failure of pollen wall synthesis in the intermediate microspore stage. The molecular nature of the corresponding genes is mostly unknown. In contrast to maize, several delayed dehiscence mutants have been described in Arabidopsis (for review, see Patterson, 2001). Anthers in the DEFECTIVE IN ANther DEHISCENCE mutant, for example, do not dehisce, and pollen grains do not germinate either on medium or on fresh stigmata (Ishiguro et al., 2001). Another mutation, $ms35$, generates lack of secondary wall thickening in the endothecium; thus, anthers do not open, although pollen are fully fertile (Dawson et al., 1999).

The temporally coordinated degeneration of anther tissues seems to be regulated by a sequential gene expression cascade. Several transcription factor genes have been reported to be expressed in anthers, including those encoding MYB-related proteins, zinc finger transcription factors, and MADS box transcription factors, namely DEFH125, AGL15, AGL18, ZmMADS1, and ZmMADS2 (Zachgo et al., 1997; Kobayashi et al., 1998; Alvarez-Buylla et al., 2000; Fernandez et al., 2000; Heuer et al., 2000, 2001; Robson et al., 2001; Yang et al., 2001). Kobayashi et al. (1998), for example, reported seven zinc finger transcription factor genes sequentially expressed during anther development in petunia ($Petunia~hybrida$) and suggested that they act in a regulatory cascade. The maize MADS box genes ZmMADS1 and ZmMADS2 may be candidates of a similar system regulating anther and pollen development. ZmMADS1 expression peaks in young microspores and decreases during microgametogenesis (pollen development), whereas ZmMADS2 transcripts accumulate in mature pollen and pollen tubes, when ZmMADS1 expression is almost completely switched off (Heuer et al., 2000). Here, we report the functional analysis of the ZmMADS2 gene of maize, which is required for regulating anther dehiscence and pollen maturation and discuss its role in nuclear degradation during anther dehiscence.

**RESULTS**

*ZmMADS2 Antisense Plants Exhibit Anther Dehiscence and Pollen Maturation Defects*

To study the function of the late pollen gene ZmMADS2, we have used an antisense approach to generate loss-of-function mutants. The constitutive maize ubiquitin promoter was chosen to drive full-length ZmMADS2-cDNA transgene expression. This promoter was previously shown to be expressed at much higher levels in pollen and other tissues compared with the ZmMADS2 promoter (Schreiber and Dresselhaus, 2003).

Eighteen BASTA resistant transgenic maize lines were generated of a total of 1,783 bombarded immature maize embryos (transformation efficiency of 1%). Genomic Southern blots showed integrations of the ZmMADS2-cDNA antisense construct pDNS-4 in nine transgenic plants (cotransformation efficiency of 50%). Four plants (all independent lines) showed a full-length integration of the pUbi::ZmMADS2-AS construct, whereas the other five plants, representing two independent transgenic lines, showed a partial integration. All transgenic plants containing full-length integrations showed strong transgene expression in leaves, whereas the other plants showed a lower expression because of an incomplete integration of the ubiquitin promoter (data not shown). Two of the four full-length integration plants and their T1 and T2 progenies showed a wild-type (WT) phenotype, whereas the other seven T0 plants were male sterile. As shown in Figure 1a, all seven sterile plants (representing four independent lines) showed the same phenotype: fully developed tassels but no opening of male florets. Tassels of transgenic plants remained green for several weeks without occurrence of anthesis (Fig. 1a), whereas tassels of WT plants of the same age reached maturity (Fig. 1b). Development of anthers was arrested shortly before opening of the anterior pore without elongation of the filament. Figure 1c shows the development of anthers from T0 plants compared with WT plants from 3 d before until anthesis. Anthers of transgenic plants were arrested at stage VIII of development, whereas anthers of WT plants dehisced and released mature pollen. Anthocyanin levels indicate an arrest between middle and late stage VIII of anther development. Maturation of transgenic pollen corresponded to an arrest of development at 1 d before anthesis. Starch granules were visible inside the still partly vacuolated pollen grain, and nuclei of sperm cells appeared round instead of the sickle-shaped form of mature WT pollen (Fig. 1, d and e). A few hundred pollen of different anthers of both transgenic and WT lines were analyzed. Some 2% pollen of transgenic and WT lines were arrested at the microspore stage, whereas about 98% of WT pollen reached maturity. In contrast, more than 90% pollen of anthers from independent male sterile lines showed the arrested phenotype (see description above), and few pollen reached maturity. In contrast to WT pollen, arrested and fully developed pollen of male sterile plants neither germinated in vitro nor led to progeny kernels after selfing or outcrossing to A188 WT plants. Expression of ZmMADS2 in pollen of male sterile plants could not be detected. Later pollination of cobs from male sterile plants with pollen of WT plants also
did not result in progeny kernels. This female “sterility” effect of male sterile plants was probably caused by the late pollination as silks became dry.

Genomic Structure of the ZmMADS2 Gene

ZmMADS2 is a member of the MIKC type (without N-terminal extension) of MADS box transcription factor genes. As shown in Figure 2a, the ZmMADS2 gene (GenBank accession numbers AY227363 and AY264885) contains eight exons and seven introns, with exon as well as intron positions and sizes comparable with other typical MADS box genes (Riechmann and Meyerowitz, 1997). The second intron (I2 in Fig. 2a), which was cloned incompletely, is an exception, because with more than 2.3 kb, it is unusually long. The MADS box is located within exon 1 (E1 in Fig. 2b) and contains a bipartite nuclear localization signal. Exon 2 contains the linker or intervening sequence (E2 in Fig. 2b). Functional domains are separated by relatively large introns, whereas shorter introns are inserted inside both the K box and C terminus. The context sequence of the translational initiation codon is typical for monocots (Joshi et al., 1997). The transcription start point was defined by primer extension analysis (data not shown) and is located 311 bp upstream of the ATG START codon.

The ZmMADS2 promoter was isolated as a 1,502-bp fragment upstream of the transcription start point. A TATA box some 20 to 30 bp upstream of the transcription start point could not be detected. Sequence analyses using the PLACE database (Higo et al., 1999) and the MatInspector software (Genomatix Software, München, Germany; Quandt et al., 1995) revealed numerous putative cis-acting elements (Fig. 2c) within the promoter sequence, including late pollen-specific elements of tomato and tobacco (Bate and Twell, 1998; Rogers et al., 2001), which are almost equally distributed within the promoter sequence. Root-specific elements (Elmayan and Tepfer, 1995) occur from position −1,301 to −419, sugar starvation-induced elements (Grierson et al., 1994; Hwang et al., 1998; Toyofuku et al., 1998) within boxes 0 and II to IV, and stress/abscisic acid-induced elements (Lopez-Molina et al., 2002) in boxes I, II, IV, and V (Fig. 2c). Sequence analysis using the Gene Quest Software (LASERGENE software, DNASTAR, Inc., Madison, WI) showed multiple repetitive elements within the promoter sequence. Interestingly, the only box lacking repetitive elements, box II, has been shown in transient expression studies (Fig. 3b) to contain the most important cis element(s) for pollen-specific marker gene expression in maize and tobacco.

**Figure 1.** Phenotypes of transgenic maize plants expressing ZmMADS2 cDNA in antisense orientation under control of the constitutive ubiquitin promoter of maize. Development of tassels, anthers, and pollen is arrested at 1 d before anthesis. a, Tassel of a transgenic plant at the latest stage of development. b, Tassel of a WT plant at anthesis. c, Anthers of a WT plant (left) and that of a transgenic antisense (AS) plant (right) at 2 d before anthesis (middle stage VIII), 1 d before anthesis (late stage VIII), and at anthesis (stage IX). Light microscopic (d) and UV microscopic (e) images of pollen of transgenic plants at the latest stage of development. Pollen were stained with 4',6-diamino-2-phenylindole dihydrochloride (DAPI) and acetic orcein. Note that a vacuole is still visible (asterisk), and sperm cells (arrowheads) are round instead of sickle shaped. Light microscopic (f) and UV microscopic (g) image of a mature WT pollen stained with DAPI and acetic orcein. Vacuoles are no longer visible, and sperm cells (arrowheads) are sickle shaped. Arrows point toward the vegetative nucleus. Bars = 20 μm.
Transient Expression of ZmMADS2 Promoter Deletion Constructs

Transient transformation of mature maize and tobacco pollen and young leaves was performed with seven ZmMADS2 promoter deletion constructs to elucidate pollen-specific promoter elements of monocot genes. A promoterless luciferase construct was used as a negative control (de Wet et al., 1987). As shown in Figure 3a, pDNS 6b (approximately 1 kb upstream of transcription start point plus 5’ untranslated region [UTR]) showed highest expression in both maize and tobacco pollen. In maize, expression levels of pDNS-5, -6, and -7 were only slightly lower, ranging on average between 80% to 95% compared with the relative expression level of pDNS-6b. Relative expression levels of pDNS-8 ranged from 35% to 70% compared with pDNS-6b (Fig. 3a and b). In tobacco, pDNS-6 showed an average expression level of 96%. Expression levels of pDNS-7 ranged between 30% and 70% with an average of 66%, and those of pDNS-8 were between 10% and 50% with an average of 24% (Fig. 3b). In contrast to maize, expression levels of pDNS-5, the longest promoter fragment, were very low in tobacco, ranging from 3% to 25% (average of 7%). This may be because of a repression by cis-acting elements located in Box V functioning in tobacco but not in maize (Fig. 2c). Expression levels of pDNS-9 and -10 were only slightly above background in maize compared with the promoterless LUC construct and were not determined in tobacco. Luciferase expression was not measurable above background levels in young leaves.

**Figure 2.** Genomic structure of the ZmMADS2 gene. a, The ZmMADS2 gene consists of eight exons (E1–E8) and seven introns (I1–I7) with an unusually large second intron (I2). The transcription start point was determined using a primer extension assay. The ATG START codon is located 312 bp downstream of the transcription start point. The context sequence around the translational initiation codon ATG is typical for monocots. Intron sizes are given in base pairs above the sequence. b, ZmMADS2 belongs to the MIKC type of MADS box proteins. ZmMADS2 contains of a highly conserved MADS box at the N terminus, a linker sequence, a keratine box, and a highly variable C terminus. A putative bipartite nuclear localization signal is found inside the MADS box. Distribution of exons is shown above protein domains. c, Clustering of putative cis-acting elements and repetitive sequences upstream of the ATG START codon. Putative cis elements responsible for sugar starvation induction are displayed in green (ACGTA or AATAGAAA), those responding to water stress and abscisic acid are displayed in blue (TAACTG and ACACNNG, respectively), putative root-specific elements are shown in red (ATAA), and sequences homologous to motifs responsible for late pollen-specific expression are given in orange (AGAAAA or GTGA). Sequence repeats are shown below the horizontal bar. Yellow, Direct repeats (GTATGTAACATA…); brown, inverted repeats (TAATAATTATTA); dark orange, dyad repeats (ATTGAAAAGTTA). Arrow, Transcription start site; arrowhead, position of the start codon.
of both plants, indicating that the ZmMADS2 promoter is pollen specific. The most important functional cis-acting elements are located in box II between positions −327 and −210 relative to the transcription start point. This region could now be used to identify pollen-specific cis elements functioning in both monocots (maize) and dicots (tobacco).

ZmMADS2 Is Transcribed in Anthers at 1 d before Dehiscence and during Pollen Tube Growth

Northern-blot analyses and reverse transcription (RT)-PCR was performed to study the expression of ZmMADS2 during anther maturation and pollen development. As shown in Figure 4a, transcripts were first detected during anther development, in the middle of stage VIII, when anthers reach their final size but are still green. Transcripts are most abundant in anthers at late stage VIII (shortly before opening of the anterior pore, see also Fig. 1c) and in mature pollen. Very weak signals were observed in roots with and without tips but not in other vegetative tissues. To investigate whether the signals obtained from anthers were derived from the maternal tissues of the anther or maturing pollen, we have micro-dissected anther tissues at different developmental stages and removed microspores/pollen. Figure 4b shows expression of ZmMADS2 starting at stage VIII of anther development in both connective tissue and endothecium, which also contained epidermal cells. Interestingly, relatively strong transcript amounts were
still detectable in degenerating endothecium and connective tissues after anthesis (stage IX).

**ZmMADS2 Forms Homodimers and Localizes to Nuclear Fragments in Endothecial and Connective Cells**

MADS box proteins are known to form dimers in the cytoplasm, which then enter the nucleus. To investigate whether ZmMADS2 forms homodimers in vivo, a prerequisite to enter the nucleus without an additional binding partner, we have used the yeast (*Saccharomyces cerevisiae*) two-hybrid system with ZmMADS2 both as bait and prey. Of 60 independent yeast clones growing on selective media, all carrying both bait and prey, 44 clones showed a blue staining using the β-galactosidase assay. Figure 5 shows that auto-activation of ZmMADS2 fused to the LexA-binding or B42 activation domain was not detectable. The selection (Fig. 5b) and the β-galactosidase assay (Fig. 5c) demonstrate that ZmMADS2 proteins interact and form homodimers in vivo.

To investigate the subcellular localization of ZmMADS2 and its tissue-specific localization within the anther, transgenic maize plants expressing a ZmMADS2-green fluorescent protein (GFP) fusion protein under control of the ZmMADS2 promoter were generated. Immature embryos (728) were bombarded, and 12 BASTA plants were regenerated (transformation efficiency of 1.6%). Two independent lines containing a full-length integration of the pZmMADS2::ZmMADS2-GFP construct were used for further studies. Both lines showed a strong expression of the chimeric gene (data not shown) and the pattern of GFP fluorescence as identical. Strong GFP fluorescence was detectable exclusively in nuclei of cells of the connective tissue and the endothecial layer of anthers at 1 d before anthesis (Fig. 6, a, b, e, and g). Restricted localization of the fusion protein to the nucleus indicates that ZmMADS2 acts as a DNA-binding protein. At the stage of maximum GFP flu-
Fluorescence, the tapetum was completely degenerated, whereas inter-microsporangial stripes at the site of separation were still attached to the connective tissue (Fig. 6a). GFP fluorescence was never observed at other developmental stages of the anther nor in WT anthers at comparable stages of development (Fig. 6c). Longitudinal sections showed that the ZmMADS2 fusion protein is expressed in endothecium and connective tissue and that localization of the protein is not restricted to the unopened anterior pore (Fig. 6e). Additional DAPI staining of cross or longitudinal sectioned anthers revealed that nuclei showing brightest GFP fluorescence showed faint DAPI signals, whereas nuclei showing strong DAPI staining gave weaker or no GFP fluorescence. This result indicates that ZmMADS2 protein accumulates during the final stages of anther maturation, when stabilizing anther cells degenerate before merging and dehiscence of the anther locules. Figure 7 illustrates the nuclear degradation process during the final stage of anther maturation within representative endothecial cells and cells of the connective tissue. As cell death in these cells is proceeding, the compartmented structure of the nuclei (Fig. 7, a and b) disintegrates (Fig. 7, c and d). Apoptotic bodies, containing almost completely degraded DNA, appear at the final stage of degeneration (Fig. 7, e and f).

**DISCUSSION**

The majority of plant MADS box genes have been shown to be involved in flowering and flower organ development, although some are also expressed during vegetative development. We have reported here about the functional analysis of the MADS box gene ZmMADS2 from maize, which belongs to the AGL17 subfamily of MADS box transcription factors (Heuer et al., 2000). ZmMADS2 shares highest amino acid sequence identity with DEFH125 (Zachgo et al., 1997). Both genes are predominantly expressed in anthers and pollen, but most other members of this monophyletic clade, such as AGL2, AGL17, and ANRI, are exclusively expressed in roots (Zhang and Forde, 1998; Burgeff et al., 2002). ANRI is the only member of this clade whose function is known, because it is a regulator of a signal transduction pathway linking the external NO3− concentration in the soil with an increase in the rate of lateral root elongation (Zhang and Forde, 1998).

Many late pollen genes such as ZmMADS2 have been reported to be expressed both in sporophytic anther tissues and in the male gametophyte (Xu et al., 1993). The LAT52 gene of tomato, for example, shows an expression pattern similar to ZmMADS2. This gene is expressed in sporophytic tissues of the anther, mature pollen, and root caps, but also in the endosperm (Twell et al., 1991) and was shown recently to be a ligand of the pollen receptor kinase LePRK2 (Tang et al., 2002). Translational enhancement of the late pollen genes LAT52 and NTP303 has been reported to be mediated by their 5′-UTRs (Bate et al., 1996; Hulzink et al., 2002). Transient transformation assays with ZmMADS2 promoter deletion constructs revealed only slight differences in expression levels of the luciferase reporter gene either with or without 5′-UTR, indicating that the functional role of the 5′-UTR of ZmMADS2 is different.

Distribution of functional domains and localization of intron-exon boundaries within the ZmMADS2 gene resemble those of a typical MADS box gene, except for the large second intron (>2.3 kb). An unusual large second intron (2,985 bp) is also present in the AG (AGAMOUS) gene of Arabidopsis, which is required for tissue-specific expression (Sieburth and Meyerowitz, 1997). This also seems to be different for the ZmMADS2 gene, where pollen-specific elements are present some 330 to 210 bp upstream of the transcription start point.
Bioinformatical analysis of the ZmMADS2 promoter resulted in the identification of numerous putative regulatory elements. Already known cis elements required for pollen- and root-specific expression are distributed equally within the ZmMADS2 promoter sequence, indicating that these known motifs might not be significant for regulation of pollen- and root-specific expression in maize. Regulatory elements identified in the promoter of the pollen-specific maize gene ZM13 (Hamilton et al., 1998), a maize homolog of the previously mentioned LAT52 gene of tomato (Bate et al., 1996), could not be identified in the ZmMADS2 promoter region. Transient transformation studies with the luciferase reporter gene under control of seven ZmMADS2 promoter deletion constructs revealed that cis-acting elements required for pollen-specific expression are located in boxes II and III. Putative cis-acting elements inside this fragment include two G box-like sequences mediating sugar repression in box III (ACGTA), an additional one in box II (AATAGAAAA), and a coupling element of G boxes is located in box II (CGAC; Hwang et al., 1998; Toyofuku et al., 1998). In addition, a Suc-responsive element, an auxin-responsive element, and elements responding to water stress and abscisic acid were identified in this region of the promoter, suggesting a role of ZmMADS2 e.g. in water export of the stabilizing anther tissues (endothecium and connective), which causes the opening of anther locules. Dehydration of the anther is an active process with water being exported along an osmotic gradient generated by starch/sugar conversion (Bonner and Dickinson, 1989). Jasmonic acid was described recently as a factor that might regulate synchronous regulation of pollen maturation, anther dehiscence, and flower opening in Arabidopsis because of active water transportation (Ishiguro et al., 2001), because e.g. water uptake of the filaments from the anther wall (endothecium and epidermis) results in filament elongation and anther dehiscence. Similar to ZmMADS2 antisense plants, the DEFECTIVE IN ANther DEhiscence jasmonic acid-insensitive mutant of Arabidopsis shows a block in anther dehiscence leading to male sterility (Ishiguro et al., 2001). DELAYED DEHISCENCE encodes another enzyme of the jasmonic acid pathway necessary for timely release of pollen in Arabidopsis (Sanders et al., 2000). Whether ZmMADS2 is a jasmonic acid-responsive gene has to be shown in further experiments.

Increasing amounts of the ZmMADS2 protein were observed in nuclei of endothecial and connective cells during degeneration culminating with highest amounts in apoptotic bodies containing little amounts of DNA. Programmed cell death (PCD) is a very active process occurring during anther maturation (Ku et al., 2003) and requires tight genetic regulation. ZmMADS2 might represent an example of a regulator protein associated with PCD. Interestingly, AGL17, a member of the same monophyletic clade, has been shown to be localized in sloughing cells of the cap of primary and lateral root tips (Burgeff et al., 2002). These cells undergo a process partly similar to PCD.

The finding that the ZmMADS2-GFP fusion protein could not be observed in maize pollen tubes, although transcript amounts have been shown previously to be present (Heuer et al., 2000), indicates that either the transcript is not translated, or GFP signals have not been strong enough for measuring significant fluorescence levels. Maize pollen grows very fast, and cytoplasm is not only found in the tip of the tube, which is the case e.g. for tobacco pollen tubes, but instead along the whole tube. ZmMADS2-GFP fusion protein might be diluted in the large cytoplasm amounts of the tube resulting in insufficient GFP fluorescence. A ZmMADS2-specific antibody is now required to study the presence and localization of the protein in mature pollen and during pollen tube growth.

The role of ZmMADS2 during pollen tube growth is still unclear. Zachgo et al. (1997) suggested that the Antirrhinum majus homolog of ZmMADS2, DEFH125, is secreted from pollen tubes and transported into nuclei of the transmitting tissue. When a pollen tube has passed, cells of the transmitting tract are no longer needed and usually die by desiccation. It remains to be shown in further experiments if ZmMADS2 is secreted from pollen tubes in vivo to move toward cells of the transmitting tissue or other target cells like the degenerating synergid, which is another example of a plant cell undergoing PCD. The identification of ZmMADS2 target genes and possible interacting partners will now be the next step to elucidate the functional network of this transcriptional regulator during reproduction in both male sporophytic and male gametophytic tissue.

MATERIALS AND METHODS

Plant Material and Stable Transformation of Maize (Zea mays)

Maize plants of the inbred line A188 and tobacco (Nicotiana tabacum; line SRI) were grown in the greenhouse under standard greenhouse conditions at 26°C with 16 h of light and relative humidity of 60%. Anthers of WT and transgenic plants were collected at 1, 2, and 3 d before anthesis and at anthesis. Before DAPI staining, anthers were cut into slices with a razor blade. Isolation and transformation of immature maize embryos of the inbred line A188 was carried out as described by Brettschneider et al. (1997).

Genomic Sequence Analyses, Primer Extension, and DNA Sequencing

The promoter sequence of the ZmMADS2 gene was amplified by genomic walking using the GenomeWalker Kit (CLONTECH Laboratories, Palo Alto, CA). Gene-specific primers TnorF1 (5’-CCTATA GCTAGCTCTCTCTCTTG-ACCCT-3’) and TnorR2 (5’-TAAAGGAGCGAGGTGTGGTG TGG-3’) were used with a Dra I and a Pvu I genomic library of maize. DNA fragments were fully sequenced and aligned. The first intron and part of the second intron (see I1 and I2 in Fig. 2a) were amplified by genome walking from a HindIII library of maize. Introns 3 to 7 were amplified from genomic DNA of maize inbred line A 188. All fragments were cloned into pCR-Blunt II TOPO (Invitrogen) and sequenced using the ABI PRISM 377 Sequencer (PE-Applied
The ZmMADS2 transcription start point was determined using the Primer Extension System (Promega, Madison, WI). According to the manufacturer’s protocol, a (6-fluorescein-6-carboxamido)hexanoate-marked primer (5'-FAM-CAAAAGAGTTAAGGAGGAGGAGAT-3') was annealed to 1 μl of RNA extracted from mature pollen and the resulting extension product compared with standard markers using an ABI PRISM 377 sequencer.

The nucleotide sequence data reported are available in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under the accession numbers AY227363 and AY264885.

Genomic Southern Blots and Expression Analyses

Extraction of genomic DNA from maize was performed according to Dellaporta et al. (1983). Ten micrograms of genomic DNA was digested with the restriction enzymes indicated and resolved on 0.8% (w/v) agarose gels. DNA was transferred to Hybond N+ membranes (Amersham Biosciences Europe, Freiburg, Germany) with 0.4 μl NaOH. Blots were hybridized overnight with radioactive probes prepared using the Prime-it II Random Primer Labeling Kit (Stratagene, La Jolla, CA) in QuickHyb (Stratagene) or Church buffer (7% [w/v] SDS, 0.5 M NaH2PO4 [pH 7.2], and 1 mM EDTA) containing 100 μg ml−1 salmon sperm DNA. Filters were washed with decreasing concentrations of SSC with a final wash at 65 °C in 0.2× SSC/0.1% (w/v) SDS. Filters were washed at 70 °C to Kodak X-omat AR (Eastman-Kodak, Rochester, NY) or Hyperfilm MP (Amersham-Pharmacia Biotech) films using intensifier screens. Plant material for northern-blot analyses was collected in the greenhouse from different tissues and organs of the maize inbred line A188. RNA was extracted from all samples with TRIzol (Invitrogen) according to the manufacturer’s specification. RNA was separated in 1.5% (w/v) denaturing agarose gels and transferred with 10× SSC onto Amersham Hybond N+ membranes. RNA was cross linked to membranes with 300 ml of radiation in a UV Stratallinker 1800 (Stratagene). Hybridization, washing, and exposure were performed according to the procedure described for DNA gel blots. For RT-PCR analysis, RNA was prepared using the mRNA DIRECT Micro-Kit (Dynal Biotech, Hamburg, Germany). Anthers stages were freshly collected from the greenhouse, prepared in a petri dish containing Tri-EDTA buffer using fine forceps and a binocular, and immediately frozen in liquid nitrogen. Tissues were collected in 1.5-mL Eppendorf tubes (Eppendorf AG, Hamburg, Germany) and grinded with plastic mortar and pestle under liquid nitrogen. RNA preparation was performed following the Dynal protocol. RT was performed using Moleney murine leukemia virus RT (MBI, Fermentas, St. Leon-Rot, Germany) for 1 h at 42°C followed by 10 min at 70°C. Two to 4 μL of the RT reaction was used for a standard PCR with 1 unit of Taq DNA Polymerase (MBI) per reaction.

Constructs for Transformation

ZmMADS2 cDNA was amplified with primers M2Bcl (5'-TGATCATGGG-GAGGGGAAA GATC-3') and M2Xho (5'-CTCGAGGAATTCAATTGGA-3'). This open reading frame was inserted into the pDNS-5 promoter in a slightly modified promoterless luciferase construct (de Wet et al., 1987). An NheI adapter was integrated in the promoterless construct (R. Brettschneider, unpublished data), and the Pin III genome walker fragment (see above) was inserted using the Hind III restriction sites. The resulting construct, pDNS-5, was used as a template for amplification of shorter fragments for generation of constructs pDNS-6, -7, and -8. Depending on the template sequence at bp −1,036, −497, and −327, respectively, primers Prom1 (5'-AAGCTTTAGGTTAAGCCTGCGCCG-3'; pdNS-6), Prom2 (5'-AAGCTTTAGGCTCATAATCTGTAAGGAGGAG-3'; pdNS-7), and Prom3 (5'-AAGCTTTAGGCTCATAATCTGTAAGGAGGAG-3'; pdNS-8) were used to introduce either a Pin or HindIII restriction site. The counterpart primer LUC2 (5'-GCCCATTGACGTCCTGCTC-3') is located in the luciferase coding sequence behind an XbaI site. PCR products were restricted with XbaI and either HindIII or PstI and ligated into the corresponding linearized vector. The ZmMADS2 5'-UTR (255 bp) was isolated with NheI from a cDNA library of a maize mature pollen library (Heuer et al., 2000) and inserted in correct orientation in pDNS-6. The resulting plasmid, pDNS-6b, was used as a template for amplification of the ZmMADS2 promoter deletion constructs pDNS-9 and -10, respectively. pDNS-9 and pDNS-10 were generated analogously to pDNS-6 to -8 using primers Prom4 (5'-TAGGCTTAGGTTAAGCCTGCGCCG-3') and Prom5 (5'-GGGAGAAATCTGTAAGGAGGAG-3'), introducing an XbaI site at −210 and −67, respectively. The ZmMADS2-gfp fusion construct used for transient transformation of tobacco pollen was generated by amplification of a ZmMADS2 promoter fragment (corresponding to pDNS-8), the 5'-UTR, the ZmMADS2-CDS, gfp, and terminator sequence with Prom2 and a vector primer using pDNS-1 as a template. The resulting product was integrated into the pBlunt II TOPO (Invitrogen) described above. All constructs were fully sequenced before usage. Only constructs without errors were used for transformation experiments.

Transient Maize of Maize and Tobacco Pollen

Pollens of maize and tobacco were collected from nonstressed greenhouse plants and transformed as described by Schreiber and Dresselhaus (2003). A relatively even spreading of maize pollen was achieved by placing petri dishes containing solid pollen germination medium (PGM) side by side on a table about 30 cm under tassels, which were shaken. Best results were obtained when dry pollen was collected in the afternoon from plants that have not been in contact with any pest control after anthesis. Tobacco pollen was collected directly from mature flowers into microfuge tubes and mixed with 2 mL of liquid PGM. We pipetted 200 μL on PGM plates and left them at room temperature for 45 to 60 min. until germination started. PGM was prepared as two times concentrated solution containing 20% (w/v) Suc (Roth), 0.001% (w/v) H3BO3 (Sigma-Aldrich, St. Louis), 20 mM CaCl2 (Sigma-Aldrich), 0.1 mM KH2PO4 (Merck KGaA, Darmstadt, Germany), and 12% (w/v) polyethylene glycol 4000 (Merck KGaA, Darmstadt, Germany). PGM containing all components was heated to 70°C on a stirring heater, and temperature was held for another 10 min until there was a complete solution of polyethylene glycol. After sterile filtration of 2× PGM, an equal volume of autoclaved 0.6% (w/v) noble agar (Agar Molecular Biology Grade, AppliChem, Darmstadt, Germany) to a final concentration of 0.3% (w/v) was added and poured into petri dishes 3 cm in diameter. Plates were left in a sterile bench with opened lids until PGM was solid and no remnants of liquid remained on the surface of PGM. It was important to stir 2× PGM for at least 10 min at 70°C and to use noble agar instead of other gelling agents like agarose or phytagel. Biologic transformation and marker gene studies were carried out as described by Schreiber and Dresselhaus (2003).

Analysis of Protein-Protein Interaction

Detection of homodimerization of ZmMADS2 was carried out using the yeast (Saccharomyces cerevisiae) Hybrid Hunter Two Hybrid System, Version A (Invitrogen) according to the manufacturer’s specifications. Yeast transformation was carried out as described by Agatep et al. (1998).

Acknowledgments

We like to thank Reinhold Brettschneider for providing the promoterless luciferase construct and for fruitful discussions. We acknowledge Sigrid Heuer for critical reading of the manuscript, Patricia Lauert for helping with the primer extension analysis, Dr. Rebecca Favaro for helping with the yeast
two-hybrid experiments, and Dr. Hermann Schmidt (DNA Cloning Service, Hamburg, Germany) for preparing the pDNS-1 and pDNS-4 constructs.

Received July 22, 2003; returned for revision August 22, 2003; accepted December 12, 2003.

LITERATURE CITED


Beadle GW (1932) Genes in maize for pollen sterility. Genetics 17: 413–431


Cheng PC, Greyson RI, Walden DB (1979) Comparison of anther development in genic male-sterile (ms10) and in male-ferile corn (Zea mays) from light microscopy and scanning electron microscopy. Can J Bot 57: 578–596

Christensen AH, Quial PH (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res 5: 213–218


eder and spikelet development and is co-expressed with ZmMADS1 during flower development, in egg cells, and early embryogenesis. Plant Physiol 127: 33–45


Sieburch LE, Meyerowitz EM (1997) Molecular dissection of the AGAMOUS
control region shows that cis elements for spatial regulation are located intragenically. Plant Cell 9: 355–365


