Isolation and characterization of a flowering plant male gametic cell-specific promoter[1]

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Received 8 January 2003; revised 17 March 2003; accepted 24 March 2003

First published online 8 April 2003

Edited by Marc van Montague

Abstract **Flowering plant male gametic cell-specific gene expression has been reported recently but the regulatory elements controlling specificity of such genes expressed in generative cell and sperm cells have not been identified and studied. Here, we report the 0.8 kb promoter sequence upstream of the start of the transcription site of the generative cell-specific gene, *LGC1*, sufficient to regulate the expression of reporter genes in a cell-specific manner. In addition, the diphtheria toxin A-chain-(DT-A)-coding region under the control of the *LGC1* promoter sequence confirmed unequivocally the lack of *LGC1* expression in vegetative tissues. Transgenic tobacco plants carrying the *LGC1-DTA* construct showed normal phenotype except for anthers of these plants that contained sterile and aborted pollen. Truncation and internal deletion analysis of the *LGC1* promoter identified −242 bp as the minimal sequence necessary for male gametic cell-specific expression. In addition, a regulatory sequence required for determining generative cell-specific expression of *LGC1* was identified. Deletion of this regulatory sequence led to loss of the generative cell specificity resulting in activation of this promoter in other tissues where it is normally repressed. Therefore, male gametic cell specificity of the *LGC1* gene seems to be regulated by factors that suppress its activation in other plant cells. This is the first report of a male gametic cell-specific promoter, hence can be used as a novel tool in molecular analyses and experimental manipulation of flowering plant spermatogenesis and fertilization.**

Key words: Male gamete; Pollen; Plant reproductive biology; Generative cell; Promoter

1. Introduction

In plants, differentiation of vegetative and generative cells as a result of pollen mitosis-I is a unique event in the development of male gametophyte and the gametic cells. Pollen mitosis-I is a highly asymmetric division, which marks the generative cell for a developmental fate very different from that of the vegetative cell. While the vegetative cell aids the process of fertilization through pollen germination and pollen tube growth, the generative cell divides to form male gametes. Until recently, the generative cell was thought to play a passive role in pollen differentiation, being dependent upon a functional vegetative cell for its development and transport through the pollen tube [1]. The highly condensed state of chromatin in the generative cell and lack of clones specific to generative cell from mature pollen cDNA libraries led to the view that the generative cell was transcriptionally quiescent [2]. However, it is reasonable to think that the generative cell is likely to express genes that are involved in the second mitotic division and possibly in the process of fertilization. The demonstration of the presence of translatable mRNAs in generative cells [3] led to identification of generative cell-specific clones [4,5]. *LGC1* is one such clone, the expression of which can be localized exclusively in the male gametic cells of lily [4]. Protein prediction models suggest that the LGC1 protein might be associated with the plasma membrane of the generative cell and hence play a role in cell-to-cell recognition. Northern hybridization and reverse transcription-polymerase chain reaction (RT-PCR) analysis confirmed the generative cell specificity of this clone. Further, in situ hybridization experiments clearly showed that the transcription of *LGC1* in the generative cell is initiated soon after its movement into the cytoplasm of the vegetative cell. Immunolocalization studies showed that the LGC1 protein is localized on the plasma membrane of the generative cell [5].

Previously, several pollen-specific genes have been described and the promoters of many pollen-specific genes have been isolated and characterized [6,7]. These genes belong to a class that are likely to be expressed only in the vegetative cell of pollen. The vegetative cell-specific promoters have served as important tools in studying the development of the male gametophyte, particularly the regulation of genes in the vegetative cell following asymmetric division [1,8]. Promoter analyses of these vegetative cell-specific genes have led to identification of sequence motifs that are conserved in various pollen promoters. However, no regulatory sequences and mechanisms have been reported to explain generative cell-specific gene expression.

We therefore examined regulation of *LGC1* expression in generative cells by characterization of its promoter region by using reporter genes such as GUS and *GFP*. The production of male sterile but otherwise normal transgenic plants carrying *LGC1* promoter-DTA fusion constructs provided unequivocal confirmation of the generative cell specificity of *LGC1* promoter. For determining the nature of *cis*-regulatory elements that direct generative cell-specific gene expression, series of 5′ deletion promoter-*GFP* fusion constructs were tested in transient expression assays. Here, we show that the *LGC1* gene

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[1] GenBank accession number: AY207012.

0014-5793/03/$22.00 © 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00335-1

promoter contains a *cis*-regulatory element, the removal of which leads to loss of generative cell specificity of expression. Our results suggest that cell specificity of *LGC1* in generative cells is controlled by regulatory factors that repress its expression in other plant cell types.

2. Materials and methods

2.1. Isolation of the LGC1 promoter

To clone the 5′ flanking sequence of the *LGC1* gene, a modified PCR strategy, 'uneven PCR' [9] was used. This method that utilizes a combination of gene-specific primer and an arbitrary oligonucleotide is modified to utilize two different annealing temperatures that favor the amplification of specific products. Genomic DNA from *Lilium longiflorum* was used as template for the 'uneven' PCR protocol. Three gene-specific nested primers L13B (CACGTCACTAAGGTCTGATAAT), L13E (TCCGTCACTAAGTGGTCTGATAAT) and L13F (TCCGTAACCATACAGAAGAGAACGC) correspond to the *LGC1*-coding region. The arbitrary primers used were 10-mers from OPA and OPB kits supplied by Operon Technologies (Alameda, CA, USA). For the first round of uneven PCR, 20 ng DNA was used along with 0.25 μM of gene-specific primer (GSP-1) and 0.05 μM of 10-mer primer, 200 μM dNTP (final concentration) and two units of Amplitaq (Stratagene) in a final volume of 40 μl. Cycling conditions of uneven PCR were 94°C for 1 min, then for cycle 1, 94°C for 30 s, 55°C for 1 min, 72°C for 1 min; for cycle 2, 94°C for 15 s, 42°C for 1 min, 72°C for 1 min; cycles 1 and 2 were then repeated two times. Then for cycle 7, 94°C for 15 s, 57°C for 30 s; 72°C for 30 s; for cycle 8, 94°C for 15 s, 45°C for 30 s, 72°C for 30 s; cycles 7 and 8 were repeated 20 times. Finally the samples were held at 72°C for 5 min.

0.5 μl of the products from the first round of PCR were used as templates for the second round of uneven PCR. In the second round of uneven PCR all the components were the same as in the first round except that a nested specific primer (GSP-2) was used. The cycling conditions for the second round of reaction were: 94°C for 1 min; then for cycle 1, 94°C for 15 s, 57°C for 30 s, 72°C for 30 s; for cycle 2, 94°C for 15 s, 45°C for 30 s, 72°C for 30 s; cycle 1 and 2 were repeated 20 times; finally samples were held at 72°C for 5 min. Following electrophoresis of half of the PCR, gel was blotted and probed with a *LGC1* cDNA. PCR products that showed a positive signal were cloned into pGEM T-easy vector (Promega) following electrophoresis of the remaining PCR product and elution from agarose gel. Cloned fragments were sequenced and sequences aligned with *LGC1* cDNA using CLUSTAL W (Baylor College of Medicine) alignment program.

2.2. Plasmid constructs

Standard molecular biology procedures [10] were followed. A 946 bp *LGC1* promoter sequence including 140 bp of *LGC1* cDNA (the whole original uneven PCR fragment) was spliced to the *GFP* gene in pBluescript to produce the p*LGC1-GFP* construct. This fragment was originally cloned in pGEM T-easy vector as a PCR product. It was later cloned into pBluescript SK+ at the *Eco*RI site after removal from T-easy with *Eco*RI. This fragment was then excised with *Hin*dIII and *Xba*I from pBluescript and fused with a *Xba*I and *Eco*RI GFP-nos fragment in pBluescript SK+ at *Hin*dIII and *Eco*RI.

For pLGC1:uidA the construct was cloned into pBI101 (Clontech). The plasmid pBI101 contains a promoterless *GUS* [11] cassette fused to the nopaline synthase polyadenylation region in the binary vector pBIN19 [12]. For the *LGC1*-DTA construct, *GUS* gene in plasmid pBI101 was replaced with the DTA gene [13] to form the plasmid p*LGC1*-DTA. Deletion fragments were amplified by PCR using *LGC1* gene-specific primers and cloned in pGEM T-easy vector for sequencing. Following sequence verification these constructs were spliced to the *GFP* gene in p*LGC1-GFP* by replacing the *LGC1* promoter.

2.3. Transient expression and stable transformation

The regulation of expression of the *LGC1* gene by putative promoter sequences in the 5′ fragment was initially assayed in a transient expression using *GFP* reporter gene. Mature pollen and petals of *L. longiflorum* Thumb., and *Allium* epithelium were transformed via microprojectile bombardment. *Allium* was used because of its taxonomic relatedness to *Lilium* and the convenience of this material for microscopic detection of GFP expression. A CaMV 35S-*GFP* construct was used as control. For bombardment, 5–10 μg of test plasmid was coprecipitated onto 1.25 mg of gold microprojectiles in the presence of calcium chloride and spermidine (free base) in a 45 μl volume. 2–5 μl of this particle suspension was introduced into 25 mg of pollen or appropriate quantities of other tissues using a Bio-Rad He-particle gun. Prior to bombardment, pollen was hydrated in pollen germination medium+ (300 mM sucrose, 1.6 mM HBO_3, 3 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.8 mM $MgSO_4 \cdot 7H_2O$, 1 mM KNO_3, 25 mM MES–KOH, pH 5.9) and spread over a nylon membrane on agar plates. The epithelium and petal tissue was placed on agar solidified MS medium (MS medium supplemented with 30 g/l sucrose). After bombardment the tissues were incubated for 3–16 h at 25°C. The transformed tissues were then observed under a fluorescence microscope for *GFP* expression. p*LGC1*-gus and p*LGC1-DTA* were introduced into tobacco through *Agrobacterium*-mediated transformation [14].

2.4. Histochemical detection of GUS activity

Whole root segments, leaf sections and pollen were incubated in 300 μl of reaction buffer (50 mM sodium phosphate buffer pH 7.0, 1 mM Na_2-ethylenediamine tetraacetic acid (EDTA), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.1% Triton X-100 and 1 mM X-gluc) and 37°C for 4–5 h (anther sections and pollen) or 12–16 h (vegetative tissues). Stained sections were observed using a light microscope.

3. Results and discussion

3.1. LGC1 promoter sequence shows generative cell specificity in transient and stable transformation assays

A blast-N search of a 0.8 kb *LGC1* 5′ sequence did not reveal any significant homology to any known nucleotide sequence in the GenBank database indicating that this is a novel genomic sequence. A detailed analysis of this fragment, however, revealed several promoter and enhancer elements that are common to other eukaryotic promoters. The presence of a typical TATA box sequence TATAAA categorizes this promoter as a TATA box-containing promoter. This sequence is located at −26 in relation to the predicted transcription initiation site in the *LGC1* 5′ flanking region. Another motif in this *LGC1* 5′ flanking region is an enhancer element called the GC box located at position −160 in relation to the transcription initiation site. The GC box has the consensus sequence GGGCGG and is found at about position −110 in many eukaryotic promoters [15]. A sequence with resemblance to either a G box (CACGTG) or a T box (AAGCTT), and made up of an ACGT core sequence is located at position −135. The ACGT core sequence is the target sequence for bZIP DNA binding regulatory proteins, identified in diverse eukaryotic species ranging from higher plants to mammals [16,17].

Following bombardment with the *LGC1-GFP* construct, *GFP* expression was detected in the mature pollen only. No expression was detected either in petals or in the onion epithelium. Expression of *GFP* in the mature pollen was first detected in the 3–4 h following bombardment. Very bright expression of the *GFP* could be visualized in the generative cell (Fig. 1A) while bombardment with *CaMV* 35S-*GFP* construct resulted in *GFP* expression in petals and epithelium (Fig. 1E and F) but not in pollen. The inability of the *CaMV 35S* promoter to drive pollen-specific expression has been reported earlier [7]. The transformation frequency of *GFP*-expressing pollen was considerably lower compared to those reported for vegetative cell-specific promoters such as *LAT 52*. For generative cell-specific expression, the coated

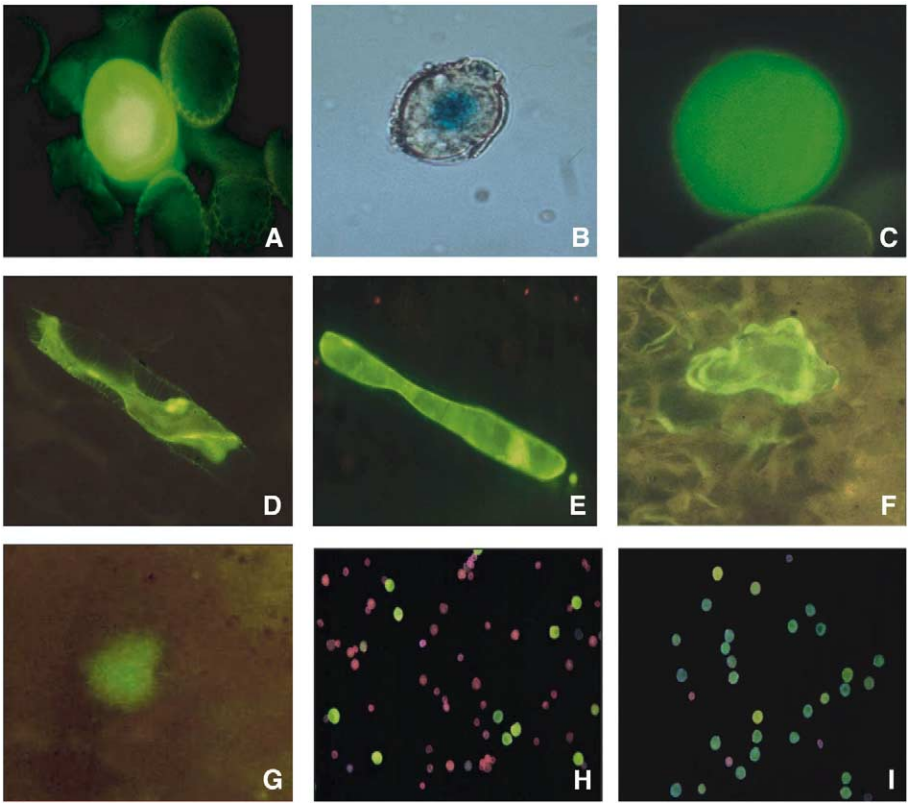

Fig. 1. Expression of the LGC1 promoter in transient and stable transformation experiments. A: Mature lily pollen bombarded with the *LGC1-GFP* construct showing *GFP* expression in generative cell. B: Pollen from a *LGC1*-GUS transgenic tobacco plant showing GUS activity in the generative cell of pollen. C and D: *GFP* expression in lily pollen and onion epithelial cells following bombardment with the truncated *LGC1* (−50)-*GFP* fusion construct. E and F: GFP expression in onion epithelium and lily petals following bombardment with the 35S-*GFP* construct. G: *GFP* expression in lily petal tissue following bombardment with the truncated *LGC1* (−50)-*GFP* fusion construct. H: Pollen ablation in the *LGC1-DT-A* construct carrying transgenic tobacco plant. Red propidium iodide fluorescence indicates sterile ablated pollen while green fluorescence shows viable FCR positive pollen. I: FCR positive viable pollen from wild-type tobacco plant.

particles need to penetrate the generative cell in the pollen. Since the small generative cell is enclosed within the larger vegetative cell, the probability of a coated particle entering the generative cell is low compared to those retained in the larger vegetative cell.

To study the expression of *LGC1* promoter in heterologous species, *Nicotiana* was transformed with *LGC1-GUS* chimeric construct. *Nicotiana*, like *Lilium*, has bicellular pollen and thus is ideal for studying gene expression in the generative cell.

Transgenic *Nicotiana* plants were tested by Southern analysis to confirm the copy number of T-DNA insertions and plants with single and multiple T-DNA insertions were analyzed. Histochemical staining for *GUS* on various tissues including leaves, roots, floral tissue and various stages of pollen development showed that GUS is expressed only in the generative cell of the binucleate pollen (Fig. 1B). No GUS expression was observed in the uninucleate microspores, vegetative cell in the mature pollen or any other vegetative and floral tissue. Localized staining of the generative cell was observed after 4 h of incubation in GUS substrate.

A detailed analysis of various developmental stages of pollen showed that the *GUS* expression is first detected soon after microspore mitosis when the generative cell detaches from the pollen wall. The expression increases as the generative cell moves to the center of the vegetative cell. Maximum expression, however, is observed in the mature pollen where the generative cell has migrated to the center of the vegetative cell. The expression pattern of the *GUS* gene in the transgenic plants driven by the *LGC1* promoter was very similar to *LGC1* gene expression as detected on the basis of Northern analysis and in situ hybridization [4]. *LGC1* promoter is thus able to direct high level of *GUS* expression in the generative cell of tobacco pollen. These studies about functional conservation of *LGC1* promoter in heterologous species point towards conservation of transcriptional factors required to control the specificity of expression of this promoter in the male gametic cells.

3.2. LGC1-*DTA expression in transgenic tobacco plants provides unequivocal evidence for male gametophytic expression of* LGC1 *promoter*

Examination of *LGC1* promoter-*GUS* and promoter-*GFP* constructs in transgenic plants and transient assay respectively, showed that this promoter directs generative cell-specific expression of the reporter genes. However, any leaky non-specific expression below the detection limits of these reporter gene assays cannot be ruled out. To ascertain the expression of the *LGC1* promoter, fusion was made to DT/A cytotoxin gene and transformed into *Nicotiana*. DTA has been shown to be a very potent cytotoxin and a few molecules are enough to cause cell death [13]. Therefore, any leaky ex-

pression of the *LGC1* promoter in tissues other than the generative cell should result in ablation of tissues where this gene is expressed.

Southern analysis of *LGC1-DT-A* transgenic plants indicated variable number of T-DNA inserts. The transgenic plants carrying *LGC1/DT-A* constructs exhibited normal vegetative and floral growth firmly establishing that this promoter is not active in the vegetative tissues. However, the anthers from the *LGC1-DT-A* plants were reduced in size and shed less pollen upon dehiscence indicating decreased pollen production as compared to the untransformed control. Microscopic analysis of pollen showed a varied degree of abortion. Pollen lethality in the transgenic plants varied from 50% in the plants with single copy insertions, to more than 90% in the plants with multiple copy insertions. The aborted pollen were small and irregular in shape as compared to the wild-type pollen and failed to hydrate and germinate in pollen germination media. Pollen viability was also assessed using fluorescein diacetate (FDA) and propidium iodide double staining [18]. While the large round viable pollen showed high fluorochrome retention and fluoresced green, the aborted, shriveled pollen appeared red with distinct staining of nuclei indicating a degraded cytoplasm (Fig. 1H and I). In the aborted pollen the generative cell nucleus is still seen attached to the pollen wall indicating that the DTA is expressed soon after microspore mitosis when the generative cell is still attached to the pollen wall. DTA expression data in conjunction with expression of GUS and GFP showed that the *LGC1* promoter is specifically active in the male gametic cells.

3.3. *Identification of a* cis*-acting region that confers repression of promoter activity in other plant cells*

A transient assay with lily pollen and stable transformation of *Nicotiana* show a 811 bp *LGC1* upstream promoter sequence to be sufficient to direct high levels of reporter gene expression in the generative cell. To delineate the minimal promoter sequence that can direct generative cell-specific expression and to identify regulatory elements, 5′ deletions of the *LGC1* promoter were tested in a transient assay. In total, 10 deletions were analyzed along with full-length *LGC1* and *CAMV 35S* promoter as control (Fig. 2). The deletions and the full-length promoters were fused to the *GFP* gene and analyzed by microprojectile bombardment of mature lily pollen, petals and onion epithelium.

Truncations of the *LGC1* promoter up to −242 showed a generative cell-specific expression similar to the full-length promoter (Fig. 2). No expression was detected in the onion epithelium or lily petal tissue. However, further deletion of the promoter to −183 produced three dramatic changes in the expression pattern of the *GFP*. Firstly, the pollen transformed with these deletion constructs exhibited a diffused pattern of *GFP* expression in the pollen that no longer appeared to be concentrated in the generative cell (Fig. 1C). This expression did not appear to be generative cell specific. Instead this pattern resembled expression of pollen-specific (vegetative cell-specific) promoters. Secondly, the number of pollen expressing these deletion constructs increased 10-fold as compared to those bombarded with the full-length promoter construct. This can be expected if the generative cell-specific expression non-specifically changes to vegetative cell specific. Finally, the expression of these truncated constructs was also detected in the petal and epithelium tissues. The smallest truncated form

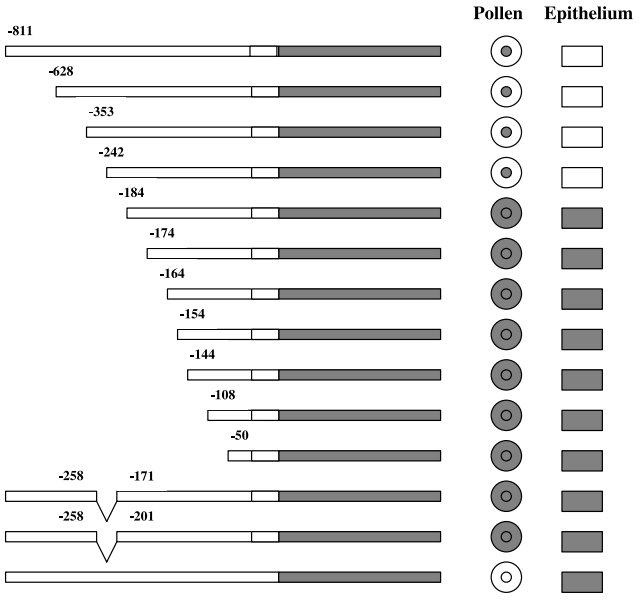

Fig. 2. Expression pattern of *LGC1-GFP* promoter constructs. Schematic representation of the reporter gene constructs used for transient expression following microprojectile bombardment of lily pollen and onion epithelium tissue. Promoter truncations up to −242 retain generative cell specificity with no expression recorded in epithelial cells. Further truncations to −50 and promoter fragments with internal deletions show expression in the vegetative cell of pollen and onion epithelium cells. The 35S-*GFP* construct shows *GFP* expression in epithelial cells with nil expression in pollen. In all bombardments lily petals were also used in parallel to onion epithelial tissue and in all cases the pattern of *GFP* expression in petal tissue matched with that obtained with onion epithelial tissue.

of the promoter at −50, which still retains the TATA box, also showed expression in the pollen, petals and epithelium (Fig. 1D and G). The expression pattern of *CaMV 35S* promoter was opposite to that of *LGC1* promoter and expression of this construct was limited to the vegetative tissues only, i.e. petals and epithelium and no expression was detected in pollen. The lack of *35S* promoter activation in pollen has been reported in literature [7].

Our present results indicate that the generative cell specificity of the *LGC1* promoter is maintained down to −242 bp. This truncated promoter fragment exhibited an expression pattern that is similar to the 811 bp fragment. The −242 bp fragment therefore includes the minimal promoter necessary for the generative cell-specific expression. Since the deletions −183 and onwards lose cell specificity, it indicates that generative cell-specific regulatory sequences are present between the region of −242 and 183. To test the role of the sequences between −242 and −183 in regulating tissue specificity, an internal deletion construct was made by deleting this 58 bp putative regulatory region from the full-length *LGC1* promoter and fusing this deleted fragment to *GFP*. Following bombardment, the expression of this construct was detected both in the mature pollen and onion epithelium indicating a constitutive expression. The expression pattern of this construct was similar to that observed with the −183 bp and smaller deletion constructs. The data from these studies indicate the presence of a regulatory element in the region −241 to −183 that regulates the generative cell specificity of *LGC1* promoter.

3.4. LGC1 *gene appears to be generative cell specific via its selective repression in other cells*

Deletion analysis of the *LGC1* promoter showed that the minimal promoter region required for generative cell-specific expression of the *LGC1* gene is −242 bp with respect to the transcription initiation site. This region appears to contain all the regulatory sequences that are required for generative cell-specific expression of this gene. Deletion of this region affects the specificity of the promoter and it exhibits a non-specific pattern of expression. These deleted promoter fragments show expression in the vegetative (epithelium) and floral (petal) tissues besides vegetative cell of pollen. Expression in the generative cell at this stage, however, cannot be ruled out. For the correct expression of the *LGC1* promoter in the generative cell, the coated particle needs to penetrate this cell of pollen. Although most of the bombarded particles that enter the pollen end up in the vegetative cell, they do not express GFP because of the specificity of the *LGC1* promoter. With the deletion of this regulatory region however, the promoter becomes active in the vegetative cell as well, and hence the number of pollen expressing *GFP* increases nearly 10-fold compared to those bombarded with full-length promoter.

Results from transient expression studies indicate the presence of a silencer element in the −242 to −183 region that inhibits the expression of this promoter in all other plant tissues, including the vegetative cell. The removal of this silencer region also eliminates the specificity of this promoter, which then shows non-specific expression. Silencers, which form an intrinsic part of many eukaryotic promoters, can direct an active repression mechanism and hence can regulate tissue specificity, have been widely studied in mammalian genes [19,20]. It has been proposed that the repressors bind to the silencer elements and interact with the core promoter to inhibit transcription. When the repressor does not bind to the silencer element there is no interaction with the core promoter RNA polymerase complex and transcription can proceed. With the deletion of the silencer element sequence the repressor does not bind and no inhibitory complex is formed with the core promoter thus allowing transcription to occur. This may be the case with truncated forms of *LGC1* promoter lacking the silencer region that also express in tissues where it should be normally repressed.

The differential regulation of *LGC1* gene in generative and vegetative cells of pollen can be explained on the basis of the repressor exclusion model. In the proposed model a repressor protein with specificity towards the putative silencer element represses the expression of this gene in the vegetative cell of pollen (and all other plant cells). This putative repressor is expected to be present in the microspore but its exclusion from the generative cell pole following asymmetric cell division leads to de-repression of *LGC1* gene in generative cell and appearance of *LGC1* transcripts soon after migration of generative cell into vegetative cell cytoplasm. The phenomenon of asymmetric division which has been studied in many diverse organisms, has a common mechanism, i.e. the unequal partitioning of gene products to the opposite poles and subsequent inheritance of these diverse products by the two cell types that arise from this division [21,22]. Similar partitioning of gene products can also be expected during the asymmetric division of the microspore. Thus, according to our proposed model a repressor protein with a binding specificity for the silencer element might regulate LGC1 suppression in all other cells except generative/sperm cells. Regulation of cell-specific genes via suppressing their transcription in other cells has been reported in mammalian neuronal [23] and spermatogenic tissues [24]. The male gametic cell-specific gene expression of *LGC1* appears to be controlled through negative regulation by a repressor protein that specifically interacts with the silencer element and selectively represses transcription in the vegetative cell of pollen and other plant tissues. This is in contrast to other reported pollen-specific (vegetative cell) genes, in which specificity is achieved, in large part by specifically expressed enhancer binding factors [7]. We have obtained preliminary evidence for this repressor protein by gel mobility shift assay in which a 43 bp DNA fragment containing the putative silencer sequence was incubated with nuclear extract prepared from *Lilium* petal cells. The mobility of the labeled DNA fragment was retarded by formation of a protein–DNA complex in the presence of nuclear proteins (data not shown). The specificity of this DNA–protein interaction was confirmed by competitive inhibition of binding by excess of the same unlabeled fragment (data not shown).

Identification and cloning of the putative repressor protein present in plant cells that negatively regulate *LGC1* expression through interaction with the putative silencer region of *LGC1* (and possibly other coordinately expressed genes) should provide novel insights into flowering plant male gamete-specific gene expression. Promoters of generative cell-specific genes such as *LGC1* can serve as useful germ line tools that can be potentially used to study the developmental processes of the male gametic cells and hence fertilization in flowering plants.

Acknowledgements: We gratefully acknowledge financial support provided by the Australian Research Council (ARC) for this project.

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