GENETIC TRANSFORMATION AND HYBRIDIZATION

The *PsEND1* promoter: a novel tool to produce genetically engineered male-sterile plants by early anther ablation

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Abstract *PsEND1* is a pea anther-specific gene that displays very early expression in the anther primordium cells. Later on, *PsEND1* expression becomes restricted to the epidermis, connective, endothecium and middle layer, but it is never observed in tapetal cells or microsporocytes. We fused the *PsEND1* promoter region to the cytotoxic *barnase* gene to induce specific ablation of the cell layers where the *PsEND1* is expressed and consequently to produce male-sterile plants. Expression of the chimaeric *PsEND1::barnase* gene in two Solanaceae (*Nicotiana tabacum* and *Solanum lycopersicon*) and two Brassicaceae (*Arabidopsis thaliana* and *Brassica napus*) species, impairs anther development from very early stages and produces complete male-sterile plants. The *PsEND1::barnase* gene is quite different to other chimaeric genes previously used in similar approaches to obtain male-sterile plants. The novelty resides in the use of the *PsEND1* promoter, instead of a tapetum-specific promoter, to produce the ablation of specific cell lines during the first steps of the anther development. This chimaeric construct arrests the microsporogenesis before differentiation of the microspore mother cells and no viable pollen grains are produced. This strategy represents an excellent alternative to generate genetically engineered male-sterile plants, which have proved useful in breeding programmes for the production of hybrid seeds. The *PsEND1* promoter also has high potential to prevent undesirable horizontal gene flow in many plant species.

Keywords Anther ablation · Male-sterility · Oilseed rape · *PsEND1* promoter · Tobacco · Tomato

Abbreviations GUS: β-Glucuronidase · *nptII*: Neomycin phosphotransferese II gene · PCR: Polymerase chain reaction · *uidA*: β-Glucuronidase gene

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Introduction

Stamens are the male reproductive organs of flowering plants. They consist of an anther and, in most species, a stalk-like filament. The basic pattern of anther differentiation is mostly conserved among angiosperms (Scott et al. 1991). Developmental events leading to anther formation and pollen release occur in a precise and chronological order that correlates with floral bud size (Koltunow et al. 1990; Goldberg et al. 1993). Anther development can be divided into two phases. The phase 1 initiates with the emergence of the stamen primordia which are composed of three concentric layers of cells: L1, L2 and L3. The L1 gives rise to the epidermis, the L2 to most of the anther cells types, including the sporogenous cells, and the L3 contributes to the vasculature and connective tissues. The founder cells of the microsporangia are single L2 archesporial cells, each of which divides periclinally to form a primary parietal cell subjacent to the L1 (epidermis) and the primary sporogenous cell facing inward (Canales et al. 2002). This

cell undergoes a small number of divisions to generate the microspore mother cells (MMCs), which will differentiate in microspores after meiosis. The primary parietal cells divide periclinally to form endothecial cells subjacent to the epidermis and secondary parietal cells, which divide again to generate a middle layer next to the endothecium and the tapetal cells adjacent to the MMCs (Scott et al. 2004). During phase 2, pollen grains differentiate, the anther enlarges and is pushed by filament extension to reach the stigma and finally dehiscence occurs (Goldberg et al. 1993).

Genetic and molecular analyses have already uncovered a number of important regulators of anther development, including anther cell differentiation, tapetum function, and microspore development (Ma 2005). Several genes have been identified that are critical for early anther development. They were detected first in the archesporial cells of the anther primordium, and successively their transcripts were restricted to the sporogenous cells and/or to the anther wall layers, fundamentally in the tapetal cells. Mutations of these genes lead to impair anther development from very early stages and produce male-sterile plants. Unfortunately, the expression of these genes was not only observed through anther development, they are also expressed in other floral or vegetative organs (Yang et al. 1999, 2003; Shiefthaler et al. 1999; Canales et al. 2002; Nonomura et al. 2003).

Genetic improvement of a crop plant could take advantage of the isolation and characterization of genes involved in the development of the floral reproductive organs and their use in transgenic plants to manipulate the flowering process and/or the resulting fruit. Genes that are expressed exclusively in male reproductive organs might be used to produce genetically engineered male-sterile plants with potential applications in breeding programmes (i.e. hybrid seed production) or to avoid in some cases undesirable horizontal gene transfer.

The fusion of cytotoxic genes to tissue-specific regulatory sequences can result in the programmed destruction of specific cell types during development. Cytotoxins used in this manner are powerful tools to study cell functioning and signalling within specific organs or tissues. Also, they have been used to study the tissue specificity and timing of regulatory sequences because cells/tissues ablation can be directly observed (Koning et al. 1992). Genetic cell ablation has been used to study male gametogenesis and as a biotechnological tool to produce male-sterility (Mariani et al. 1990; Nasrallah et al. 1991; Paul et al. 1992; Hird et al. 1993; Dennis et al. 1993; Roberts et al. 1995; Zhan et al 1996; Beals and Goldberg 1997; De Block et al. 1997; Lee et al. 2003). Basically, tapetum-specific promoters fused to cytotoxic genes were used for this purpose. Expression of the cytotoxic genes at specific stages of the anther development destroys tapetal cells, produces microspore abortion and to prevent normal pollen development. The cytotoxic gene must be expressed early to microspore stage to obtain efficient male-sterility (Roberts et al. 1995). In contrast to the destruction of tapetum cells, an approach to alter the programmed cell death (PCD) so as to allow the tapetal cells to live longer has been recently reported. The expression of *AtBI-1*, which suppresses Bax-induced cell death, in the tapetum at the tetrad stage inhibits tapetum degeneration and subsequently results in pollen abortion and male-sterility (Kawanabe et al. 2006).

F1 hybrids show a better performance than inbred lines regarding yield and vigour. To produce F1 hybrids it is necessary to devise a system for pollination control and engineered male-sterility seems to be one of the most reliable systems to prevent self-pollination. The ability to produce hybrid plants by genetic engineering would be greatly facilitated by the availability of a dominant male-sterility gene that could be introduced into different plants species.

The *PsEND1* (*Pisum sativum ENDOTHECIUM 1*) is a pea anther-specific gene that displays expression in the epidermis, connective and parietal cells of the anther primordium. Later on, *PsEND1* expression becomes restricted to the epidermis, connective, endothecium and middle layer until anther dehiscence. *PsEND1* is not expressed in other floral organ or vegetative tissues. Using a construct driving the *uidA* (GUS) gene controlled by the *PsEND1* promoter, we have proved that this promoter is fully functional in the anthers of transgenic *Arabidopsis*, tobacco and tomato plants, showing an expression pattern very similar to the one displayed by the *PsEND1* gene in pea (Gómez et al. 2004).

We report here the use of the *PsEND1* promoter region as a novel tool to produce engineered male-sterile plants. A chimaeric construct of the *PsEND1* promoter fused to a ribonuclease gene (*barnase*) from *Bacillus amyloliquefaciens* (Hartley 1988), produced selective genetic ablation of the cell lines involved in anther architecture at early stages of development and, subsequently, efficient male-sterility in both Brassicaceae (*Arabidopsis thaliana* and *Brassica napus*) and Solanaceae (*Nicotiana tabacum* and *Solanum lycopersicon*) transgenic plants.

Materials and methods

Plant material

Arabidopsis thaliana cv. Columbia (Nottingham *Arabidopsis* stock centre, UK) and oilseed rape (*Brassica napus* L.) cv. Drakkar (Centrum Grüne Gentechnik, Germany) plants were grown in cabinets at 21°C under long-day (16 h light) conditions, illuminated by cool-white fluorescent lamps (150 μmol quanta m^{-2} s^{-1}), in a (1:1:1) mixture of sphagnum:perlite:vermiculite. Tobacco plants (*Nicotiana tabacum* cv. Petite Havana SR1; IBMCP seed collection, Spain) were grown in a mixture (1:1) of sphagnum:vermiculite under 16 h

photoperiods in a greenhouse at 25°C (day) and 18°C (night). Supplementary lighting was provided by 400 W Phillips HDK/400 HPI [R] [N]. Tomato plants (*Solanum lycopersicon* cv. Micro-Tom; IBMCP seed collection, Spain) were grown under the same conditions in the greenhouse. Tobacco and tomato plants were irrigated with a Hoagland No. 1 solution (Hewitt 1966) supplemented with oligoelements. *Arabidopsis* and oilseed rape plants were irrigated with water and, once a week, with the same mineral solution.

Light microscopy (LM) and scanning electron microscopy (SEM)

Floral buds and stamens from *Arabidopsis*, oilseed rape, tobacco and tomato were freshly harvested and dissected using forceps and scalpel. Specimens were fixed in 4% *p*-formaldehyde in 100 mM Na-phosphate buffer at pH 7.0. After dehydration in a graded ethanol series the tissue was infiltrated and embedded in paraffin (Paraplast Plus, Sigma) or Historesin (Leica) as described previously (Gómez et al. 2004; Cañas et al. 2002). Sections, 2–4 µm thick, were obtained in an Ultracut E (Reichert-Jung) microtome and mounted onto slides with TESPA (3-aminopropil-trietoxisilane, Sigma) as adhesive. The sections were stained with 0.5% Safranin-O, 1% Alcian blue or 0.02% Toluidine blue and mounted with Merckoglas (Merck). A bright-field microscope was used for sample visualization and photography (Eclipse E600, Nikon). Light photographs of dissected flowers and stamens were obtained using a stereomicroscope (MZ8, Leica). For SEM, fresh floral buds and inflorescences were vacuum infiltrated with a FAE solution (ethanol:acetic acid:formaldehyde:water; 50:10:3.5:26.5, v/v/v/v) for 15 min and fixed with fresh solution during 4–16 h at 4°C. The samples were dehydrated in an ethanol series and critical point dried in liquid CO_2 (Polaron E300). The specimens were mounted on stubs and, if necessary, several outer whorl organs of individual flowers were removed manually. The inflorescences were then coated with gold–palladium (4:1) particles (200 nm) in a Sputter Coater SC500 (Baltec). SEM was performed with a Jeol JSM-5410 microscope (10 KV) and the images were processed using the programme Autobeam (ISIS, Oxford Instruments).

Chimaeric gene constructs

For DNA cloning, we followed the methods described in Sambrook et al. (1989). The promoter region of the *PsEND1* gene was previously cloned into the binary vector pBI101 where the 2731 bp promoter fragment was fused to the coding sequence of the β-glucoronidase (*uidA*, *GUS*) reporter gene (Gómez et al. 2004).

PsEND1::barnase chimaeric gene: primers Ribo1 (5′-*TAGGATCCC*GACCATGGCACAGGTTATC-3′) and Inhi2 (5′-*GCGAGCTC*TTAAGAAAGTTGATGGTGATG-3′) were designed based on the published sequence of *barnase* and *barstar* genes (Hartley, 1988) to amplify the *barnase–barstar* fragment previously cloned into the *Bam*HI site of pBluescript KS(+). Barnase is a very active ribonuclease. Even a low level of expression from aberrant promoter sequences or run-off expression from neighboring genes during manipulations in *E. coli* or *Agrobacterium* would have prevented the survival of the bacteria. Therefore, the *barstar* gene which encodes an inhibitor of barnase is included in the construct. The Ribo1 and Inhi 2 primers introduce *Bam*HI and *Sac*I restriction sites, respectively. The PCR resulting fragment was cloned into the pGEM-T Easy (Promega) and later released with the *Bam*HI and *Sac*I enzymes. The *Bam*HI-*Sac*I fragment was cloned by replacement of the *uidA* coding sequence into the binary vector pBI101 generating the pBI101-*PsEND1::barnase* construct (Fig. 1 upper case). The nos-Km plant selectable marker gene, which confer resistance to kanamicyn in transgenic plants, are also introduced in the T-DNA.

PsEND1::barstar chimaeric gene: Primers Inhi1 (5′-*GAGGATCC*ATGAAAAAAGCAGTCATTAAC-3′) and Inhi2 were used to amplify the *barstar* sequence (Hartley, 1988). These primers introduce *Bam*HI and *Sac*I restriction sites, respectively. The PCR resulting fragment was cloned into the pGEM-T Easy (Promega) and later released with the *Bam*HI and *Sac*I enzymes. The *Bam*HI-*Sac*I fragment was cloned by replacement of the *uidA* coding sequence into the binary vector pBI101 generating the pBI101-*PsEND1::barstar* construct (Fig. 1 upper case).

PCR analysis

For PCR analysis of the transgenic lines, genomic DNA was extracted following the method of Rogers and Bendich (1984). The presence of the *barnase* transgene was detected by PCR using the primers: Pro3 (5′-TGCTCACAACATAACCTTCCTTG-3′; nucleotides −26 to −4 of the *PsEND1* promoter sequence, GenBank accession number: AY324651) and Inhi3: (5′-CGCAGCCTTCCGCTTTCGC-3′; complementary sequence nucleotides 373–355 of the *barstar* gene, GenBank accession number: X15545). DNA sequences were analysed following the method described by Sanger et al. (1977) in an ABI PRISM 377 (Perkin Elmer) automatic sequencer.

Genetic transformation of *Arabidopsis thaliana*, *Brassica napus*, *Nicotiana tabacum* and *Solanum lycopersicon*

The *Arabidopsis thaliana* cv. Columbia transformation was performed by vacuum infiltration and selection of the plants resistant to kanamycin described by Bechtold et al. (1993), using the strain C58 of *A. tumefaciens*.

The kanamycin resistant plants were transferred to pots with vermiculite:perlite:peat (1:1:1) and grown in culture chambers at 22°C in long-day conditions until the seeds were collected. Control plants were also transformed only with the plasmid pBI101.

Spring oilseed rape (*Brassica napus* L.) cv. Drakkar was transformed with *Agrobacterium tumefaciens* strain C58C1 ATHV (Hood et al. 1986) following the protocol described by Damgaard et al. (1997) with minor modifications. After a precultivation period of 2 days, the hypocotyl explants were cut in 5 mm segments and incubated for 60 min in MS-CIM-G medium (MS combined with 20 g l^{-1} glucose, 1 mg l^{-1} 2,4-D, 0.1 mg l^{-1} IAA) containing 1×10^8 *Agrobacterium* cells ml^{-1}. Then the medium was replaced by fresh liquid MS-CIM-G medium and the co-cultivation was continued for 2 days at 22°C under continuous light. Selection was carried out on DKW medium (Duchefa) containing 20 g l^{-1} sucrose, 1 mg l^{-1} BAP, 0.01 mg l^{-1} IBA, 0.01 mg l^{-1} GA$_3$, 25 mg l^{-1} kanamycin and 500 mg l^{-1} carbenicillin. Regenerating plantlets were transferred to MS-medium (Murashige and Skoog 1962) with 250 mg l^{-1} carbenicillin and 25 mg l^{-1} kanamycin for further regeneration and rooting. Primary transformants (T1) were transferred into soil in small pots (multitrays) and acclimatised in the greenhouse. After 2 weeks, the plants were planted into 1.5 l pods with Floraton-3 soil. Slow release fertilizer (PlantoSan Compact, Aglukon) was given after 4 weeks. The light–dark period was 16 h light and 8 h darkness. For simulation of long-day conditions supplementary light was provided (Son-T Agro 400).

To produce transgenic tobacco plants, the cv. Petite Havana SR1 was used. The transformation was conducted with leaf disks in co-culture with *A. tumefaciens* (strain LBA4404) for 3 days at 24°C in the darkness using the MSS medium (Murashige and Skoog Medium 4.4 g l^{-1}, sucrose 2%, MES 100 mg l^{-1}, phytagel 3.5 g l^{-1}, pH 5.9) following the method described by Horsch et al. (1984) with the modifications of Fisher and Guiltinan (1995). After incubation, the leaf disks were transferred to plates with regeneration and selection medium (MSS medium with IAA 0.2 mg l^{-1}, 6-BAP 2.2 mg l^{-1}, carbenicillin 400 mg l^{-1} and kanamycin 100 mg l^{-1}), and the buds that appeared were transferred to the rooting medium (MSS medium with IAA 0.2 mg l^{-1}, carbenicillin 200 mg l^{-1} and kanamycin 100 mg l^{-1}). The regenerated plants were transferred to pots with peat:vermiculite (1:1) where they were kept until they produced seeds. The culture conditions during the whole process were 12 h of light and a temperature of 24°C.

To obtain transgenic tomato plants (cv. Micro-Tom), we followed the method of Ellul et al. (2003) that uses cotyledons from germinating seeds (12 days) as starting material, co-culture with *A. tumefaciens* (strain LBA4404) and the *nptII* marker gene to carry out selection of the transformants in a kanamycin medium.

Evaluation of the ploidy level in the tomato transgenic plants

Ploidy variations have been reported in both regenerated and transgenic tomato plants, affecting in most cases to the plant phenotype. The confirmation of the ploidy level in transgenic material is particularly important when a polysomatic tissue (e.g. cotyledons) is to be used as an explant source (Ellul et al. 2003). To avoid such a problem, we checked the ploidy level of our transgenic lines and only the diploid ones were transferred to the greenhouse and retained for male-sterility analysis. The ploidy level was evaluated by flow cytometry (Smulders et al. 1995). Nuclei were extracted from leaves of transgenic plants propagated in vitro or from plantlets obtained by germination of transgenic seeds. Leaf tissues (1 cm^2) were cut off into small pieces using a razor blade and 200 μl of nuclei extraction buffer (Partec) were added to homogenize. The mixture was filtered through a 50 μm nylon mess (Nyblot) and finally 800 μl of a DNA-stain solution containing 1 mg l^{-1} of DAPI (4,6-diamino-2-phenyl-indole) were added. The DNA of the isolated nuclei was measured by fluorescence with a Ploidy Analyser Partec PAS-II flow cytometer equipped with a mercury lamp. Data were distributed in a semi-logaritmic scale in which the 2C to 32C picks were assessed in abcises. The calibration was made with the 2C pick of young leaves from plantlets obtained by germination of diploid seeds of the cultivar Micro-Tom.

Pollen viability and germination tests

Pollen viability tests were performed in squeezed anthers using a solution of carmine acetate (0.5 g) in 45% acetic acid and diluted 1:1 with 30% glycerol. Viable pollen grains (stained in red) were observed and counted with an Eclipse E600 (Nikon) microscope.

Pollen germination tests were performed in a medium composed of 0.292 M sucrose, 1.27 mM Ca(NO$_3$)$_2$, 1.62 mM H$_3$BO$_3$, 1 mM KNO$_3$, 0.1 mM KH$_2$PO$_4$ and 0.5% agarose. The pollen grains were incubated on glass slides coated with the solid medium in the dark during 2 h at 25°C. The germinated pollen grains were observed and counted using a Diaphot (Nikon) inverted microscope.

Results

The *PsEND1*::*barnase* construct produces efficient anther ablation at early stages of development and consequently male-sterility in Brassicaceae spp. (*Arabidopsis thaliana* and *Brassica napus*) transgenic plants.

We fused a 2.7 Kb *PsEND1* gene regulatory fragment to the ribonuclease gene *barnase* (Hartley 1988) (Fig. 1 upper
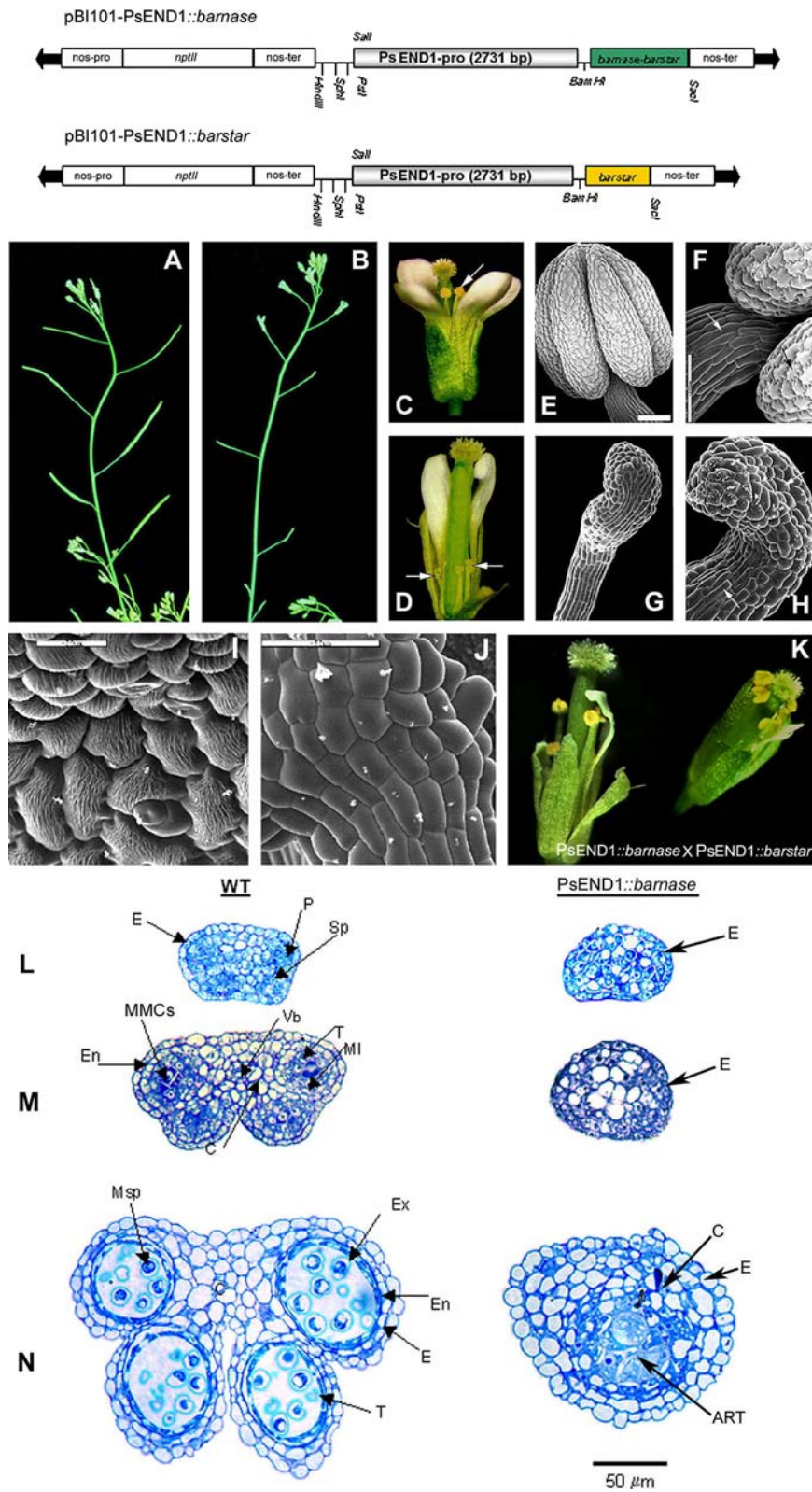
case). With this chimaeric construct we wanted to take advantage of the transcriptional activity of the *PsEND1* promoter through the anther development to arrest the anther differentiation at early stages (anther primordium), and subsequently to produce male-sterile plants. The chimaeric gene was introduced into *Arabidopsis thaliana* plants via in planta transformation. The analysis of 17 kanamycin resistant transformants showed that 16 were male-sterile when compared with wild-type or pBI101control plants. PCR analysis demonstrated the presence of the transgenes in all the primary transformants (data not shown). The transformants showed a phenotype essentially identical to untransformed control plants in vegetative and floral organs morphology, height, growth rate and flowering time. All the *PsEND1::barnase* plants failed to produce siliques and seeds and their floral

◀ **Fig. 1** Male-sterile *Arabidopsis thaliana* (Col) transgenic plants carrying the *PsEND1::barnase* chimaeric gene. *Upper case:* Chimaeric constructs used to produce engineered male-sterile plants (*PsEND1::barnase*) and to restore fertility (*PsEND1::barstar*). **A** Close-up of an *Arabidopsis* wild-type (WT) stem with normal seed pods (siliques). **B** Transgenic *Arabidopsis* plant showing the unpollinated ovaries that remain in the floral limbs. **C** WT flower showing normal anthers (*arrow*). **D** Flower (with two sepals and two petals removed) of a transgenic plant at the same stage showing the barnase effects on the anther development. The ablation of the anther is almost complete, with no pollen sacs being formed (*white arrows*). The filament does not undergo the lengthening process. **E** and **F** Scanning electron microscopy (SEM) analysis of an *Arabidopsis* WT stamen. The *black arrow* indicates the cell types present in the epidermis of the anther (toothed edges) and the *white one* indicates those of the filament (lengthened). **G** and **H** SEM analysis of a *PsEND1::barnase* stamen. The cellular types present in the filament can be observed in the hook-shaped structures (white arrows) but not those of the epidermis of the WT pollen sacs. **I** Epidermal cell types of a WT anther. The toothed cells are well ornamented. **J** Epidermal cell types of a *PsEND1::barnase* anther. The lengthened cells do not show ornamentation in their surface. **K** Flowers with restored anthers and filaments produced by crossing of fertile plants bearing the *barstar* gene (*PsEND1::barstar*) with a male-sterile line bearing the *barnase* gene (*PsEND1::barnase*). **L** Comparative study of the WT and *PsEND1::barnase Arabidopsis* anther development. Transverse section of a wild-type and a *PsEND1::barnase* anther at stage 3 (defined by Sanders et al. 1999). Arquesporial cells have differentiated parietal (P) and sporogenous (Sp) cells in the wild-type anther primordium. The epidermis (E) has been differentiated from the L1 layer. The *PsEND1::barnase* anther primordium shows an oval shape like an undifferentiated anther primordium where the defined four regions of mitotic activity are not observed. **M** Comparative transverse sections of a wild-type and *PsEND1::barnase* anther at stage 5. Four clearly defined locules are established in the WT anther. Now all the anther cell types are present: connective (C), endothecium (En), middle layer (MI), tapetum (T), the vascular region (Vb) and the microspore mother cells (MMCs). In the *PsEND1::barnase* anther there are not defined locules and cell lineages are still undifferentiated. **N** Transverse section of a wild-type and a *PsEND1::barnase* anther at stage 9. All the anther tissues are already formed in the WT anther. The microspores (Msp) can be observed into the pollen sacs and the tapetum (T) is not yet degenerated. At a similar stage, the morphology of the *PsEND1::barnase* anther is quite different to those showed by the WT and it is only possible to see a unique pollen sac with a mixture of abnormal reproductive (ART) and non-reproductive tissues

organs senesced and felt off with the exception of the carpel that remained attached to the pedicel (Fig. 1A and B). In the 16 male-sterile transformants the anther development was arrested at early stages and the formation of the pollen sac and pollen grains also failed. Hook-shaped structures were formed in the place of normal anthers. These structures never reached the stigma because the filament did not elongate (Fig. 1C and D). The cross-pollination of the transformed plants with pollen from wild-type plants produced normal fruits and seeds. These results indicate that female fertility was unaffected in the *PsEND1::barnase* plants. Segregation studies indicated that the inheritance and stability of the transgenes in the progeny were maintained in the next generation (Table 1).

The malformed stamens of the *PsEND1::barnase* plants were characterized by scanning electron microscopy (SEM) to identify defects in anther morphology and/or in the different cell types. The four-lobed structure of a wild-type *Arabidopsis thaliana* anther is shown in Fig. 1E and F. In contrast, stamens of the *PsEND1::barnase* plants consist of a short filament which terminates in a hook-shaped tip in the place of a four-lobed anther (Fig. 1G and H). The cell types mainly present in the epidermis of the *PsEND1::barnase* plants were elongated cells without ornamentation that reminded the cell types of an anther filament (Fig. 1I, J). However, the toothed cells with ornamentation present in the epidermis of the wild-type pollen sacs were absent from these structures (Fig. 1F, H–J).

To determine the histological differences and to identify the cellular types affected by the cytotoxic gene, we analysed both wild-type and *PsEND1::barnase* transverse sections at different developmental stages. At early stages of development (stages 3–4; Sanders et al. 1999), it was already possible to observe differences among both types of anthers. In the wild-type anther, we observed four regions containing the parietal and sporogenous layers, while we never observed these defined four regions of mitotic activity in the *PsEND1::barnase* anther primordium (Fig. 1L). Later on, the wild-type anther has established four clearly defined locules. The locules contained all the cell types of the different anther walls and the microspore mother cells (MMCs). However, at a similar stage, the transgenic anther does not show before mentioned cell types (Fig. 1M). When the wild-type anther shows all the differentiated tissues and tetrads into their pollen sacs (stage 9), the *PsEND1::barnase* structures lacked of a bilateral symmetry shape and contained an outer layer of epidermal cells that surrounded several rows of parenchymal cells and a single pollen sac. This pollen sac included abnormal reproductive and non-reproductive tissues (Fig. 1N).

An additional advantage related to the use of the *barnase* gene is the possibility to restore male fertility of the genetically engineered male-sterile plants by means of crosses with

Table 1 Segregation analysis of the male-sterile phenotype in four *Arabidopsis* transgenic lines

| Line | No. of analysed plants (T) | Phenotype | | | Segregation | χ^2 | Signification level (*P*) | No. of copies |
		S[a]	F[b]	(%)[c]				
B_2	28	4	24	14.0	1:1	14.285	0	–
B_8	48	21	27	43.7	1:1	0.750	0.39	1
B_{14}	21	10	11	47.6	1:1	0.048	0.83	1
B_{21}	29	19	10	65.5	3:1	1.38	0.24	2

The results obtained for the transgenic lines B_2, B_8 and B_{14} were the expected (relation 1:1 of sterile plants with respect to fertile plants in the F1 generation) after crossing a wild-type plant with a hemizygotic plant containing a transgene inserted in an unique *locus*. Progeny segregation data (3:1) of the transgenic line B_{21} suggest transgene insertions in two different *loci*.

[a]Sterile plants obtained.

[b]Fertile plants obtained.

[c]Percentage of sterile plants with respect to the total of analysed plants.

transgenic plants carrying the *barstar* gene, an inhibitor of the barnase activity (Mariani et al. 1992). To demonstrate the reversibility of the barnase–barstar system when used under the control of the *PsEND1* promoter, transgenic plants containing the *PsEND1::barstar* chimaeric gene were generated. After crossing with the *PsEND1::barnase* plants, fertile plants with restored anthers were generated as expected (Fig. 1K). The restored anthers showed viable pollen grains which produced normal silliques and seeds after fertilization.

We also transformed oilseed rape plants (*Brassica napus*) with the chimaeric *PsEND1::barnase* construct to find out whether the pea *PsEND1* gene 5′ regulatory region could function in a distantly related and agronomically important crop, leading to male-sterility. We obtained 14 primary transformants showing abnormal stamen phenotypes. The stamens showed short filaments and abnormal anthers (Fig. 2A, B, D, E). The absence of pollen grains into the transgenic anthers was corroborated by light microscopy (data not shown). Like in the transgenic male-sterile *Arabidopsis* plants, the unpollinated carpels remained attached to the floral pedicel and finally senesced and felt off (Fig. 2F), in contrast with the untransformed carpels that were fertilized and formed normal fruits and seeds (Fig. 2C). Backcrosses of the male-sterile phenotypes with untransformed pollen to maintain these transgenic lines were successful (data not shown). The PCR analysis demonstrated the presence of transgenes in all the primary transformants selected (Fig. 2G).

The *PsEND1::barnase* construct also produces efficient anther ablation and male-sterility in Solanaceae spp. (*Nicotiana tabacum* and *Solanum lycopersicon*) transgenic plants.

The chimaeric *PsEND1::barnase* gene was also introduced into both Solanaceae species to prove whether the chimaeric construct could produce to male-sterility and to determine how the *barnase* gene expression affects the tobacco and tomato anther development.

In tobacco, three independent lines of transformants were selected for further analysis. All the transgenic plants were identical to the untransformed control plants with respect to growth rate, height, morphology of vegetative and floral organs and flowering time. The resulted transgenic flowers showed abnormal stamens with short filaments and malformed anthers showing an arrowhead shape (Fig. 3D). In a wild-type plant at the same stage of development, the anther dehiscence is completed and the pollen grains are fertilizing the stigma (Fig. 3A).

The morphological differences among wild-type and *PsEND1::barnase* anthers were observed by scanning electron microscopy (SEM). The characteristic paddle-like anther shape and the pollen grains into the pollen sacs of a wild-type anther are shown in Fig. 3B and C, in contrast with the arrowhead shape with trichomes adopted by the transgenic anther (Fig. 3E). Also, no pollen grains were found into the transgenic pollen sacs (Fig. 3F).

Paraffin cross-sections showed that the different tissues involved in the anther architecture are altered and collapsed, especially the connective tissue (Fig. 3H) and no pollen grains were detected into the abnormal pollen sacs when compared with a wild-type anther (Fig. 3G).

We also transformed tomato (cv. Micro-Tom) plants with the chimaeric construct carrying the *barnase* gene under the control of the *PsEND1* promoter. We generated 72 primary transformants from which we selected 25. To avoid artefactual results, the ploidy level of the transgenic lines selected was investigated, 24 were diploid and one resulted tetraploid. The PCR analysis demonstrated the presence of transgenes in the diploid lines selected (Fig. 4I). The transgenic plants were grown in the greenhouse to produce flowers. The development of these plants was normal when compared with non-transformed plants, but the flowers were male-sterile showing phenotypes with different degrees of severity. The anthers with a more severe phenotype showed an altered morphology and the pollen sacs were collapsed bearing necrotic tissues. The carpel was not covered by the anthers (staminal cone) like happens in the wild-type flowers (Fig. 4A and C or 4B and D). Two transgenic lines showing weak

male-sterile phenotypes, contained several pollen grains into the malformed pollen sacs, but the germination analysis of these pollen grains showed that they were no viable (data not shown).

Both wild-type and *PsEND1::barnase* transverse tomato anther sections were examined at different developmental stages to identify the cellular types affected by the cytotoxic gene and to investigate how the *PsEND1::barnase* gene expression affects the tomato anther development. The four corners of wild-type anther primordium are well defined at the first stage showed in Fig. 4E. The parietal and sporogenous cells have been differentiated from archesporial cells and the epidermis, connective and vascular tissues were present. At a similar stage, the *PsEND1::barnase* anther primordium was surrounded by an epidermal layer but

the four typical regions of the anther primordium are not well defined (Fig. 4E). When the wild-type anther begins to differentiate all the wall layers, including the endothecium, in the transgenic anther it was impossible to distinguish the above mentioned cell types (Fig. 4F). At the next observed stage anthers showed the four locules and the tetrads of microspores have been established. The microspores are within each locule surrounded by the callose walls and the tapetum. In contrasts, the *PsEND1::barnase* anther at a similar stage of anther development showed different features: the connective tissue has collapsed, the pollen sacs have an abnormal shape and contain possible tetrads surrounded by callose walls and tapetum (Fig. 4G). Finally, while in the wild-type anther has occurred the disruption of the connective tissue separating the pollen sacs, the transgenic anther
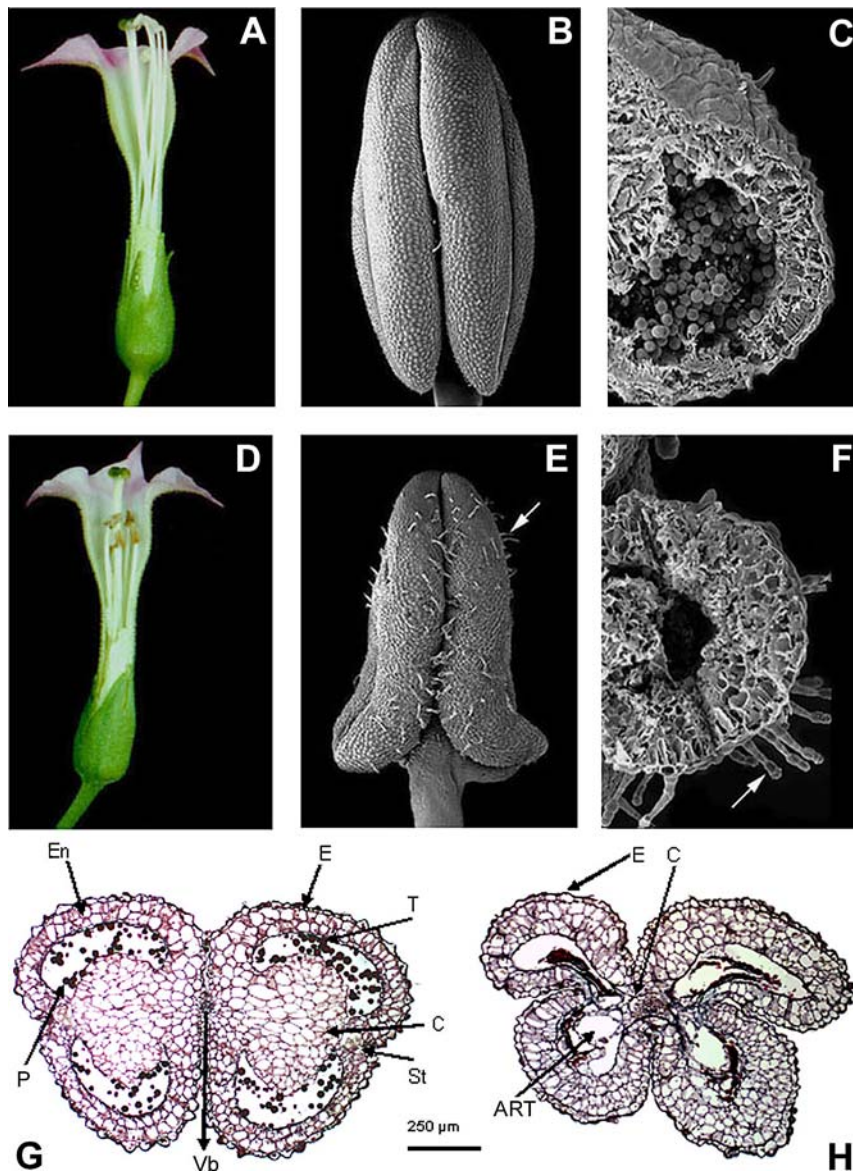
Fig. 3 Tobacco (*Nicotiana tabacum*, cv. Petite Havana SR1) *PsEND1::barnase* transgenic plants. **A** Wild-type tobacco flower after anthesis. The anthers reach the stigma and release the pollen grains. **B** Scanning electron microscopy (SEM) analysis of a tobacco anther from untransformed plants at stage 3 of anther development (Koltunow et al. 1990). The anther takes its characteristic paddle-like shape. **C** SEM analysis of a wild-type tobacco pollen sac with mature pollen grains at stage 3 of anther development. **D** PsEND*1::barnase* tobacco flower after anthesis. The stamens show reduced filaments and the anther shape resemble an arrowhead. **E** SEM analysis of a *PsEND1::barnase* anther at stage 3 of anther development. The anther shows an arrowhead shape. Note the large number of trichomes in their epidermis (white arrow) and the collapsed pollen sacs. **F** SEM analysis of the *PsEND1::barnase* pollen sacs without pollen grains at stage 3 of anther development. **G** Transverse section of a WT tobacco anther at stage 3 of anther development (defined by Koltunow et al. 1990). All anther tissues are present: the epidermis (E), the endothecium (En), the connective (C), the vascular bundle (Vb), the tapetum (T) begins to degenerate and the pollen grains (P) are visible. **H** Transverse section of a *PsEND1::barnase* tobacco anther at stage 3 of anther development. Note that the connective (C) tissue is almost absent producing the abnormal anther shape and the different cellular layers conforming the pollen sacs, like the epidermis (E) and endothecium (En) are altered in their cell number. A mixture of abnormal reproductive tissues (ART) was observed into the pollen sacs

does not shows the typical signals of anther dehiscence. The wild-type pollen sacs contained the pollen grains whereas the *PsEND1::barnase* locules contained abnormal reproductive tissue (Fig. 4H).

The female fertility of tobacco and tomato male-sterile plants was unaffected. Backcrosses of the *PsEND1::barnase* plants with wild-type pollen to maintain these transgenic lines were successful (data not shown).
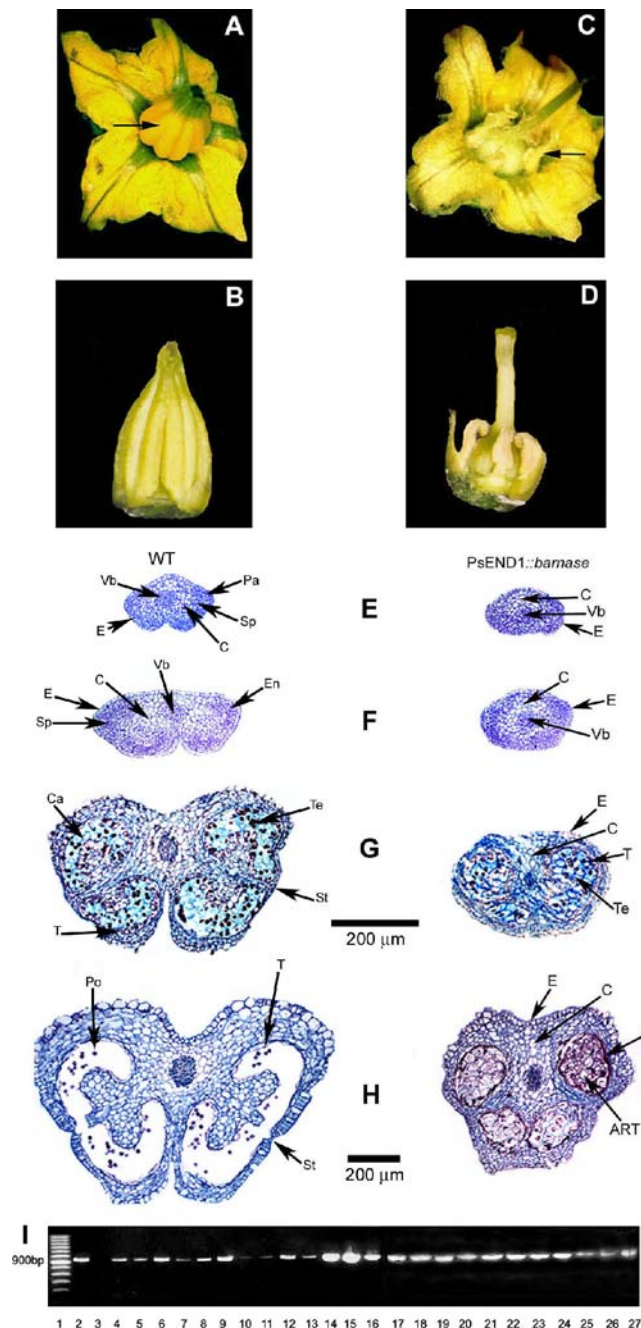
Fig. 4 Tomato (*Solanum lycopersicon*, cv. Micro-Tom) *PsEND1::barnase* transgenic plants. **A** Wild-type tomato flower at anthesis. The stamens surround the style and form the staminal cone (black arrow). **B** Wild-type staminal cone. **C** *PsEND1::barnase* flower at anthesis. The ovary and style are visible by the anthers absence. **D** The absence of the staminal cone being visible the style and ovary. **E** Transverse section of a wild-type anther: Parietal (Pa) and sporogenous cells (Sp) are already differentiated. The epidermis (E), connective (C) and vascular bundle (Vb) are also present. In contrast, the four typical regions of the anther primordium are not well defined in the transverse section of the *PsEND1::barnase* anther. The epidermis, connective and vascular bundle are visible at this stage. **F** Transverse wild-type anther section, the wall layers begin to be formed. The endothecium (En) and sporogenous cells (Sp) are visible. In the transverse section of a *PsEND1::barnase* anther the four typical regions are not properly formed and the anther wall layers are absent. **G**: Transverse section of a wild-type anther, the tetrads of microspores (Te) into the locules surrounded by callose walls (Ca) and the tapetum (T) are visible. In the *PsEND1::barnase* anther the connective is collapsed (C) and several structures that look to be similar to tetrads are visible into the pollen sacs surrounded by the callose wall and tapetum. **H** Transverse section of a wild-type anther. Residual tapetum can be seen into the pollen sacs (T), there is a degradation of the connective tissue in the stomium region to communicate the locules, and pollen grains (P) are visible into the pollen sacs. In the transverse section of a *PsEND1::barnase* anther a mixture of abnormal reproductive tissues (ART) can be seen into the pollen sacs. **I** PCR analysis to identify the chimaeric *PsEND1::barnase* gene (868 bp) in the different transgenic lines. *Lane 1:* 100 bp ladder, *lane 2:* plasmid (control +), *lane 3:* non-transgenic plant (control −), *lanes 4–27:* transgenic plants used for further analysis in the greenhouse

Discussion

The anther plays an important role in crop production because it is responsible for carrying out the male reproductive processes necessary for generating the seeds that will produce the next plant generation. Gene expression studies during anther cell differentiation had lead to develop different genetically engineered systems for male fertility control. Most of these systems are based on the ability to inhibit pollen viability by interfering with critical functions in the tapetum, a glandular cell layer surrounding the anther locule that is responsible for provide nutrients, cell-wall components and enzymes to the developing microspores (Bedinger 1992). Genetic ablation techniques have been used as a tool to produce male-sterility, a desirable feature in the process of obtaining hybrid seeds (Mariani et al. 1990; Denis et al. 1993; De Block et al. 1997; Lee et al. 2003). Also, the fusion of cytotoxic genes to anther-specific promoters has been used to study male gametogenesis by visualization of the developmental events in the absence of specific cells (Mariani et al. 1990; Paul et al. 1992; Hird et al. 1993; Roberts et al. 1995; Beals and Goldberg 1997).

We have used the early anther-specific promoter *PsEND1* fused to the cytotoxic *barnase* gene to produce male-sterility in two Brassicaceae (*Arabidopsis thaliana* and *Brassica napus*) and two Solanaceae (*Nicotiana tabacum* and *Solanum lycopersicon*). Also, we have used the chimaeric *PsEND1::barnase* gene to study the timing of the regulatory *PsEND1* sequences analysing the barnase effects through the anther development.

Progeny analysis of the transgenic plants showed that, under greenhouse conditions, the expression of the *PsEND1::barnase* construct does not affect the vegetative and floral development, so confirming the anther specificity of the *PsEND1* promoter region previously observed by means of *uidA* expression studies (Gómez et al. 2004). The potential biotechnological applications of the *PsEND1* promoter largely depends on both its spatial and temporal expression pattern since the ectopic expression of the cytotoxic agent would damage other plant tissues and organs, decreasing the agronomic value of hybrid plants. The common characteristic of the *PsEND1::barnase* plants was the failure in producing fruits and seeds, as a consequence of the anther defects. Macroscopically, the *Arabidopsis*, oilseed rape and tobacco stamens showed reduced filament size and anthers that reminded "hook-shaped" or "arrowheads" structures. The tomato stamens were practically absent, being visible the style and ovary of the carpel. The formation of short filaments is commonly associated with male-sterility or reduced fertility (e.g. Theis and Robbelen 1990; Mariani et al. 1990; Denis et al. 1993; Worral et al. 1992). All the transgenic flowers were fully female fertile and normal fruits containing seeds were obtained when the transgenic plants were cross-pollinated with pollen from untransformed plants. These results showed that the *PsEND1::barnase* plants were male-sterile but their pistils were able to recognize and transmit pollen normally.

The effect of the *barnase* cytotoxic gene through *Arabidopsis* and tomato anther development was studied at histological level. Microscopic studies confirmed the early and specific expression of the *PsEND1* promoter. The histological differences were clearly observed from very early stages of the *Arabidopsis* and tomato anther development. When the wild-type anther primordium are composed by four regions containing the parietal and sporogenous cell layers, in both *PsEND1::barnase* primordia we never observed the four typical regions of mitotic activity. The effects of the cytotoxic gene were visible through the anther development, leading to abnormal anthers with no viable pollen grains.

The *PsEND1::barnase* gene is quite different to other chimaeric genes previously used in similar approaches to produce male-sterility by genetic anther ablation. The *PsEND1* gene begins to be expressed in the pea anther primordium in the epidermis, connective and parietal cell layers that will differentiate the endothecium and middle layer. *PsEND1* expression can be detected in these tissues until the anther dehiscence, but it was never observed in the tapetum or in the microsporocytes (Gómez et al. 2004). The ablation of the target cells correlated with the failure of neighbouring cells to differentiate normally. The *PsEND1::barnase* gene could be ablating the parietal cells, and therefore affecting the differentiation of the contiguous sporogenous cells within the developing microsporangial territories. This fact, could lead to impair microsporogenesis development before the microspore mother cells (MMCs) differentiation. At the same time, the expression of the cytotoxic gene in the connective tissue produces their ablation, resulting in the collapse of this anther tissue. The immediate consequence of this fact is a reduction in size of the stamens and morphological changes in the transgenic anther.

In order to improve commercially available varieties, breeders generate hybrids to increase crop yields and production efficiencies. Traditionally, the hybridization of autogamous plants (e.g. oilseed rape and tomato) has been done by manual emasculation followed of fertilization with the selected pollen. However, emasculation is an intensive labour process and does not guarantee 100% sterility. Engineered male-sterility seems to be the most reliable system to prevent self-pollination in both species. The oilseed rape is a 30% allogamous and a 70% autogamous; for this reason, it is necessary to devise a system for pollination control to produce F1 hybrids. As we show here, the *PsEND1* promoter works as an efficient tool to produce male-sterile oilseed rape plants in combination with the ribonuclease barnase. No fruit and seeds were produced in any case, demonstrating the efficiency of the *PsEND1::barnase* construct in this crop

species. Tomato is a major crop in many parts of the world. There are many male-sterile systems available in tomato; however they are generally not used on commercial scale because of difficulties with maintaining pure male-sterile lines. The production of male-sterile plants in tomato using the *PsEND1::barnase* construct showed also high efficiency.

In summary, the tightly regulated *PsEND1::barnase* gene could be useful to provide valuable information with respect to cell function, to cell lineage relationships and to interactions between developing anther cell types. Here, we have showed that the *PsEND1* promoter is fully functional not only in model species (*Arabidopsis*, tobacco) but also in plants of agronomical interest (oilseed rape, tomato); therefore, it could serve as a novel tool to efficiently generate male-sterile plants and to prevent undesirable gene flow between sexual compatible plant species.

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