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Seed-specific promoters direct gene expression in non-seed tissue

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Abstract

The organ specificity of four promoters that are known to direct seed-specific gene expression was tested. Whereas the phaseolin (*phas*)- and legumin B4 (*leB4*)-promoters were from genes encoding 7S and 11S globulins from *Phaseolus vulgaris* and *Vicia faba*, respectively, the *usp*- and the *sbp*-promoters were from non-storage protein genes of *V. faba*. The expression of different promoter–reporter gene fusions was followed either by RT-PCR or by registering the reporter enzyme activity in organs of transgenic tobacco, pea, narbon bean, or linseed. In addition to seeds, the promoters directed reporter gene expression in pollen and in seed coats. USP-, vicilin- and legumin-mRNA were detected by RT-PCR in pollen of *Pisum sativum* and *V. faba.* Expression during microsporogenesis and embryogenesis seems to be a general character of various seed protein genes.

Key words: Gene expression, organ specificity, pollen, promoters, seed, seed coat.

Introduction

Genes encoding seed storage proteins, like zein of maize (*Zea mays* L.), phaseolin of kidney bean (*Phaseolus vulgaris* L.), or legumin of faba bean (*Vicia faba* L.), were among the first deciphered plant genes (Wienand *et al.*, 1981; Pedersen *et al.*, 1982; Slightom *et al.*, 1983; Bäumlein *et al.*, 1986). Their promoters were functionally analysed by expressing the complete genes or promoter–

reporter gene fusions in foreign plants (Sengupta-Gopalan *et al.*, 1985; Bäumlein *et al.*, 1987, 1991*a*; Murai *et al.*, 1983). These studies led to the conclusion that the promoters of seed storage protein genes direct seed-specific gene expression. Later on, promoter dissection analysis revealed controlling elements for this seed specificity (Bäumlein *et al.*, 1991*a*; Wobus *et al.*, 1995). Promoters, like that of *phas*, soon became favourite tools for directing seed-specific expression of foreign genes in the genetic engineering of plants (Altenbach *et al.*, 1989).

Seed-specificity was also attributed to the promoters of genes encoding a sucrose- binding protein (SBP) (Heim *et al.*, 2001) and a so-far unknown seed protein (USP) of *V. faba* L. (Bäumlein *et al.*, 1991*b*; Fiedler *et al.*, 1993). SBP is closely related to the class of 7S storage globulins (Shutov and Bäumlein, 1999). PCR revealed only that low levels of SBP-mRNA can be detected in seed coat and sink leaves of *V. faba* (Heim *et al.*, 2001) as well as in the phloem of leaves, stems, and roots of soybean (*Glycine max*) (Contim *et al.*, 2003). Whereas in *G. max* the function of SBP could be related to sucrose uptake in sieve tubes, its function in *V. faba* is less clear. The *usp*-promoter directs the transcription of the quantitatively predominating mRNA in mid-maturation cotyledons of faba bean seeds (Bassüner *et al.*, 1983, 1988). Its expression starts earlier than that of the genes encoding vicilin and legumin, the major storage proteins in developing seeds of this grain legume. Expression of a *usp*-promoter-controlled *uidA* was not only observed in developing seeds but also in root tips of *Nicotiana tabaccum* and *Arabidopsis thaliana* seedlings (Bäumlein *et al.*, 1991*b*). Nevertheless, the *usp*-promoter was successfully used to mediate strong seed-specific gene expression in several foreign plants (Fiedler and

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Conrad, 1995; Phillips *et al.*, 1997; Conrad *et al.*, 1998; Czihal *et al.*, 1999; Saalbach *et al.*, 2001).

However, when pea plants expressing the *usp*-promoter-controlled gene of a thermostable bacterial xylanase were grown in the greenhouse, decreased fertility of the transgenic plants was observed, which could be explained best by assuming *usp*-promoter-mediated xylanase gene expression in pollen (I Saalbach, unpublished results). Similarly, van der Geest *et al.* (1995) found that the *phas*-promoter-directed expression of a Diphtheria Toxin A gene affected microsporogenesis in tobacco transformants. In addition, a *phas*-promoter-controlled *uidA* was expressed in tobacco pollen. This led to the suggestion that genes encoding seed-storage proteins might be expressed in general, not only during seed maturation but also during pollen development (Hall *et al.*, 1999), although phaseolin or its mRNA have not been found in kidney bean pollen (van der Geest *et al.*, 1995).

Using the thermostable bacterial α-amylase (Conrad *et al.*, 1995) and xylanase (Herbers *et al.*, 1995) as reporters, the specificity of the *usp*- and *sbp*-promoters, respectively, was re-investigated in transgenic tobacco and pea. Some representatives of the families of 7S and 11S storage globulin gene promoters were included in this study. The presence of USP- and storage globulin-specific mRNAs was detected in grain legume pollen. It is concluded that, in Angiosperms, several storage globulin and non-storage protein gene promoters are not only active in the cotyledons and seed coat of developing seed, but in pollen too. The implications of this finding are discussed with regard to organ development and biotechnology of plants.

Materials and methods

All experiments and analyses were repeated at least twice with independently grown plant material.

Plant material

The following plant species were used (provider of seeds in parenthesis): *Linum usitatissimum* L. cv. Flanders (Dr Voss, Hamburg); *Nicotiana tabaccum* L. var. SNN (IPK, Gatersleben); *Phaseolus vulgaris* L. cv. Valja (commercial); *Pisum sativum* L. cv. Greenfeast, Erbi or Eiffel; *Vicia faba* L. ssp. *minor* cv. Fribo (IPK, Gatersleben); *Vicia narbonensis* L. (Dr T Pickardt, Berlin). Transgenic plants are listed in Table 1.

Except for mature seeds and samples that were used immediately, all other organs and tissues were frozen in liquid nitrogen directly after harvest and stored at −70 °C. A list of the different organs that have been analysed is given in Table 1.

Plant cultivation

Wild-type and transgenic plants were always simultaneously raised from seeds in pots with soil, either in controlled growth chambers (narbon bean, pea) (16/8 h light/dark, 22/18 °C day/night temperature, 70% humidity in the air) or in greenhouses with supplementary illumination from September to May (field bean, flax, tobacco). In addition, material was harvested from plants grown in field trials (pea).

For root analysis tobacco plants were grown in pots with sand and Gamborg B5 nutrient solution (commercially supplied by Duchefa Biochemie, Haarlem, The Netherlands). After raising the seedlings in soil for 10–14 d, they were transferred to pots (13 cm diameter) with sand and watered with 50 ml d^{-1} and 100 ml d^{-1} of nutrient solution up to 35 d after transfer and during later growth periods, respectively. In this substrate, tobacco plants grew slower than in soil. If both types of cultivation were started simultaneously, material was harvested at different times when plants had reached similar growth stages.

Four-day-old tobacco seedlings were harvested after germinating seeds on moist filter paper in small covered photo trays at greenhouse temperature in the dark. Sand culture was used to obtain material from later stages of seedlings with primary leaves grown in the light under greenhouse conditions. Seeds were always surface-sterilized before starting germination.

Promoter–reporter gene fusions and plant transformation

Transgenic plants are listed in Table 1. References for promoter–reporter gene constructs as well as for the transformation techniques and raising of transgenic plants are also given in this table.

Table 1. *Transgenic plants that were used in the present investigation*

Promoters, reporter genes and analysed organs are listed. References in the first lane indicate where promoters have been described. In the last lane references for promoter–reporter gene constructs (first reference) and transformation techniques (second reference) are given. SBP, sucrose binding protein; *uidA*, β-glucuronidase (GUS) gene; USP, unknown seed protein.

Promoter, source	Reporter gene	Species	Organs	Reference
USP (*Vicia faba*) (Bäumlein *et al.*, 1991*b*)	Thermostable α-amylase	*Nicotiana tabacum*	Petals, anthers, filaments, pollen, ovaries, seeds, seedlings, vegetative organs	Conrad *et al.* (1995); Saalbach *et al.* (1994)
	Thermostable α-amylase	*Pisum sativum*	Anthers, pollen, seed coat	Conrad *et al.* (1995); Saalbach *et al.* (2001)
	uidA	*Linum usitatissimum*	Pollen	Voss (2001)
Phaseolin (*Phaseolus vulgaris*) (Slightom *et al.*, 1983)	Thermostable xylanase	*Pisum sativum*	Anthers, pollen	Herbers *et al.* (1995); Saalbach *et al.* (2001)
SBP (*Vicia faba*) (Heim *et al.*, 2001)	*uidA*	*Pisum sativum*	Anthers, pollen, seed coat	Heim *et al.* (2001); Saalbach *et al.* (2001)
Legumin B4 (*Vicia faba*) (Bäumlein *et al.*, 1986)	*uidA*	*Vicia narbonensis*	Anthers, pollen, seed coat	Pickardt *et al.* (1998); Pickardt and Saalbach (2000)

Table 2. *List of primers used for detecting specific mRNAs by RT-PCR*

Gene	Accession no.	Species	Primer pairs (5′ to 3′) sense/antisense
α-amylase	M38570	*Bacillus licheniformis*	CT TCT TGC TGC CTC ATT CTG CAG/ CGA TGC AGC ATG GAA CTG ATA ATG
Legumins A1, A2, B4 consensus	X55013 X55014 X03677	*Vicia faba*	GAT TYA GRV ARG GTG ATA TCA TTG C/ AAY ASC AGC TCT RTC ATT KGT CTT G
Vicilin	Y00462	*Vicia faba*	AGC TAT ACT CAC AGT GTT ATT ACC C/ TAT CTA CCT CTT GAG CTG ATC CAG G
usp	X56240	*Vicia faba*	AAT CAC TGC GAC ATC ATC CGG AG/ ATG ACA CAA AGA TTG TCT GCG GTA TG
Actin	X63603	*Nicotiana tabacum*	ATG GCA GAC GGT GAG GAT ATT C/ CTT TGC AAT CCA CAT CTG TTG G

Segregation analysis and Southern blotting

Segregation analysis for selecting homozygous single insert lines of the different transformants was based on reporter enzyme detection in extracts from at least 100 different offspring seedlings per F₁ mother plant. In some cases, analysis was repeated with the same number of plants. In selected tobacco transformant lines with the USP-promoter α-amylase construct, results of segregation analysis were verified by Southern blotting.

DNA extraction and Southern blotting were performed according to Sambrook *et al.* (1989). Tobacco leaf DNA was restricted with *Hin*dIII (MBI Fermentas, Vilnius, Lithuania) which has only one cleavage site in the T-DNA region of the vector used for transforming the plant with the USP-promoter α-amylase gene construct (Table 2).

RNA extraction

Total RNA was extracted using a phenol–chloroform method according to Chomzynsky and Sacchi (1987). This method was not suitable for seeds due to the low RNA yield. Therefore, extraction of seed RNA followed the procedure of Becker *et al.* (1995). After quantifying nucleic acids, the extract was treated with DNAse before being used for RT-PCR. Generally, 20 μg of total RNA were incubated with RNAse-free DNAse I (Roche Applied Sciences, Germany). Subsequently, RNA quality was checked electrophoretically (see below).

Starting from 20 μg of total RNA of mid-maturation seeds the mRNA fraction was isolated using affinity binding to Dynabeads (Dynal, Oslo, Norway) according to the manufacturer's instructions.

Semi-quantitative RT-PCR

Two μg of DNA-free total RNA were reacted with reverse transcriptase (MBI Fermentas, Vilnius, Lithuania) in 20 μl of reaction mixture according to the instructions of the enzyme manufacturer. The reaction was run with 4 μg of total RNA in order to detect mRNA of the thermostable bacterial α-amylase in vegetative organs of tobacco. RT-PCR of seed mRNA was performed with an amount of mRNA corresponding to 2 μg of total RNA. The absence of genomic DNA was controlled by running no-RT-PCR in parallel with RT-PCR.

Five μl of the RT-reaction mixture in a total volume of 50 μl were used in the subsequent PCR. Primers that were used for PCR amplification of mRNA-specific DNA are listed in Table 2. Actin-mRNA was amplified with 20 cyles and all other mRNAs with 30 cycles of this standard PCR programme following the instructions of the manufacturer of Takara *Taq*-DNA-polymerase (Takara Shuzo Co., Kyoto, Japan). Each cycle included the following steps: 30 s at 94 °C, 30 s at 58 °C, and 60 s at 72 °C.

Electrophoresis of nucleic acid

After sample denaturation with Glyoxal Load Dye (Ambion, Austin, Texas, USA) at 50 °C for 30 min, RNA electrophoresis was run in 0.8% agarose gels with TAE buffer. One per cent agarose gels were used for electrophoresis of DNA from 10 μl of PCR reaction mixture. Nucleic acid bands were visualized by EtBr staining.

DNA sequencing

DNA sequencing followed the dideoxy-method according to Sanger (1980) using the Megabace 1000 automatic sequencer of Amersham Bioscience Ltd. (Buckinghamshire, UK).

Controls in reporter enzyme activity analysis

In all cases, offspring without enzyme activity after segregation analysis of transformed mother plants were used as controls. They correspond to wild-type plants that passed transformation. For all species, the number of independent transgenic lines analysed for reporter gene expression is indicated in Table 3 in the Results.

α-Amylase activity assays

In general, 25 mg of plant material were ground in a mortar before 1 ml of buffer (0.1 M maleic acid, 0.1 M NaCl, 0.002 M CaCl₂.2H₂O, and 0.01% Na-azide, pH 6.5) was added in order to extract the enzyme under shaking at 40 °C for 10 min. However, immature tobacco seeds and whole pea seeds were homogenized with 200 μl of this buffer before extracting the enzyme in the same way. When endogenous heat-sensitive amylases had to be inactivated, extracts were heated to 80 °C for 30 min. Extracts were centrifuged at 9000 *g* for 10 min and aliquots of the supernatant used for assays. Seed extracts with thermostable α-amylase added formed a positive control after heat inactivation. Heat-inactivated extracts from wild-type samples and a standard flour sample provided by the manufacturer (Gammazyme) formed the negative controls. For long-term storage, extracts could be frozen at −80 °C without detectable loss in enzyme activity.

α-Amylase activity was measured using the Amylase HR Reagent (Megazyme Alpha-Amylase Assay Procedure, ICC Standard No. 303) according to the manufacturer's instructions. Extracts were diluted 1:4 with extraction buffer, before 50 μl of the diluted extract were reacted with 50 μl of HR reagent at 40 °C for exactly 5 min. The reaction was stopped by adding 750 μl of stop reagent. Spectrophotometric measurement took place with 1 cm cuvettes at 410 μm. In blanks, the stop reagent was added at time 0 of incubation. The results were expressed as Ceralpha Units (CU) which can be converted to International Units (IU) using the formula (IU of starch=4.1× Ceralpha Units).

In some cases the activity of thermostable α-amylase was semi-quantitatively determined by measuring halo sizes in agar plates containing Murashige–Skoog nutrient solution in a 1:10 dilution and 0.5% starch. One to 10 μl of original or appropriately diluted and heat-treated enzyme extract were spotted directly on the plates which were incubated overnight at 37 °C. Seeds, seed coats, and intact

Table 3. *Seed promoter-directed reporter gene expression in pollen and seed coats of different transgenic plants (see legend of Table 1 for abbreviations)*

The results are based on reporter enzyme activity measurements (compare Fig. 3). The number of analysed lines per species is given in parenthesis in the species lane.

Promoter	Reporter gene	Species	(No. of lines)	Pollen	Seed coat
USP	α-amylase	*Pisum sativum*	(3)	x	x
	α-amylase	*Nicotina tabacum*	(30)	x	n.d.[d]
	uidA	*Linum usitatissimum*[a]	(4)	x	n.d.[d]
Phaseolin	Xylanase	*Pisum sativum*	(3)	x	n.d.[d]
SBP	*uidA*	*Pisum sativum*	(3)	x	x[c]
Legumin B4	*uidA*	*Vicia narbonensis*[b]	(2)	x	x[c]

[a] Transgenic seeds kindly provided by H Voss, Hamburg.
[b] Plants kindly provided by T Pickardt, Berlin.
[c] According to Pickardt *et al.* (1998).
[d] n.d., Not determined.

pollen were placed directly on the plates followed by an incubation in the dark at 80 °C for 30 min. Subsequently, halos were made visible by staining the plates with Lugol solution (Fluka).

Histochemistry

For primary fixation, anthers were kept for 4 h at room temperature in 50 mM cacodylate buffer, pH 7.2, containing 1.0% (v/v) glutaraldehyde and 2% (v/v) formaldehyde, followed by one wash with buffer and two washes with distilled water. For the secondary fixation, samples were transferred to a solution of 1% (v/v) OsO₄. After 1 h samples were washed three times with distilled water. Dehydration of the samples was done step-by-step by increasing concentrations of ethanol: 30%, 50%, 60%, 75%, 90%, and two times 100% (v/v) for 1 h each. After 1 h dehydration with propylene oxide the samples were infiltrated step-by-step with Spurr resin (Plano GmbH, Marburg, Germany) as follows: 33%, 50%, and 66% (v/v) of the resin in propylene oxide for 4 h each and, finally, 100% Spurr resin overnight. Samples were then transferred in BEEM capsules, kept there in fresh resin for 6 h and polymerized at 70 °C for 23 h. Semi-thin sections (approximately 3 μm) were mounted on slides and stained for 2 min with 1% methylene blue in 1% aqueous borax at 60 °C prior to examination in a Zeiss Axiovert 135 microscope. Dehydration, resin embedding, and semi-thin sectioning of anthers after GUS staining (see below) were done as described above. However, the sections were used without staining for light microscopy examination.

Detection of β-glucuronidase (GUS) activity followed the protocols given in Gallagher (1992).

Results

USP-promoter-controlled reporter gene expression

The short and the long usp-*promoter*: The strong predominance of USP-mRNA in *V. faba* seeds and its temporal pattern of transcription (Bassüner *et al.*, 1988) made the original 637 bp 'short' usp-promoter attractive for strong seed-specific expression of foreign genes in biotechnology. In an attempt to increase its strength still further, an additional 512 bp fragment of the usp-promoter was sequenced, generating a 'long' usp-promoter now comprising in total 1149 bp (Fig. 1a). This long usp-promoter turned out to be more active than the 'short' one. The expression levels of the gene constructs were compared when the promoters were fused in front of a reporter gene encoding a thermostable α-amylase from *Bacillus licheniformis*. Segregation analysis indicated single gene inserts for 16 and 10 lines of short and long usp-promoter–reporter gene transformants of tobacco, respectively. The activity of thermostable α-amylase was determined in seed extracts after the endogenous α-amylase activity had been inactivated by heating. Average activity ratios of 1:3 were measured for bacterial α-amylase in lines where gene expression was directed by the short and long usp-promoter, respectively (Fig. 1b). Among 54 short promoter lines, only those with more than one segregating gene locus showed a higher α-amylase activity than 2.5 U/seed, whereas single locus lines of long promoter transformants with the lowest activity had already reached this value. With one exception (see next section) no analysis was performed whether the loci segregating as single inserts had or had not tandem inserts of the reporter gene.

usp-*promoter controlled expression patterns of reporter genes*

Both usp-promoter–reporter gene constructs were used to follow usp-promoter-controlled reporter gene expression in different tissues during the life cycle of tobacco (*Nicotiana tabacum* L. SNN) transformants (Fig. 2). Expression levels
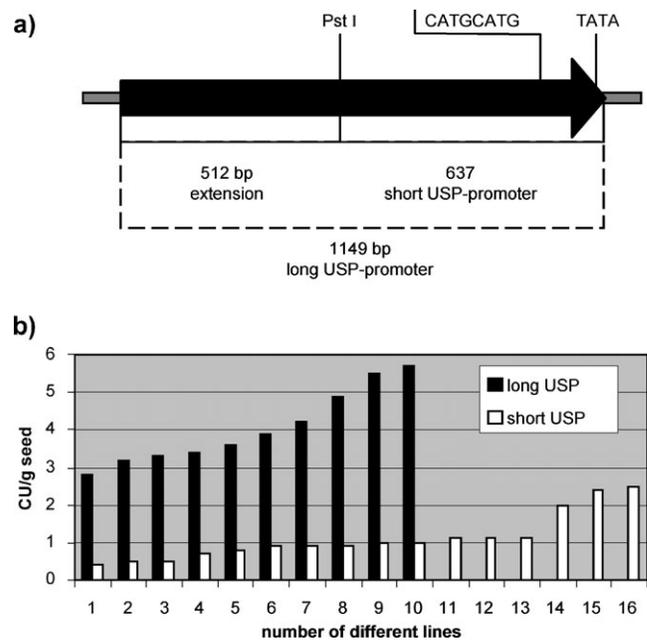

Fig. 1. The long usp-promoter is three times stronger than the short one in directing reporter gene expression in seeds. (a) The short and the long usp-promoter, (b) comparison of enzyme activities (CU, Ceralpha Units) in seeds of single gene insert lines expressing the thermostable bacterial α-amylase under control of the short and the long usp-promoter.
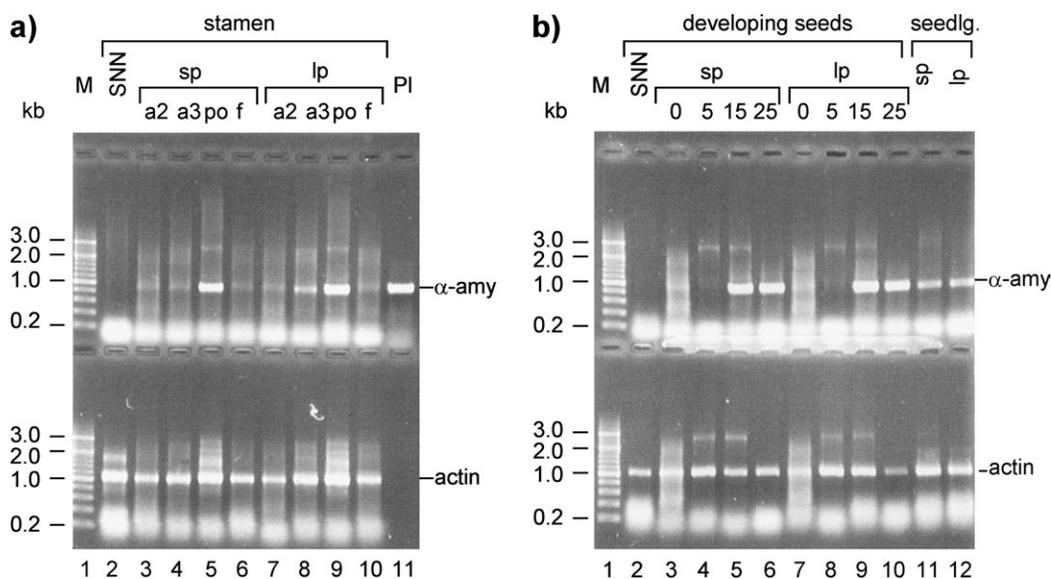
Fig. 2. RT-PCR-based analysis of patterns of *usp*-promoter controlled reporter gene expression (mRNA of thermotolerant bacterial α-amylase) in different organs of transgenic tobacco. M, molecular weight markers; SNN, wild-type tobacco; sp and lp, short and long *usp*-promoter, respectively. (a) Maturing stamens; a2, from 2 cm long flower buds; a3, from 3 cm long flower buds; p, pollen; f, filaments. (b) Developing seeds and seedlings (4 d after imbibition). Numbers above the lanes indicate days after flowering. Actin-cDNA is given as reference.

cannot be compared quantitatively since only the short promoter line contained a single gene insert. Although the long promoter line showed single locus segregation according to enzyme activity determination, subsequent Southern blotting revealed that it contained a tandem insert of two amylase genes. Samples from three and four different plants per short and long promoter line, respectively, were analysed by Southern blotting.

RT-PCR confirmed that, similar to the short promoter, the long promoter was active in directing reporter gene expression in developing seeds (Fig. 2b, lanes 3–6 and 7–10, respectively). Independent of the promoter length, 4 dai seedlings also contained USP-mRNA, (Fig. 2b, lanes 11 and 12). Among flower organs only mature pollen contained reporter enzyme-mRNA (Fig. 2a, lanes 5 and 9, respectively). But the weak signal detected in developing anthers from flowers with 2 and 3 cm petal lengths might only result from mRNA of developing pollen that is diluted by mRNA from non-pollen anther tissues free of amylase-mRNA. This interpretation is supported by histochemical analysis of *sbp*–*uidA* construct expression in pea anthers where no GUS staining was observed in non-pollen tissue (Fig. 3b).

Usp-promoter activity in pollen (Fig. 3a) and cotyledons (Fig. 3e) of transgenic pea as well as in the pollen (Table 3) of transgenic tobacco was also detected by measuring *in vitro* starch degradation due to the activity of the thermostable bacterial α-amylase. Similarly, *usp*-promoter-directed reporter enzyme formation was shown by testing for GUS activity in pollen of *Linum usitatissimum* L. (Table 3). In addition, measurements of α-amylase activity showed that the *usp*-promoter directs gene expression in seed coats of pea (Fig. 3e).

No *usp*-promoter-directed reporter gene expression could be detected in roots, leaves, or nodal and internodal axis sections of fully developed tobacco plants (not shown).

Various storage globulin promoters direct reporter gene expression in pollen and seed coat

As shown in Fig. 3b and c and Table 3, in addition to the *usp*-promoter, various fusions of other seed protein-specific gene promoters with genes of bacterial reporter enzymes (Table 1) were tested by transforming pea, narbon bean, and linseed. Gene expression was, in all cases, registered by enzyme activity detection. The *phas*-promoter-directed pollen-specific expression of a thermotolerant xylanase damaged a certain percentage of pollen grains of pea (Fig. 3c). Phaseolin from garden bean (*Phaseolus vulgaris* L.) is a 7S storage globulin. The *sbp*-promoter directed *uidA* expression in pea pollen (Fig. 3b). The promoter of a gene encoding legumin B4 from field bean, a representative of the class of 11S globulins, also mediated *uidA* expression in transgenic narbon bean (Table 3). Similar to the *usp*-promoter, the *sbp*- and the *leB4*-promoters also directed reporter gene expression in seed coats of pea (Fig. 3e; Table 3) and narbon bean (Table 3), respectively.

These results from reporter gene expression raise the question as to whether seed storage protein genes themselves are expressed in pollen (see below).

usp *and globulin gene expression in pollen of grain legumes*

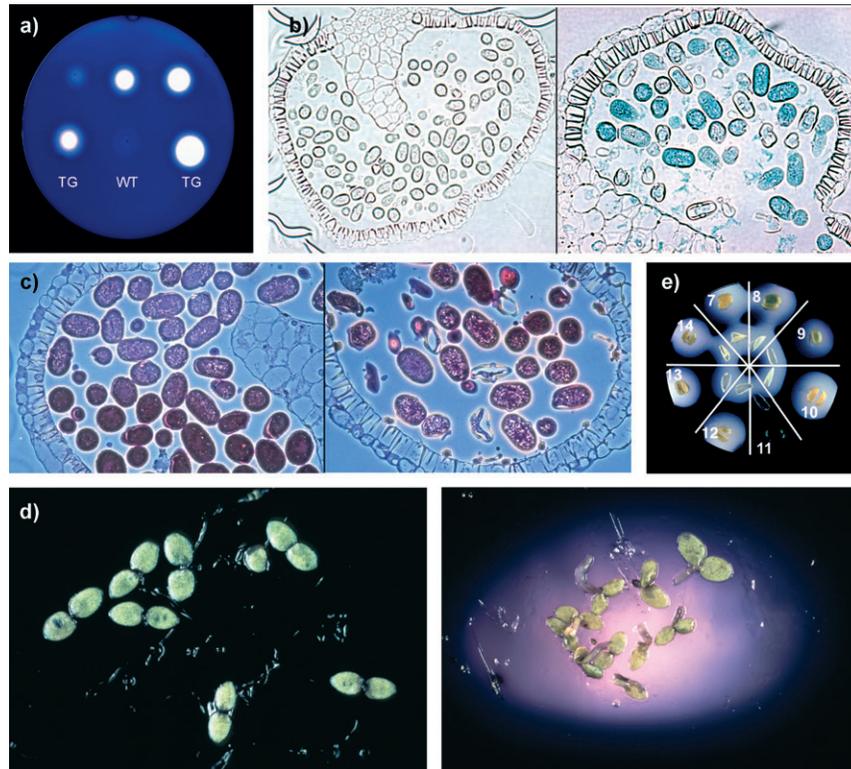Vicilin-, legumin- and USP-mRNA were detected by RT-PCR in pollen of pea and field bean (Fig. 4). Signals

Fig. 3. Analysis of the reporter gene expression by halo formation on starch-containing agar plates (thermostable α-amylase) and histochemistry (GUS, thermostable xylanase). (a) *usp*-promoter-controlled thermostable α-amylase in pollen extract of transgenic *Pisum sativum:* the different sizes of white halos result from different extract dilutions. WT, wild type; TG, transgenic. (b) *sbp*-promoter-controlled *uidA* expression in anthers of *Pisum sativum*; left, wild type; right, GUS-staining of pollen in an anther of a transgenic plant. (c) *Phas*-promoter-controlled thermostable xylanase in anthers of *Pisum sativum*; left, wild type; right, transgenic plant with a certain percentage of damaged pollen grains; methylene blue staining of pollen. (d) *usp*-promoter-controlled thermostable α-amylase in cotyledons of imbibed seeds of *Nicotiana tabacum*; heat-treated cotyledons put directly on starch-containing agar plates; left, wild type; right, transgenic plants. (e) *usp*-promoter-controlled thermostable α-amylase in cotyledons of imbibed seeds (outer circle) and seed coats (inner circle) of transgenic *Pisum sativum*. Numbers indicate different transgenic seeds, except no. 11 which is the wild type. Heat-treated organs put directly on starch-containing agar plates.

obtained in anther extracts should result from maturing pollen, but not from non-pollen tissue, where no *sbp*-promoter-directed reporter gene expression takes place (see above). Extracts from field bean cotyledons of mid-maturation seeds were used for comparison (Fig. 4, lane 1). There, legumin-mRNA and legumin predominate over vicilin-mRNA and vicilin, respectively, at that developmental stage (Bassüner *et al.*, 1983). By contrast, pollen shows a predominance of vicilin-mRNA in *V. faba* (lanes 2–5). Similarly, a predominance of vicilin over legumin was reported for embryonic axes of field bean seeds (Schlereth *et al.*, 2000; Tiedemann *et al.*, 2000).

Discussion

Promoters of various seed protein-specific genes direct gene expression not only in developing seeds but in pollen, too. Seed protein promoter-controlled reporter gene expression was also observed in the seed coat of developing seeds. This agrees with the detection of legumin-mRNA by *in situ* hybridization in seed coats of *V. faba* (Wobus *et al.*,

1995). Activity determination of various reporter enzymes confirmed translation of the corresponding mRNAs in pollen.

USP-specific primers were deduced from the same *V. faba* gene that is controlled by the *usp*-promoter. Using these primers USP-mRNA was detected in faba bean as well as in pea pollen. This indicates that the faba bean as well as the pea *usp*-promoter direct gene expression in pollen and that the same promoters, but not two different ones, direct *usp* expression in seeds and pollen. The situation is less clear for the *leB4*-promoter of *V. faba*. The expression of the legumin genes in pollen was detected by using consensus primers for three different *V. faba* legumin genes (Table 2). Therefore, it cannot be excluded that, in pollen, a legumin gene different from that encoding legumin B4 generated the signal. This would indicate that two legumin promoters direct gene expression in pollen, the unknown one and the *leB4*-promoter. The *phas*-promoter used for reporter gene expression and the vicilin-specific mRNA detected in grain legume pollen were from different species as well as from two different 7S globulin genes. By
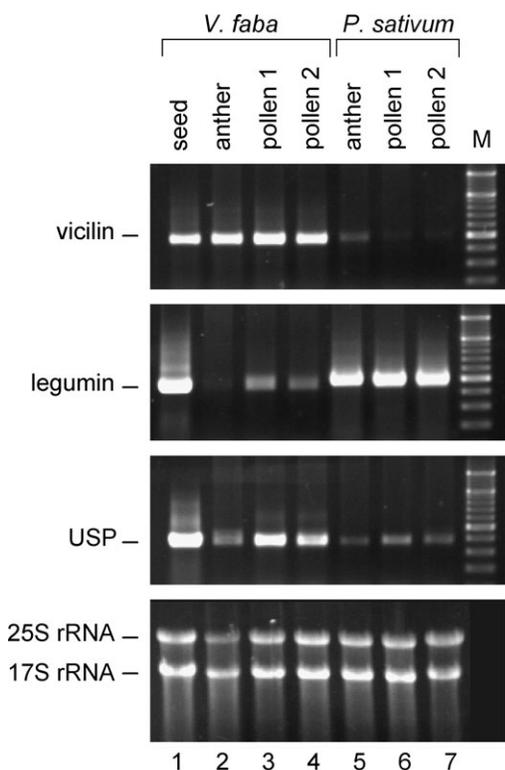
Fig. 4. Seed protein mRNA in anthers and pollen of *Vicia faba* and *Pisum sativum*. M, molecular weight markers, ribosomal RNA is given as reference. USP, unknown seed protein.

analogy to the *usp* and legumin genes it can be concluded that 7S globulin gene promoters, including the promoter of the related *sbp*, also direct 7S globulin-mRNA formation in pollen. Future dissection studies will reveal the mechanistic basis of the double specificity of seed protein promoters in seeds and pollen. If controlling elements for seed- and pollen-specificity do not overlap, the promoter might be engineered to maintain only one of the two specificities and to generate either seed- or pollen-specific promoters. This could be advantageous for basic research as well as for biotechnological applications.

Seed protein promoters are already active in reporter gene expression during pollen maturation. *Phas*-promoter activity was registered before meiosis during microsporogenesis of transgenic tobacco expressing the Diphtheria Toxin A gene (van der Geest *et al.*, 1995). Storage globulin gene expression has not been directly investigated in non-pollen anther tissue. But, tapetum cells were not affected when the Diphtheria Toxin A was expressed under control of the *phas*-promoter in anthers of tobacco (van der Geest *et al.*, 1995). This might be taken as an indication that seed protein promoters are inactive in non-pollen anther tissue which, in the present paper, was confirmed by histochemical analysis of reporter gene expression. However, the promoter of the zein gene *zE19* from *Zea mays* is active not only in pollen but in non-pollen tissue of anthers as well (Quattrocchio *et al.*, 1990). In addition, van der Geest *et al.* (1995) observed *uidA*

expression under the control of the *phas*-promoter in post-meiotic maturation stages of transgenic tobacco pollen. Taking into consideration the half-life times of GUS-mRNA the authors could not deduce whether the detection of GUS-activity was based on continued transcription/translation or on the translation of stored mRNA that had been generated already in the pre-meiotic period. It is well known that several mRNAs generated by transcription during pollen maturation can be stored and become translated later during pollen tube formation (Twell, 2002).

Comparing quantitatively reporter gene expression in seeds and pollen of transgenic tobacco van der Geest *et al.* (1995) found that, in pollen, the *phas*-promoter activity is only approximately 1% of that known from seeds. By contrast, no such activity difference was observed for the promoter of the zein gene *zE19* (Quattrocchio *et al.*, 1990). Due to the incomplete extraction from seeds by the method of Chomzynsky and Sacchi (1987) different methods had to be used for RNA extraction from seeds and other tobacco organs. In addition, polyA-RNA had to be isolated from total seed RNA to obtain good RT-PCR results. Due to these methodological differences, expression levels in seeds and pollen could not be compared quantitatively at the mRNA level in the present investigation. However, comparison of α-amylase activity levels revealed approximately 1% *usp*-promoter activity in pollen compared with seeds of transgenic pea (Giersberg *et al.*, 2002).

Activity of seed protein promoters in pollen was detected in distantly related taxonomic families as Leguminosae, Linaceae, and Solanaceae. In addition, the promoter of zein gene *zE19* directs *uidA* expression in seeds and pollen of transgenic *Petunia* plants and mRNA of this seed protein gene was detected in pollen of *Zea mays* (Quattrocchio *et al.*, 1990) indicating that seed protein genes are also expressed in pollen of monocotyledonous plants. Thus, seed protein promoter activity in pollen should be a general phenomenon in Angiosperms.

Usp encodes a non-reserve protein of developing seeds. The originally published *usp*-promoter with 637 base pairs (bp) (Bäumlein *et al.*, 1991*b*) was shown to have at least six controlling elements within the first 0.4 kb. It was assumed that all important *cis*-regulatory elements are located there and govern in complex interaction the quantitative but also spatial and temporal patterns of *usp* expression (Fiedler *et al.*, 1993). Nevertheless, in model experiments the long 1149 bp *usp*-promoter described in the present paper directs significantly higher expression of reporter genes. But, no expression pattern differences were found between the short and the long *usp*-promoter.

Seed protein promoter activity in pollen might have drastic consequences for biotechnological applications. The *phas*-promoter-controlled expression of reporter genes like Diphtheria Toxin A (van der Geest *et al.*, 1995) and thermostable bacterial xylanase (Fig. 3c; I Saalbach *et al.*, unpublished results) led to pollen sterility. On the other

hand, expression of *uidA* as well as of the gene encoding a thermostable α-amylase under the control of seed protein promoters in pollen obviously did not affect the fertility of transgenic plants from three different plant families. Taking into account the possibility of much higher activity in developing seeds than in pollen, seed protein gene promoters should be suited for high level expression of foreign genes in plant seeds. However, even at a low level of concomitant expression in pollen, genes encoding proteins that affect pollen fertility cannot be used for biotechnological production in seeds.

As proposed by Hall *et al.* (1999), storage globulins represent amino acid reserves for seed and pollen germination. This suggestion implies that, besides storage proteins, other components of the biochemical and cell biological syndrom of protein storage, for example, protein storage vacuoles/protein bodies and processing enzymes, should also be present in maturing pollen. This seems to be the case since processing legumains (Müntz *et al.*, 2002) were found in maturing tobacco pollen (Zakharov and Müntz, 2004). Seeds of tobacco are known to contain 11S globulins (Sano and Kawashima, 1983) which are most probably processed by these legumains. Components of storage protein mobilization similar to those in germinating seeds and seedlings (Müntz *et al.*, 2001) might be found in pollen as well.

Acknowledgements

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