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METHOD ARTICLE

Two novel strategies to assess *in vivo* meiotic protein expression in *Arabidopsis thaliana* [version 1; peer review: 3 approved with reservations]

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Abstract

For studies on key meiotic processes such as crossover formation and genome haploidization, the availability of portable promoter sequences for effector protein expression in meiocytes is of great importance. In this study, we present two novel strategies to facilitate screening for such promoter elements. The first strategy was based on expression of a previously constructed GFP-tagged zinc finger protein for visualization of the pericentromeric regions of chromosomes in meiocytes. Here, we show that expression of this reporter protein under control of different promoters allowed for the visualization of fluorescence foci in meiocytes, demonstrating that this is a useful tool for such purposes. The second reporter system was based on the visualization of cytotoxicity triggered by expression of the *Agrobacterium tumefaciens* virulence protein VirD5. We show that constitutive expression of VirD5 is lethal, but when driven by meiotic promoters led to reduced fertility with normal vegetative growth. We show that both strategies offer useful tools for the assessment of meiotic effector protein expression, especially when combined with available gene expression data sets.

Keywords

Arabidopsis, meiosis, gene expression, promoter, meiocytes, zinc finger, fluorescence reporter, negative selection marker

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Introduction

The cell division process of meiosis generates haploid gametes for fertilization. Cells that are primed for meiosis (meiocytes) undergo two consecutive rounds of chromosome segregation. The first division (meiosis I) results in separation of the homologous chromosomes¹, which is facilitated by the formation of physical connections (chiasmata) at double stranded break sites generated by transterases, such as SPO11-1^{2,3} and SPO11-2⁴ in the model plant species *Arabidopsis thaliana*. Resolution of these junctions can lead to the reciprocal exchange of parts of chromosome arms, a process known as meiotic crossing over⁵. The second round of division (meiosis II), results in separation of the sister chromatids¹, finally yielding four haploid daughter cells from a diploid meiocyte. In *Arabidopsis*, male and female meiosis are separated within the flower and take place in the anthers and carpels, respectively. Male meiocytes are organized as column-like structures (referred to in this study as male meiocyte columns)^{6,7}, and upon completion of meiosis give rise to four haploid pollen cells that are physically attached to each other as structures known as tetrads. Maturation of tetrads eventually leads to the release of individual pollen cells.

The process of meiosis is of great interest to plant breeders for different reasons. Most importantly, crossover events are the driving force behind genetic variation in plants^{5,8,9}. Research on crossover frequency has thus received a considerable amount of attention in literature (e.g. 10–12). To facilitate the engineering of crossover-related events in meiocytes without affecting non-meiotic tissue, there is interest in identifying gene promoters that allow for effector activity during meiosis. Although there is a vast amount of literature available on the genetics and cytogenetics of plant meiosis¹, it has been challenging to identify DNA sequences that can be used to express proteins of interest preferably in meiotic cells.

Relatively recent studies on the male meiocyte transcriptome^{6,13} have provided cues for the identification of genes that are more or less specifically expressed during male meiosis. The upstream regions of these genes (generally representing the gene control region¹⁴) are mostly referred to as ‘promoters’. Such promoters can be regarded as portable elements for the assembly of gene expression cassettes that can mediate meiotic effector protein expression. Accurately quantifying meiotic transgene expression, however, is subject to a number of practical limitations. Firstly, relative expression levels do not necessarily correlate with promoter activity, which is determined by endogenous RNA metabolism as well (e.g. 15). Secondly, the isolation of meiocytes for quantitative gene expression analysis is technically very challenging and laborious, and could easily result in contamination of RT-qPCR or transcriptome data with non-meiotic transcripts. Qualitative assessment of promoter activity using fluorescence microscopy is an attractive possibility. However, the dynamic cytoplasmic and nuclear states of meiocytes make it difficult to determine the exact localization of fluorescence, and to distinguish it from autofluorescence.

In this study, we have investigated two novel strategies to screen for portable promoter elements to express effector proteins

specifically or at high levels in meiocytes. The first strategy was based on expressing a previously constructed GFP-tagged zinc finger protein (1803F-GFP) to visualize pericentromeric fluorescence foci in meiocytes. Here, we show that 1803F-GFP expression driven by the promoter of the *Arabidopsis* *RPS5A* gene (*pRPS5A*) which is active in vegetative^{16–18} and meristematic tissue¹⁸, resulted in clear GFP foci in male meiocytes. We also selected and cloned 14 promoter sequences that could be of interest for expression of transgenes in meiocytes based on published transcriptome data, and assessed their activities in meiocytes using the 1803F-GFP reporter system. As a second strategy, we assembled constructs encoding the highly cytotoxic *Agrobacterium tumefaciens* virulence protein VirD5, which was recently shown to disturb chromosome segregation in plant cells by interacting with the kinetochores of chromosomes¹⁹. We reasoned that, due its mode of cytotoxicity, VirD5 could be used as a novel negative selection marker to assess effector protein expression in meiocytes. Here, we show that the VirD5 reporter system can be used to obtain *in planta* cues about promoter strength and specificity. Altogether, our data show that both strategies can provide valuable *in vivo* information regarding promoter activity in meiocytes.

Results

Molecular cloning of candidate promoters for meiotic protein expression analyses

To investigate the activity and specificity of promoters of interest to drive effector protein expression in meiocytes, we first cloned the promoters of a selection of genes which are involved in meiosis or highly expressed in meiocytes. In total, we cloned the promoter sequences of 14 genes based on the two available studies on the male meiocyte transcriptome^{6,13}. An overview of these 14 genes is provided in Table 1. In brief, we firstly selected five genes with important roles in meiosis (*ZYP1A*, *SPO11-2*, *NBS1*, *SPO11-1*, *MUS81*) that are expressed in male meiocytes^{6,13}, and rather specifically so based on meiocyte/anther and meiocyte/seedling expression ratios⁶ (Table 1). In addition, we selected four genes (*ASK1*, *DMC1*, *SMC1* and *MS5*) which are involved in meiosis and are expressed in male meiocytes^{6,13}, but not specifically so (Table 1). Secondly, we selected four genes (*At1g27710*, *At5g25980*, *At5g26622*, *At5g42530*) which are the most highly expressed genes in male meiocytes (13 and personal communications with the authors). Finally, we selected the promoter of the gene *At4g40020*, which has been described to be expressed specifically during meiosis I⁷.

Establishing the 1803F-GFP reporter system to visualize meiotic protein expression

Previously, we have described a GFP-based reporter to monitor chromosomes *in vivo* in vegetative tissue of *Arabidopsis* plants²⁰. This construct encoded a fusion between the fluorescent protein GFP and an array of three zinc fingers (3F) targeting a cognate 9 bp DNA sequence¹⁴ in the highly conserved pericentromeric 178 bp repeats which make up a large portion of the pericentromeric DNA in *Arabidopsis*²¹. Expression of this fusion protein, which is referred to here as ‘1803F-GFP’, can result in distinctive GFP signals that visualize the pericentromeric regions of chromosomes or the cytological chromocenters^{20,22}.

Table 1. Overview of genes with meiotically active promoters which were cloned for this study. The genes are listed in locus ID order.

Locus ID	Gene name	Annotation	Expression Level in male meiocytes (RKPM)	Expression Level in male meiocytes (RKPM)	Upstream promoter length used in present study (bp)	Reference(s) for expression in meiocytes
At1g22260	ZYP1A	Synaptonemal complex protein	1.9 ^{SD}	16.8 [0.5; 17.0]	2102	Yang <i>et al.</i> (2011) Chen <i>et al.</i> (2010)
At1g27710	N.A.	Glycine-rich protein	8847 ^{PC}	N.A.	796	Yang <i>et al.</i> (2011)
At1g63990	SPO11-2	Spo11/DNA topoisomerase VI subunit	0.43 ^{SD}	17.5 [2.3; 2.0]	2072	Yang <i>et al.</i> (2011) Chen <i>et al.</i> (2010)
At1g75950	ASK1	Arabidopsis SKP1 Homologue 1	102.9 ^{SD,NS}	96 [0.3; 1.0]	2373	Yang <i>et al.</i> (2011) Chen <i>et al.</i> (2010)
At3g02680	NBS1	Nijmegen Breakage Syndrome 1 (component MRE11 complex)	1.7	7.1 [0.6; 1.6]	1746	Yang <i>et al.</i> (2011) Chen <i>et al.</i> (2010)
At3g13170	SPO11-1	Spo11/DNA topoisomerase VI subunit	1.3 ^{SD}	1.0 [0.3; 2.9]	1088	Yang <i>et al.</i> (2011) Chen <i>et al.</i> (2010)
At3g22880	DMC1	Disruption of Meiotic Control 1	8.1 ^{SD,NS}	25.2 [0.5; 6.0]	3123	Yang <i>et al.</i> (2011) Chen <i>et al.</i> (2010) Li <i>et al.</i> (2012) Klimyuk and Jones (1997)
At3g54670	SMC1	Structural Maintenance of Chromosomes 1	14.4 ^{SD,NS}	83 [0.9; 1.8]	2545	Yang <i>et al.</i> (2011) Chen <i>et al.</i> (2010)
At4g20900	MS5	Tetrahelicopeptide repeat (TPR)-like superfamily protein	9.8 ^{SD,NS}	69.3 [2.0; = 0 in seedling]	2852	Yang <i>et al.</i> (2011) Chen <i>et al.</i> (2010) Li <i>et al.</i> (2012) Cifuentes <i>et al.</i> (2016)
At4g30870	MUS81	Arabidopsis MUS81 endonuclease homologue	3.2 ^{SD}	17.4 [0.9; 4.0]	1238	Yang <i>et al.</i> (2011) Chen <i>et al.</i> (2010)
At4g40020	N.A.	Myosin heavy chain related protein	N.A.	N.A.	1507	Li <i>et al.</i> (2012)
At5g25980	TGG2	Beta glucosidase 37	284 ^{PC}	N.A.	4777	Yang <i>et al.</i> (2011)
At5g26622	N.A.	Beta-galactosidase related protein	472 ^{PC}	N.A.	3509	Yang <i>et al.</i> (2011)
At5g42530	N.A.	Hypothetical protein	703 ^{PC}	N.A.	2214	Yang <i>et al.</i> (2011)

These signals are particularly easy to observe in non-green tissues, such as roots²². An important benefit of the formation of foci is that the GFP signal is strongly concentrated, making discrimination from autofluorescence much easier compared to the detection of diffuse GFP signal. In addition, as the 178 bp repeat regions are strongly conserved and present in the pericentromeric regions of all five *Arabidopsis* chromosomes, the 1803F-GFP reporter system also allows for ploidy determination. For these reasons, we considered the 1803F-GFP system a suitable read-out for meiotic protein expression. An overview of the 1803F-GFP strategy is provided in Figure 1.

Previously, we have used the 1803F-GFP reporter system^{20,22} under control of the promoter of the *Arabidopsis* *RPS5A* gene (*pRPS5A*) in the binary vector pRF 1803F:GFP Kana. For this study, we slightly modified the backbone of pRF 1803F:GFP Kana

to accommodate the introduction of other promoter fragments. In addition, we introduced a new, flexible hydrophilic (GGGS)₃ linker^{23,24} to possibly enhance the read-out of the 1803F-GFP system. This newly generated binary vector was named pRF 1803F:(GGGS)₃:GFP Kana. More details about the construction of pRF 1803F:(GGGS)₃:GFP Kana are provided in the Methods section. An overview of the DNA sequence and the key components of pRF 1803F:(GGGS)₃:GFP Kana is provided in Figure 2, and the amino acid sequence encoded by the *1803F:(GGGS)₃:GFP* open reading frame is provided in Figure 3.

Promoters of interest can easily be introduced into the pRF 1803F:(GGGS)₃:GFP Kana backbone, for instance by ligating *Sall*-*NotI* fragments of PCR-amplified promoter sequences into *Sall*-*NotI* digested pRF 1803F-GFP Kana, thereby directly placing the 1803F-GFP open reading frame under their

Figure 1. Screening for activity of promoters of interest using the 1803F-GFP reporter system. A promoter of interest (*promoter of interest*) is cloned into the binary vector pRF 1803F:(GGGS)₃:GFP Kana (e.g. as a PCR-amplified *Sall*-*NotI* fragment). The promoter drives expression of a fusion protein consisting of three zinc fingers (*ZF1*, *ZF2* and *ZF3*) together recognizing 9 bp of DNA which are unique to a major fraction of the 178 bp pericentromeric repeats, fused to the open reading frame of GFP (*GFP*), spaced by a flexible linker peptide (*Linker*), and with an N-terminal nuclear localization signal (*NLS*) and FLAG tag (*FLAG*). Transcription termination is under control of the NOS terminator sequence (*tNOS*). The construct is introduced into *Arabidopsis* plants as a T-DNA with left and right border sequences (*LB* and *RB*, respectively), through *Agrobacterium tumefaciens*-mediated transformation. Primary transformants are selected for by kanamycin resistance due to *NPTII* expression. Expression of 1803F-GFP under control of the promoter of interest can subsequently be visualized as nuclear GFP foci by microscopy.

^{Sall} ^{promoter of interest} ^{NotI}
 GTCTGACTCTAGAGGATCGATCCCCGGGATG**GCCGACTACAAGGACGACGACGATAAG**CGGCCGCTTGAGCCTCCGAAAAAGAACGTAAGGTCG
^{XbaI} **FLAG** **NLS**
 AGCTAGCAGGTACAGAGGCCCAAGCGGCC**CTCGAACCACCGGCGAGAAACCTACGCCTGCCCGGAATGCGGGAAGTCGTTTAGCACCTCCGG**
^{SfiI}
TAGCCTGGTGAGGCATCAGCGTACGCATACCGGCGAGAAACCGTATGCGTGTCCCGAATGCGGTAAAAGCTTTAGCCGTAGCGATGACCTTGTG
CGTCACCAGAGGACCCATACCGGCGAGAAACCTACGCCTGCCCGGAATGCGGGAAGTCGTTTAGCACCTCCGGTAGCCTGGTGAGGCATCAGC
GTACGCATACCGGCGAGCTGGGTGGCGGTTCCGGAGAAAAGCCCGGAAGAAAACCTCG**GGCCAGGCCGCCAACTCGAG****GTTGGAGGTGGATC**
^{SfiI} ^{XhoI}
CGGAGGAGGAGGTTCTGGGAGGCGGAGGTTCAACTAGTGTGAGCAAGGGCGAGGAGCTGTTACCGGGGTGGTGCCCATCCTGGTCGAGCTGGAC
GGCGACGTAAACGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCTGAAGTTCATCTGCACCACCG
GCAAGCTGCCCGTGCCCTGGCCACCTTCGTGACCACCTTCACTACGGCGTGAGTGCTTCAGCCGCTACCCGACCACATGAAGCAGCACGA
CTTCTTCAAGTCCGCCATGCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAG
TTTCGAGGGCGACACCTTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAAC
ACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACCTTCAAGATCCGCCACAACATCGAGGACGGCAGCGT
GCAGCTCGCCGACCACTACCAGCAGAACACCCCATCGGCGACGGCCCCGTGCTGTGCCCCGACAACCACCTACCTGAGCACCCAGTCCGCCCTG
AGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCTGTGAGGTTCTGTGACCGCCGCGGGATCACTCTCGGCATGGACGAGCTGTACAAGT
AAGAGCTCGAATTTCCCCGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAAT
 TTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATA
 CATTTAATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATCGGG

Figure 2. The DNA sequence of the 1803F-GFP fusion construct encoded on the binary vector pRF 1803F:(GGGS)₃:GFP Kana. The fusion construct consists of the 1803F encoding sequence (bold blue font) flanked by *SfiI* sites, fused to the eGFP encoding sequence (bold green font) through a flexible linker (bold orange font), tagged with a FLAG tag and a nuclear localization signal (NLS) at the 5' end. Other relevant unique restriction sites are also indicated. Expression of the fusion construct is under control of a promoter of interest, which can be inserted at the indicated position, and of the *NOS* terminator sequence (bold grey font).

¹ **MADYKDDDDKRPLEPPKKRRKVELAGTEAQAAL****EP**^{1803F}
^{FLAG} ^{NLS} ^{ZF1}
THT**GEKPYACPECGKSFSRSDDLVRHQRTHT****GEKPYACPECGKSFSSTSGSLVRHQRTHT****GE**
^{ZF2} ^{ZF3}
LGGSGSEKPGKKTSGQAGQLEGGGSGGGGSGGGGSTSVSKGEELFTGVVPILVELDGDVN
^{Flexible linker}
GHKFSVSGEGEGDATYGLTLKFICTTGKLPVPWPTLVTTFTYGVQCFSRYPDHMKQHDF
KSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNY
NSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSA
LSKDPNEKRDHMLLEFVTAAGITLGMD**ELYK**³⁹⁸

Figure 3. The amino acid sequence of the 1803F-GFP reporter protein. The fusion protein consists of 1803F (consisting of three ZFs; ZF1 in blue, ZF2 in red and ZF3 in purple font, respectively) fused to GFP (bold green font) through a flexible linker peptide consisting of a trimer of the aa sequence GGGGS, and tagged with a FLAG tag and a nuclear localization signal (NLS) at the N-terminus (398 amino acids in total).

control. The binary vector is also compatible with other cloning strategies, e.g. as described in this study for *pSPO11* (further details described in the Methods section).

Visualization of promoter activity in meiocytes using the 1803F-GFP reporter system driven by the *RPS5A* promoter

To investigate the 1803F-GFP system as a novel tool to visualize protein expression *in vivo* in meiocytes, we firstly

verified whether the nuclear GFP foci distinct for 1803F-GFP binding to pericentromeric regions (Figure 1) could be observed in root tip cells (diploid; $2n=10$) of transgenic plants harboring the *pRPS5A::1803F:(GGGS)₃-GFP* construct. Nuclei of root tip cells could easily be visualized as expected, and in most cases contained ten distinct GFP foci in a full Z-stack of images, corresponding to the diploid ($2n=10$) number of chromosomes (Figure 4A). The foci could be examined directly by zooming in on confocal microscopy images and did not require further image analysis.

Figure 4. The 1803F-GFP reporter system in somatic and meiotic cells. (A) Confocal fluorescence microscopy images of a root tip (T2 generation) expressing 1803F-GFP under control of *pRPS5A*. Highlighted is a nucleus in which 10 distinct GFP foci corresponding to the diploid number of chromosomes ($2n=10$) are visible. Scale bar represents 10 μ m. A contrasted black and white ('Binary') version of the GFP image is included to clearly mark GFP-fluorescent chromocenters with red arrows. **(B)** Confocal fluorescence microscopy images of male meiocytes of wild-type Col-0 plants, primary transformants (T1 generation) expressing 1803F-GFP under control of the *RPS5A* promoter (*pRPS5A::1803F-GFP*), and primary transformants expressing 1803F-GFP under control of the GAL4-based two-step expression system (*p2S-RPS5A::1803F-GFP*). Images taken with the RFP channel are presented as negative controls for autofluorescence. Scale bars represent 20 μ m. **(C)** False color images of nuclei of meiocytes harboring *p2S-RPS5A::1803F-GFP* ($n=8$ presented). Below every nucleus the number of counted GFP foci is presented. A diploid somatic cell ($2n=10$) is provided as a control. **(D)** DAPI-stained spread of wild-type Col-0 flower bud tissue. Highlighted are a diploid cell ($2n=10$) and a haploid cell ($n=5$). Scale bar represents 10 μ m.

To assess the 1803F-GFP system in meiocytes, we subsequently isolated male meiocyte columns from wild-type plants and from transgenic plants harboring *pRPS5A::1803F:(GGGS)₃:GFP*, and examined them by confocal fluorescence microscopy. Male meiocytes from transgenic plants harboring *pRPS5A::1803F:(GGGS)₃:GFP* clearly displayed GFP foci corresponding to 1803F-GFP fusions binding to pericentromeres (Figure 4B), while meiocytes isolated from wild-type Col-0 plants did not (Figure 4B). These observations demonstrated that the 1803F-GFP reporter system provides for a clear read-out for meiotic effector protein expression, and that *pRPS5A* is active during meiosis, which is a novel finding. To corroborate these observations, we constructed a GAL4-based two-step (2S) variant of the *pRPS5A* expression cassette, in which *pRPS5A* drives the expression of GAL4-VP16, which in turn can transactivate a flanking *4xUAS-minimal35S::1803F:(GGGS)₃:GFP* reporter gene (*p2S-RPS5A::1803F:(GGGS)₃:GFP* in short). This 2S construction, which was based on an earlier published enhancer trap approach^{25,26}, was expected to provide for an essentially similar expression pattern as in *pRPS5A::1803F:(GGGS)₃:GFP* reporter lines, but, due to the fact that GAL4-VP16 is a potent activator of multiple *UAS*-driven gene expression, would have higher overall strength. We subsequently isolated male meiocyte columns from transgenic plants harboring the two-step *p2S-RPS5A::1803F:(GGGS)₃:GFP* variant, and examined them by confocal microscopy. Nuclei of meiocytes harboring *p2S-RPS5A::1803F:(GGGS)₃:GFP* indeed displayed clear GFP foci which now had a slightly higher signal intensity (Figure 4B), and displayed less background GFP fluorescence than meiocytes from transgenic plants harboring *pRPS5A::1803F:(GGGS)₃:GFP*. These data showed that the 2S expression cassette is also a suitable reporter for meiotic activity of promoters of interest.

We further investigated the resolution of the 1803F-GFP system by assessing the number of visible chromosomes in the nuclei of meiocytes in male meiocyte columns (Figure 4C). When examining 1803F-GFP foci in the surrounding somatic tissue, the number of chromosomes per cell was always ten ($2n=10$). This dropped to approximately five GFP foci in meiocytes of transgenic plants harboring *p2S-RPS5A::1803F:(GGGS)₃:GFP* (Figure 4C), confirming that these cells were either haploid, or that chromocenters were in close proximity. In this case, it also has to be noted that the raw images did not require any further processing besides zooming in on nuclei. Hence, the 1803F-GFP reporter system combined with confocal microscopy had sufficient resolution to visualize chromosomes *in vivo* in meiocytes using the 2S expression cassette. To further corroborate this, we compared images taken from meiocytes expressing 1803F-GFP to confocal images of chromosome spreads which were prepared from flower bud tissue (Figure 4D). We found that images of DAPI-stained spreads of flower bud cells (Figure 4D) had a resolution which in terms of visibility of chromocenters was very similar to images obtained through squashing of 1803F-GFP expressing flower buds (Figure 4C), although the latter were captured *in vivo* without requiring any tissue fixation and staining. These observations indicated that the 1803F-GFP system had sufficient resolution to match the standard of the best available cytological images.

Visualization of the 1803F-GFP reporter system in meiocytes when driven by cloned promoter fragments

To further investigate the 1803F-GFP system as a read-out for meiotic effector protein expression, the cloned promoter sequences of meiotic genes (Table 2) were each ligated into binary vector pRF 1803F-GFP Kana, thereby replacing *pRPS5A*. *Arabidopsis* Col-0 plants were transformed with these fusion constructs

Table 2. Overview of confocal fluorescence microscopy on squashed flower buds of primary transformants (T1 generation) expressing 1803F-GFP fusions driven by the selected meiotically active promoters. Flower buds from on average five independent primary transformants ($n=5$) were examined. The promoters are listed in locus ID order. Positive fluorescence signals are indicated in grey.

Locus ID	Promoter name	::1803F-GFP GFP signal in			
		Male meiocyte column	Tetrads	Pollen	Other (within flower bud)
At1g22260	<i>pZYP1A</i>	No	No	No	No
At1g27710	<i>p1g27710</i>	No	No	No	Tapetum
At1g63990	<i>pSPO11-2</i>	No	No	No	No
At1g75950	<i>pASK1</i>	No	Low	Yes	No
At3g02680	<i>pNBS1</i>	No	No	No	No
At3g13170	<i>pSPO11-1</i>	Low	No	No	No
At3g22880	<i>pDMC1</i>	No	No	No	No
At3g54670	<i>pSMC1</i>	Low	No	Yes	Meristem ovule primordia
At4g20900	<i>pMS5</i>	Low	Low	Low	No
At4g30870	<i>pMUS81</i>	No	No	No	No
At4g40020	<i>p4g40020</i>	No	No	No	No
At5g25980	<i>pTGG2</i>	No	No	No	Vascular cells
At5g26622	<i>p5g26622</i>	Yes	No	No	No
At5g42530	<i>p5g42530</i>	No	No	No	Epithelium

using the floral dip method. Male meiocyte columns were then isolated from young flower buds of kanamycin-resistant primary transformants and examined by confocal fluorescence microscopy, as was done for *pRPS5A*. As controls, we also examined tetrads and pollen, and other tissues of flower buds which were released during the isolation of meiocytes. An overview of the results of the confocal fluorescence microscopy is provided in [Table 2](#). Confocal microscopy images of tissues in which fluorescence foci were observed, are provided in [Figure 5](#).

Using the 1803F-GFP system, we could show that the activity of the promoters of *SPO11-1*, *SMC1*, *MS5* and *p5g26622* indeed resulted in GFP foci in male meiocytes ([Table 2](#) and [Figure 5](#)). For *pSPO11-1* and *p5g26622* we did not observe foci in any other tissues of the flower bud ([Table 2](#)), indicating that these promoters are indeed specifically active during meiosis. In the cases of seven other promoters (*pZYPIA*, *pSPO11-2*, *pNBS1*, *pDMC1*, *pMUS81*, *p4g40020*, and *p5g42530*) we could not detect GFP foci in any tissue type ([Table 2](#)), while transcriptome

Figure 5. Confocal fluorescence microscopy on flower bud squashes of primary transformants (T1 generation) expressing 1803F-GFP under control of the cloned promoters. Confocal images of male and female meiocytes (male meiocyte columns and ovule primordia, respectively) are presented, along with tetrads and pollen. For the sake of clarity of the figure only the cases where GFP signals could be observed are presented. Images of all four tissue types in Col-0 are presented as a negative control, with white arrows indicating the location in case of low background fluorescence. Scale bars represent 20 μm.

data for five of these genes (*ZYP1A*, *SPO11-2*, *NBS1*, *DMC1*, and *MUS81*) are indicative of rather specific expression during meiosis (Table 2)⁶. These observations suggested that the expression levels of these genes in meiocytes are too low to be detected using the 1803F-GFP system as a reporter, which is in accordance with their low expression values in meiocytes (Table 2)^{6,13}. For three promoters (*p1g27710*, *pTGG2*, and *p5g42530*) we did not observe expression in meiocytes, tetrads, or pollen, but noted GFP signals in other tissues in the flower bud, such as the tapetum, epithelium, and vascular cells (Table 2; Figure 5), suggesting that these promoters have very low activities in meiocytes and are not at all meiosis specific.

An overview of promoter activities described in this study which either confirm findings described in literature or are completely novel is presented in Figure 6.

Establishing VirD5 expression as a novel negative selection marker for meiotic promoter activity

As a second strategy to assess the strength and specificity of meiotic promoter activity, we assembled reporter constructs with the *Agrobacterium tumefaciens* virulence gene *virD5*. Recently, the virulence protein VirD5 was described as being cytotoxic to plant cells by binding to kinetochores through an interaction with the protein Spt4, thereby disturbing mitotic cell division and leading to cell death and aneuploidy¹⁹. Transgenic plants expressing VirD5 under control of an inducible promoter could be obtained, but these died upon induction of expression¹⁹. In the present study, we hypothesized that VirD5 could also be cytotoxic to meiotic cells, and that plants expressing VirD5 under control of a meiotic promoter would grow normally, but would show partial or full sterility due to abortion of meiosis. In that manner, VirD5 expression might act as a dominant

Figure 6. False color confocal microscopy images displaying the promoter activities found in this study. Presented are the most clear examples of 1803F-GFP expression in meiocytes and pollen under control *pRPS5A*, the GAL4-based two-step expression system (*p2S-RPS5A::1803F-GFP*) and of the meiotically active promoters verified in this study. Fluorescence intensity and size of the confocal images was optimized to qualitatively show the nuclear foci representing 1803F-GFP fusions binding to pericentromeric regions. Therefore, no scale bars are shown.

negative indicator of meiotic protein expression. We chose for VirD5 over other dominant negative selection marker systems because it specifically interferes with chromosome segregation and is thus likely to be particularly toxic to actively dividing cells, such as meiocytes. A schematic overview of this approach is presented in Figure 7. To establish a VirD5 based reporter system we cloned the *virD5* open reading frame (ORF) from *Agrobacterium tumefaciens* strain LBA1100²⁷ and generated fusion constructs for a selection of nine different cloned meiotic promoters of genes for which both expression data and specificity data were available^{6,13} (Table 1).

Assessment of meiotic promoter strength and specificity by means of VirD5 expression

Wild-type Col-0 plants were transformed with VirD5 expression constructs under control of the nine cloned promoters using the floral dip method. Viable primary transformants could be

obtained at normal transformation frequencies with all of the promoters except for *pASK1* and *pSMC1* (Table 3), which did not yield any viable primary transformants (Table 3), indicating that these promoters are not at all meiosis-specific and already active in embryos and seedlings. In case of *pASK1* this is in accordance with already observed vegetative *ASK1* expression patterns (Table 1), but was unexpected for *pSMC1*, which is, more or less, a meiosis-specific promoter based on available gene expression data (Table 1). Viable, fully developed primary transformants could be obtained at high frequencies for the other seven promoters, indicating that none of them were very active in vegetative tissue.

We further noted that VirD5 expression under control of *pZYP1A*, *pSPO11-1* and *pSPO11-2* did not impact vegetative development and flowering, but did lead to strongly reduced fertility (Table 3). Phenotyping VirD5 expressing primary transformants

Figure 7. Screening for meiotic activity of promoters of interest using the VirD5 reporter system. A promoter of interest (*Promoter of interest*) is introduced into the binary vector pVirD5 Kana (e.g. as a PCR-amplified *Sall*-*NotI* fragment). The promoter then drives expression of the *virD5* open reading frame with an N-terminal FLAG-tag (FLAG) and nuclear localization signal (NLS), under control of the NOS terminator sequence (*tNOS*). The construct is introduced into Arabidopsis plants as a T-DNA with left and right border sequences (LB and RB, respectively), through *Agrobacterium tumefaciens*-mediated floral dip transformation into wild-type Col-0 plants. Primary transformants (T1 generation) are selected as kanamycin-resistant individuals due to expression of the *NPTII* gene. Depending on the strength and tissue specificity of the activity of the promoter of interest, the viability, growth and fertility of the primary transformants can be affected. Possible examples of these effects are illustrated. In case of meiosis-specific activity, vegetative and floral development are unaffected, but the plants are sterile.

Table 3. Overview of the output of transformations with *virD5* gene constructs driven by a selection of meiotically active promoters. The promoters are listed in locus ID order.

Locus ID	Promoter name	Meiosis specificity (meiocyte/seedling expression ratio) Based on Chen <i>et al.</i> (2010)	:: <i>virD5</i>			
			Transformation efficiency (%)	Number of primary transformants obtained (from X floral dip seeds)	Viable primary transformants (%)	Primary transformants with reduced fertility (%)
At1g22260	pZYP1A	Specific (17.0)	0.31	62 (20,000)	98	21.2
At1g63990	pSPO11-2	Rather specific (2.0)	0.15	30 (20,000)	93	14.3
At1g75950	pASK1	Not specific (1.0)	0.02*	3* (20,000)	0*	N.A.
At3g02680	pNBS1	Rather specific (1.6)	0.16	32 (20,000)	94	6.7
At3g13170	pSPO11-1	Rather specific (2.9)	0.32	63 (20,000)	95	34.1
At3g22880	pDMC1	Specific (6.0)	0.18	36 (20,000)	100	5.6
At3g54670	pSMC1	Rather specific (1.8)	0.01*	2* (20,000)	0*	N.A.
At4g20900	pMS5	Not specific** (N.A.)	0.16	32 (20,000)	100	0.0
At4g30870	pMUS81	Rather specific (4.0)	0.20	39 (20,000)	97	2.6
	pRPS5A::1803F-GFP (negative control)		0.54	54 (10,000)	100	7.4

* Primary transformants could be obtained at low frequencies, but died when transferred to soil

** Based on Yang *et al.* (2011)

thus provided experimental evidence that these promoters are largely inactive during vegetative development and therefore might indeed be meiosis specific. In the case of *pZYP1A* this would be in accordance with a meiocyte/seedling expression ratio of 17.0 (Table 1 and Table 3), indicating meiosis-specific expression. Furthermore, these data indicated that both *pSPO11-1* and *pSPO11-2* could be more meiosis-specific than might be expected based on the meiocyte/seedling expression ratios (Table 1). We decided to further analyze the effects of *pZYP1A* and *pSPO11-1* in more detail, because these primary transformants were the most viable and at the same time displayed the most reduced fertility, indicating meiosis specificity.

VirD5 expression under control of meiosis specific promoters *pZYP1A* and *pSPO11-1*

To further assess meiosis specificity of *pZYP1A* and *pSPO11-1* we stained siliques of representative primary transformants with cottonblue-lactophenol to examine the quality of seed set and the viability of the embryos. Whereas siliques of control plants expressing 1803F-GFP under control of *pRPS5A* exhibited normal silique development with all positions in the siliques producing viable seeds (Figure 8A), siliques of transformants expressing VirD5 under control of *pZYP1A* and *pSPO11-1* were mostly much shorter and also contained many fewer viable seeds (Figure 8A). As the flowers did not show any obvious defects, these observations indicated that VirD5 is indeed cytotoxic to meiocytes or to their precursor cells, leading to substantially reduced fertility. In the case of *pSPO11-1*, loss of fertility was accompanied by substantial heterogeneity in seed size among the T2 seed pool (Figure 8B). Remarkably, most small seeds still germinated and finally produced normal plants. It has

to be noted, however, that even though expression of VirD5 under control of *pSPO11-1* severely affected fertility, it never completely abolished seed set, indicating that *pSPO11-1*, even though meiosis-specific, is not a very strong promoter. As an alternative possibility, it might be that interference with meiotic chromosome segregation by VirD5 could still allow for the generation of a small fraction of balanced gametes with 5 chromosomes, just as observed for *spo11-1* mutants²⁸. To further address this, we constructed a 2S expression construct (placing expression of GAL4-VP16 under control of *pSPO11-1*, and transactivating a flanking *4xUAS-minimal35S::virD5* reporter construct) to artificially enhance the activity of *pSPO11-1*, as we observed for the *p2S-RPS5A* construct (Figure 8B). Primary transformants expressing VirD5 under control of this 2S construct were only slightly hampered in vegetative rosette development but were fully sterile compared to control plants (Figure 8C). This again corroborated that *pSPO11-1* is likely to be a very meiosis-specific promoter with at most low activity in other tissues. To further investigate the cause of the reduced fertility observed in plants harboring *pSPO11-1* and *pZYP1A*, we analyzed pollen production and pollen viability in five different plant lines for each construct. Staining of stage 12 flower buds^{29,30} showed that pollen production as well as viability were indistinguishable from wild-type plants. Hence, using the promoters mentioned, VirD5 expression did not distort the development of cells prior to the formation of pollen, neither of pollen development itself. It might have been that female gametogenesis was partially distorted, but further experimental exploration of this possibility has not yet been investigated.

Complete raw imaging data are available on [Figshare](#)³¹.

Figure 8. The effects of VirD5 expression under control of the cloned promoter sequences of *SPO11* (*pSPO11*) and *ASK1* (*pASK1*) on fertility and seed set. (A) Cotton blue staining of siliques of primary transformants expressing VirD5 under control of *pZYP1A* and *pSPO11*. Two stained and unstained siliques, respectively, are presented for three individual primary transformants. Siliques of primary transformants harboring the construct *pRPS5A::1803F-GFP* are presented as a negative control. Scale bars represent 2 mm. **(B)** Photos of seeds of three independent primary transformants expressing VirD5 under control of *pSPO11*. Seeds of a representative primary transformant harboring *pRPS5A::1803F-GFP* are presented as a negative control. The seeds were spread on grid paper, with each square representing 1 mm². **(C)** Photos of primary transformants expressing VirD5 under control of the GAL4-based two-step expression system (*p2S-RPS5A::1803F-GFP*). Primary transformants harboring the construct *pRPS5A::1803F-GFP* are presented as a control. Scale bar represents 10 mm.

Discussion

Here, we have described two novel strategies to assess the suitability of portable promoter elements to drive effector protein expression in meiocytes. The first strategy, based on expression of 1803F-GFP fusions under control of promoters of interest, has proven to be a suitable method to investigate such activity in

meiocytes *in vivo* without the requirement of tissue lysis, fixation or staining. The second strategy, based on expression of the cytotoxic *Agrobacterium tumefaciens* virulence protein VirD5, proved to be an additional and partly complementary tool to gather *in planta* cues regarding promoter strength and specificity, especially when combined with available gene expression data sets.

The 1803F-GFP strategy allows for the relatively simple introduction of PCR-amplified promoter fragments into the binary vector pRF 1803F-GFP Kana. Visualization of GFP signal in meiocytes can then be performed directly in the T1 generation after floral dip transformation. In addition, an important advantage of this approach over other available approaches is that promoter activity can be studied *in vivo* without the requirement of disruptive procedures such as lysis, fixation or staining. Finally, the formation of GFP foci localized to the pericentromeric regions of all five chromosomes allows for the discrimination of true GFP signals from autofluorescence as well as for ploidy determination. The 1803F-GFP system could also be combined with other techniques (e.g. chromosome spreading) to draw further conclusions with respect to expression levels or cytology. Of course, just as for any other reporter of gene expression, claims regarding promoter specificity should be interpreted with care. GFP signals in meiocytes could in theory also be due to inheritance of 1803F-GFP transcripts or proteins from progenitor cells.

In the present study we could not detect GFP foci when 1803F-GFP expression was driven by the promoters of *ZYP1A*, *SPO11-2*, *NBS1*, *DMC1* and *MUS81* (Table 2). These genes are by their function known to be expressed during early prophase I of meiosis I¹, but their mRNA levels are relatively low in meiocytes (Table 1). It could therefore be that low and subphase-specific promoter activity is difficult to detect with the 1803F-GFP system. In addition, it is known that meiocytes progress through prophase I of meiosis I relatively quickly³², making the isolation of meiocytes columns which are in a specific subphase of prophase I a very challenging task. The usefulness of the 1803F-GFP reporter system is therefore to some extent limited by the ability to isolate meiocytes in the correct meiotic subphase of interest.

The VirD5 reporter system in principle allows for the screening of promoter elements with tissue-type specific activities without the requirement of any *a priori* knowledge. Valuable biological cues regarding promoter strength and specificity can already be gathered by phenotyping primary transformants expressing VirD5 under control of a promoter element. In addition, when gene expression data sets are available, the VirD5 reporter system can be used to further verify promoter activity in a tissue type of interest, or to confirm that expression patterns are indeed limited to a specific tissue type. It must be realized that expression of VirD5 in cells that support reproductive tissues could also lead to sterility. Further investigation of promoter activity might thus be required when VirD5 expression is observed to trigger sterility.

The strategy of using VirD5 expression as a dominant negative indicator for cell ablation offers an interesting alternative to the use of other negative selection markers, because it specifically interferes with chromosome segregation¹⁹. Its expression is therefore only detrimental to dividing cells, and less likely to be toxic to other types of cells when expressed at low levels. By comparison, the protein synthesis inhibiting diphtheria toxin from the pathogenic bacterium *Corynebacterium diphtheriae*,

which is widely used as a potent negative selection marker (e.g. 33–35), is highly toxic to all cells undergoing mRNA translation and therefore lethal to every cell type, also when expressed at low levels. The VirD5 strategy might therefore offer an interesting alternative by excluding lethality due to biologically insignificant levels of transgene expression.

Conclusions

Altogether the data presented here demonstrated that effector protein expression in meiocytes can be visualized *in vivo* using the 1803F-GFP reporter system, which facilitates the assessment of meiotic promoter activity. The promoters investigated in this study were selected primarily based on published studies on male meiosis^{6,13}, but for future studies the activity and strength of any promoter could in principle be assessed in meiocytes (or other tissue types of interest) using the 1803F-GFP reporter system. In addition, we showed that expression of the *Agrobacterium tumefaciens* virulence protein VirD5 can be used as a novel negative selection marker and can also provide for experimental cues on tissue specificity and strength of cloned promoter fragments of interest. Combining the 1803F-GFP and VirD5 reporter systems with available gene expression data sets could greatly facilitate the identification of portable promoter elements with desired tissue specificity and/or strength.

Methods

Plant material and growth conditions

All plants in this study were grown on soil in a climate-controlled growth chamber at a constant temperature of 20°C, 70% relative humidity, a light intensity of approximately 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of photosynthetically active radiation from SPAR tubes, and at a 16 h photoperiod. All transgenic plants were generated in the Col-0 background.

Molecular cloning of promoter sequences

The selected cloning sequences (Table 1) were PCR-amplified from genomic DNA of Col-0 plants isolated with the CTAB protocol³⁶, using nested PCR with a gene-specific forward primer (Table 4) and four overlapping reverse primers (three gene-specific and one universal reverse primer; Table 4). The PCR conditions are listed in Table 5. Where possible, PCR primers were designed to amplify the full genomic region between the ATG start codon of the gene of interest and the first or last exon of the adjacent gene (depending on the ORF of that gene lying on the positive or negative strand). Due to a lack of suitable primer target sites, the cloned intergenic regions were 300 bp shorter for At1g75950 (*ASK1*) and 170 bp shorter for At3g54670 (*SMC1*). For At1g22260 (*ZYP1A*), the cloned 2102 bp fragment reached up to At1g22275 (*ZYP1B*) and included the complete At1g22270 (*SMO2*) sequence. The *DMC1* gene (At3g22880) is separated by 7280 bp from the neighboring At3g22886 (*MIR167A*) sequence. In this case, we amplified a 3123 bp upstream sequence which includes an 1881 bp *LIMPET1* transposable element. The amplified sequence is 177 bp longer than the reportedly active Col-0 sequence used in a previous study⁷ but might be effectively shorter than the ~3200 bp upstream sequence from the Landsberg *erecta* ecotype which does not contain the *LIMPET1* element³⁷. The forward primer and the universal

Table 4. Primers used to PCR-amplify the promoter sequences of the selected meiotic genes from genomic DNA of Col-0. The genes are listed in locus ID order.

Locus ID	Primer name	Oligo sequence (5'-3')	Restriction site underlined
All	Universal REV	<u>AGCGGCCG</u> CTTATCGTCGTCGTCCTTGTAGTC	<i>NotI</i>
At1g22260	FW	<u>GGTCGAC</u> CTAGATCGGAGATCGAGAAAGTAAGCTACAAAAATG	<i>SaI</i>
	REV1	CGGAGATAAAATTGAAATGAAAAAGATGACGAAATCGATCTATCG	
	REV2	AGTCGGCCATCTCTAGATCGGAGATAAAATTGAAATGAAAAAGATGACG	
	REV3	CTTATCGTCGTCGTCCTTGTAGTCGGCCATCTCTAGATCG	
At1g27710	FW	<u>GGTCGAC</u> TTGAGCAGATGAAAAAACAACAACTAATTCCTAATCCTTGGAAG	<i>SaI</i>
	REV1	TGCTTAGTGACACAATTTGTTGCTTAGAATCTGAAAAAGGAAGTA	
	REV2	GTCGGCCATGATGGCTGCTTAGTGACACAATTTGTTGCTTAGA	
	REV3	CTTATCGTCGTCGTCCTTGTAGTCGGCCATGATGGCTGCTT	
At1g63990	FW	<u>GGTCGAC</u> CGCTTTCACATCTTGCTAGATCTCTCTCTCTCTT	<i>SaI</i>
	REV1	CCGATTCCGCTTTTTTGAATTTTGATTTGAATTTGATTGAGAG	
	REV2	GTCGGCCATTTTGAGATTTTTTCCGATTCCGCTTTTTTGAATTTTGA	
	REV3	CTTATCGTCGTCGTCCTTGTAGTCGGCCATTTTGAGATTTTTTTCC	
At1g75950	FW	<u>GGTCGAC</u> AGATGGTCGTATGATCCAAATCTCTAACTATTTTAAATGTG	<i>SaI</i>
	REV1	TGGAAACGAAGAGAGAAGAGAAAGATTTAGATTTGGGAGATTTG	
	REV2	CTTATCGTCGTCGTCCTTGTAGTCGGCCATGGTTATGGA	
	REV3	CTTATCGTCGTCGTCCTTGTAGTCGGCCATGGTTATGGA	
At3g02680	FW	<u>GGTCGAC</u> AGGTGTTIAGTGCTTGTATATAGGCGCCTATATTAG	<i>SaI</i>
	REV1	CTTCGCTCCAAGAAATCCTTGTTGAAAGGG	
	REV2	TAGTCGGCCATTTTGCTTCGCTCCAAGAAATCCTTGTTG	
	REV3	CTTATCGTCGTCGTCCTTGTAGTCGGCCATTTTGCTTCG	
At3g13170	FW	<u>GCTCGAG</u> CTTACCAAACCTGACTAAGTATGAGACCTCT	<i>XhoI</i>
	REV1	GTTTCAAACTGAAAAATGCAATGGTCGACGAATTTTGTAG	
	REV2	TAGTCGGCCATCTCTTTGAGTTTCAAACTGAAAAATGCAATGGTC	
	REV3	CTTATCGTCGTCGTCCTTGTAGTCGGCCATCTCTTTGAG	
At3g22880	FW	<u>GGTCGAC</u> CACTCTTTTGATATAGGGAGATTCGTTGAGCA	<i>SaI</i>
	REV1	CGCTCTAAGAGTCTCTAAGCTTGAAGAGTGAG	
	REV2	TGTAGTCGGCCATTTTCTCGCTCTAAGAGTCTCTAAGCTTGA	
	REV3	CTTATCGTCGTCGTCCTTGTAGTCGGCCATTTTCTCG	
At3g54670	FW	<u>GGTCGAC</u> CGCTACCAAACCTGACAATACTCCAC	<i>SaI</i>
	REV1	TGGGGGAGAGATCTCGAGTTTGGTTTCAGAG	
	REV2	TGTAGTCGGCCATGATTGGGGGAGAGATCTCGAGTT	
	REV3	CTTATCGTCGTCGTCCTTGTAGTCGGCCATGATTGGG	
At4g20900	FW	<u>GGTCGAC</u> TAGGGCATGGGTTTAGATGTGAAGTGAAGA	<i>SaI</i>
	REV1	TCGATTCTCTGTCAATTTACTCAGATAAAGTTCGAGAAAGC	
	REV2	GTCGGCCATTTCTTTTTCGATTCTCTGTCAATTTACTCAGAT	
	REV3	CTTATCGTCGTCGTCCTTGTAGTCGGCCATTTCTTTTCGATT	
At4g30870	FW	<u>GGTCGAC</u> TGTTGGTTGTATCTTGGGGAGCA	<i>SaI</i>
	REV1	CACTACCCACTCCAAATAGTTTCAATTTATCAAGCG	
	REV2	TGTAGTCGGCCATCACTACCCACTCCAAATAGTTTCAAT	

Locus ID	Primer name	Oligo sequence (5'-3')	Restriction site underlined
At4g40020	REV3	CTTATCGTCGTCGTCCTTGTAGTCGGCCATCACTACC	
	FW	<u>GTCGACT</u> GTTACTGTCTCCGCGATCTGATTTTACGA	<i>Sall</i>
	REV1	TCGGCCATAGATGATAGAATGTTTTTATTCAGCGT	
	REV2	CCTTGTAGTCGGCCATAGATGATAGAATGTTTTTATTCAGCGT	
At5g25980	REV3	CTTATCGTCGTCGTCCTTGTAGTCGGCCATAGATGA	
	FW	<u>GGTCGAC</u> CCAAACTTTTGAGTTCTTGAGTAGGTAACGTCAC	<i>Sall</i>
	REV1	CTTGTGCCAATCTAGGGTTTATATAGCCTTTGTAAAGAAGAGT	
	REV2	GTCGGCCATGGAAGCCTTGTGCCAATCTAGGGTTTATATAGC	
At5g26622	REV3	CTTATCGTCGTCGTCCTTGTAGTCGGCCATGGAAGCCT	
	FW	<u>GGTCGAC</u> CATTGATGACATTCTCTGTAGTTATTGAGCACAAG	<i>Sall</i>
	REV1	AACTTAAAGCTAAGAGAGGACAACAGACGCTTCAC	
	REV2	GTAGTCGGCCATGAATCGACACAACCTTAAAGCTAAGAGAGGACAACAG	
At5g42530	REV3	CTTATCGTCGTCGTCCTTGTAGTCGGCCATGAATCGACAC	
	FW	<u>GGTCGAC</u> CTCTGGATAAGCCATCTGTAGAACGACG	<i>Sall</i>
	REV1	ATTTTGCTTATCAGTGCCACTCATGCTTATGCC	
	REV2	TAGTCGGCCATCTTAATATCCCTCTTATTTTGCTTATCAGTGCCACTCA	
	REV3	CTTATCGTCGTCGTCCTTGTAGTCGGCCATCTTAATATCCCTCT	

Table 5. PCR program used to amplify promoter fragments from genomic DNA with Phusion High Fidelity DNA polymerase (Thermo Scientific) using primers listed in Table 4.

Step	Temperature (°C)	Time (min:s)	Cycles
Initial denaturation	98	3:00	1x
Denaturation	98	0:10	2-4x
Annealing	68	0:20	
Extension	72	1:30	
Denaturation	98	0:10	39x
Extension	72	1:30	
Final extension	72	5:00	1x

reverse primer were added to the PCR mix at the final concentration advised by the manufacturer of Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific). Reverse primers 3, 2, and 1 were added to 1/5, 1/10, and 1/10 of the final concentration advised by the manufacturer, respectively. In this manner, relatively large PCR products of the promoter sequences fused to a FLAG tag and a nuclear localization signal (NLS) at the 3' end, flanked by *Sall* and *NotI* restriction sites (5' and 3' ends, respectively, except for the promoter sequence of *SPO11-1*, which was flanked by *XhoI* and *NotI* sites) could be generated in a single PCR reaction. Although the FLAG tag did not serve a direct purpose in the present study, it can be useful for immunological purposes such as pulldown of meiotic proteins interacting with FLAG-tagged proteins of interest. The PCR

products were ligated into the pJET Blunt cloning vector using the CloneJet PCR Cloning Kit (Thermo Fisher Scientific) and their identity was confirmed by Sanger sequencing (Macrogen Europe).

Construction of pRF 1803F:(GGGGS)₃:GFP Kana

For the construction of pRF 1803F:(GGGGS)₃:GFP Kana, the previously published binary vector pRF EAR Kana¹⁶ was taken as a cloning scaffold. The oligo DNA fragments 'Kpn EAR Sac FW' and 'Kpn EAR Sac RV' (Table 6) were annealed and ligated into *KpnI* and *SacI* digested pRF EAR Kana, thereby removing the *KpnI* site and introducing (among others) *XhoI* and *SpeI* sites into the backbone. Subsequently, oligo DNA sequences encoding the (GGGGS)₃ flexible linker (GGGGS

Table 6. Sequences of DNA oligos that were used for the construction of pRF 1803F:(GGGS)₃:GFP Kana.

Oligo name	Oligo sequence (5'-3')
Kpn EAR Sac FW	AGAGGGCCAGGCGGCCAGTACTAGGCCCTGGCCAGGCCGGCCAACTCGAGTCTAGAACTAGTTTGGATTGGAAATTGAGATTGGGATTGCTTAA TAGGTACCGGAGCT
Kpn EAR Sac RV	CCGGTACCTATTAAAGCAAATCCCAATCTCAATTCCAAATCCAAATCCAAACTAGTTCTAGACTCGAGTTGGCCGGCCTGGCCAGGCCCTAGTACTGGCCGC CTGGGCCTCTGTAC
GGGS FW	TCGAGGGTGGAGGTGGATCCGGAGGAGGAGGTTCGGGAGGCGGAGGTTCAA
GGGS RV	CTAGTTGAACCTCCGCCTCCCGAACCTCCTCCTCCGGATCCACCTCCACCC

FW and RV; Table 6) were annealed and ligated into the backbone between the *XhoI* and *SpeI* sites, yielding the plasmid pRF (GGGGS)₃ Kana. The sequence encoding eGFP was PCR amplified from the plasmid pART7³⁸, and subsequently ligated between the former *KpnI* site and the *SacI* site, yielding the plasmid pRF (GGGGS)₃:GFP Kana. The 1803F encoding sequence^{20,22} was ligated as a *SfiI* fragment into *SfiI* digested pRF (GGGGS)₃:GFP Kana, finally yielding the binary vector pRF 1803F:(GGGGS)₃:GFP Kana.

Construction of binary vectors with meiotic promoters

The cloned promoter sequences were digested from pJET with *Sall* and *NotI* (or *XhoI* and *NotI* in the case of *pSPO11-1*), and were ligated into *Sall* and *NotI* digested pRF 1803F:GFP Kana (containing *pRPS5A*)²⁰. For the construction of a GAL4-based two-step ("2S") expression system (*pRPS5A* or any other promoter of choice driving the expression of *GAL4-VP16*, which transactivates a flanking *4xUAS-minimal35S::1803F:GFP* reporter construct), a 1471 bp DNA sequence with key elements essentially as described previously^{25,26}, but directly compatible with our vector systems was synthesized (BaseClear, Leiden, The Netherlands; Figure 9), and subsequently ligated as an *EagI* fragment into pRF 1803F:GFP Kana predigested with *NotI*.

Cloning the *virD5* ORF in binary vectors carrying different promoters

For the amplification of the *virD5* ORF, genomic DNA of *Agrobacterium tumefaciens* strain LBA1100 harboring the disarmed octopine-type Ti plasmid pTiB6²⁷ was extracted using the DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer's instructions, except that lysis of the cells was performed in TES (10 mM Tris HCl pH 8.0, 50 mM EDTA, 50 mM NaCl) containing 400 µg/ml lysozyme. The *virD5* ORF followed by its TGA stop codon and 54 bp of its downstream non-coding sequence was PCR amplified from the genomic DNA of LBA1100 using the forward and reverse primer combination VirD5 *NotI* FW (5'- ATTAGCGGCCGCTGACAGGAAAGTC-GAAAGTTCAC-3'; *NotI* site underlined) and VirD5 *SpeI* RV (5'- TAATACTAGTTATCAACCAGCGATCGATGC-3'; *SpeI* site underlined). The resulting PCR product was ligated into the pJET Blunt cloning vector using the CloneJet PCR Cloning Kit (Thermo Fisher Scientific) and sequenced by Sanger sequencing (Macrogen Europe). Subsequently, the *virD5* ORF was ligated as a *NotI-SpeI* fragment into pRF 1803F:GFP Kana derivatives described above harboring the cloned promoter sequences of *MUS81*, *NBS1*, *SMC1*, *SPO11-1*, *SPO11-2* and *ZYP1A*, respectively. The relevant restriction sites are depicted in Figure 2.

EagI
TAATAAGCGGCCGCATAAATTGCTGTCTCAATTGAGCAAGCTTGTGATATTTGTAGACTTAAGAAGTTGAAGTGTCAAAGGAAAAACCT
mGAL4-VP16
AAATGTGCGAAATGTTTGA AAAATAACTGGGAATGTAGATATTCGCCAAAACCAAACGAAGCCCTTTAACAAGAGCACATCTCACAGAAG
TTGAGTCAAGATTGGAGCGATTGGAACAATTATTCCTGTTGATCTTCCCAAGAGAAGACCTGGACATGATTTTGAAGATGGATTCTGTACAG
AGACATTAAGGCGCTTTTGGACCGGACTTTTCTGTCAGGACAACGTGAACAAAGATGCGGTAAGTACAGACTAGCTTCTGTTGAGACCGAC
ATGCCGCTTACTCTCAGACAACACAGAATATCCGCCACTTCATCAAGTGAAGAAAGTAGCAATAAAGGACAAAGACAGCTAACTGTCTCGA
CCGCACCGCCAACTGATGTGTCTGCTCGGAGATGAACTGCACCTAGATGGAGAGGATGTGCGAATGGCCACGCTGATGCGTTAGATGATTT
TGACCTCGATATGCTAGGAGATGGTGATTCTCCTGGTCCAGGATTTACGCCTCACGATTCTGCACCATACGGGGCCTTAGATATGGCGGAT
TTCGAGTTTGAACAAATGTTCACTGACGCCCTCGGAATTGATGAATATGGTGGTTGAAGATCTAAGCTTTCGTCCGTATCATCGGTTTCGA
tRbcS-3A
CAACGTTTCGTCAAGTTCAATGCATCAGTTTCATTGCCCCACACCAGAATCCTACTAAGTTTGAGTATTATGGCATTGGAAAAGCTGTTTT
CTTCTATCATTGTTCTGCTTGTAAATTTACTGTGTTCTTTTCAGTTTTTGTGTTTCGGACATCAAAATGCAAATGGATGGATAAGAGTTAATA
AATGATATGGTCTCTTTTGTTCATTCTCAAATTATTATTATCTGTTGTTTTTACTTTAATGGGTTGAATTTAAGTAAGAAAGGAACCTAACAG
TGTGATATTAAGGTGCAATGTTAGACATATAAAACAGTCTTTACCTCTCTTTGGTTATGTCTTGAATTGGTTTGTTCCTTCACTTATCTG
TGTAATCAAGTTTACTATGAGTCTATGATCAAGTAATTATGCAATCAAGTTAAGTACAGTATAGGCTTTTTGTGTCGAATGTTAATTAACA
AGGCGCGCCTCCGGGTGACAGCCCTCCGACGGGTGACAGCCCTCCGACGGGTGACAGCCCTCCGACGGGTGACAGCCCTCCGAATACGAGC
pUAS
ACGGTAGCCGGGATCTCTCGACCTCGAACGTGATCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTTCATTGAGAGGACACGC
pMinimal35S
TGAAAGCTACTCGACTCTAGCCTCGACACGTGTAAGTACGCTCTGTTTCTCTACACAGCCATGGCTGACTACAAGGATGACGATGACA
EagI
AACGGCCGCTAATAA

Start FLAG

Figure 9. The oligo DNA sequence (1471 bp) which was synthesized for the construction of GAL4-based two-step expression constructs.

This procedure deleted the 1803F coding domain as well as the adjacent sequence encoding the flexible linker, replacing it by the VirD5 coding region with its own TGA stop codon. Due to the presence of a *SpeI* site in the cloned promoter sequences of *ASK1* and *MS5*, a similar cloning strategy was followed, except that the *virD5* ORF was PCR amplified using VirD5 *NotI* FW combined with the reverse primer VirD5 *SalI* RV (5'-TAATGTC-GACTATCAACCAGCGATCGATGC-3'; *SalI* site underlined), and was ligated as a *NotI-SalI* fragment into *NotI-XhoI* digested binary vectors harboring the cloned promoter sequences of *ASK1* and *MS5*, respectively. Additionally, in these cases, the *virD5* ORF was followed by its TGA stop codon and 54 bp of its downstream non-coding sequence. As a small but functionally irrelevant difference with the other constructs, the short sequence encoding the flexible linker now remained downstream of the *virD5* ORF (see position of restriction sites depicted in Figure 2).

Plant transformation

Binary vectors were mobilized to the *Agrobacterium tumefaciens* strain AGL1 through triparental mating³⁹. Col-0 plants were transformed with the constructs using the floral dip method⁴⁰. The seed pools resulting from floral dip transformation were sterilized and stratified at 4°C for 3-4 days, after which they were plated on MA medium containing 35 µg/ml kanamycin. After one to two weeks of growth on selection medium, kanamycin-resistant seedlings were transferred to soil.

Dissection of flower buds, tissue preparations and confocal microscopy

Young, unopened flower buds⁶ were harvested from the primary transformants harboring *promoter::1803F-GFP* constructs. For the isolation of male meiocyte columns, anthers were sectioned from the flower buds and placed in a drop of physiological buffer solution (0.9% w/v NaCl, Tris buffered to pH 7.0) on microscope slides. Male meiocyte columns were then released by gently applying slight pressure on the cover slide, thereby also releasing tetrads and pollen into the buffer solution. Chromosome spreads of Col-0 flower tissue were performed as described previously^{41,42}, and subsequently stained with 4',6-Diamidino-2-phenylindole (DAPI). Confocal fluorescence microscopy was performed using a Zeiss LSM5 exciter (Zeiss, München, Germany) with Illuminator HXP 120V and a standard photomultiplier tube (PMT) detector (maximal gain of 1250). Excitation of the tissue was performed at a wavelength of 488 nm. Red fluorescence was collected as a negative control with a 560 nm long-pass filter; GFP fluorescence was collected with a 505-530 nm band-pass filter, with a master gain of 678 ± 66 (mean $\pm$ standard deviation). Images were collected as Z-stacks with a pixel dwell time of 1.7 ± 1.4 s (average $\pm$ standard deviation). All images were collected with the same laser power.

Confocal image analysis

The Z-stack confocal images (.lsm files) were transformed into 3D projections using the '3D project' function of the program Fiji (Image J version 1.48f). Representative projections were compressed to 2D images, and brightness of the images was slightly enhanced using Adobe Photoshop CC 2018 (19.1.5 release) for the sake of clarity of the figures. This was done in the

same way for all images. For the false color images presented in Figure 6 an overlay of the GFP channel and bright field channel was created in Adobe Photoshop, and opacity was adjusted to visualize the GFP signal inside of the tissue. The images were then merged, inverted and the intensity of black pixels was enhanced to qualitatively visualize foci of fluorescence.

Analysis of seed set and seed quality

Siliques of primary transformants harboring *pRPS5A::1803F-GFP*, *pZYP1A::virD5* and *pSPO11::virD5* constructs were harvested in Carnoy's fixative (60% ethanol, 30% chloroform and 10% glacial acetic acid, all v/v) and stored at 4 °C until further use. Siliques were then stained with cottonblue-lactophenol/lactophenol as described previously⁴³. The primary transformants were subsequently allowed to further set seeds (T2), which were collected. Seed quality and homogeneity were assessed by collecting images of T2 seeds on grid paper using a LeicaMZ16FA stereomicroscope (Leica, Wetzlar, Germany).

Data availability

Figshare: Two novel strategies to assess *in vivo* meiotic protein expression in *Arabidopsis thaliana*. <https://doi.org/10.6084/m9.figshare.7808378>³¹.

This project contains raw confocal microscopy images of meiocytes (and other flower bud tissues) isolated from transgenic plants expressing 1803F-GFP under control of the different promoter elements described in this study.

Data are available under the terms of the Creative Commons Zero "No rights reserved" data waiver (CC0 1.0 Public domain dedication).

Plant materials and bacterial strains are available from the corresponding author upon reasonable request.

Grant information

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In this method article, van Tol *et al.* explain two techniques to determine the activity of potential meiosis-specific promoters. They applied both to test 14 promoter sequences that were selected because they correspond to genes with a meiotic function or with high expression levels in meiocytes. The first technique is based on the use of a GFP reporter system to visually detect the chromocenters and the second involves the negative selection marker VirD5, which interacts with kinetochores and disturbs cell division. Both strategies might provide important tools for meiosis studies.

However, I agree with the other reviewers and consider some of the results presented are not completely clear.

In the first paragraph of the introduction there are some statements that need to be revised. The authors state “female meiosis takes place in carpels”: it is more accurate to say that it occurs in the ovary. Later in the text “...upon completion of meiosis (male meiocytes) give rise to four haploid pollen cells”: please consider that after telophase II the PMC gives rise to a tetrad of four microspores. After that, each microspore undergoes two subsequent mitotic divisions to produce one vegetative cell and two sperm cells in the mature pollen grain. At the end of the paragraph, “four haploid pollen cells that are physically attached to each other”: please consider that in dicotyledonous male meiocytes cytokinesis occurs when both meiotic divisions are finished. Do the authors refer to the fact that cytokinesis has not yet taken place at the end of meiosis, and hence the physical connection?

The interpretation of the “meiocytes” shown in Figure 4 based on a smaller number of chromocentres is not correct, I agree with the other reviewers on this. The authors should consider that the nuclei of most cells exhibit a chromocenter pattern ranging from 4 to 10 (mean $\pm$ 8) (Fransz *et al.*, 2002¹; Del Prete *et al.*, 2014²). The comment in the text about Figure 4C: “confirming that these cells were either haploid, or that chromocenters were in close proximity”

should be more specific, as the authors should know whether the images correspond to meiocytes in the first or second meiotic division.

In general, there is no appropriate quantification for the results about the GFP patterns displayed by the different promoters. Five plants (according to the caption in Table 2) have been analyzed. Did they present the same phenotype? Some variability would be expected because it is possible that more than one transgene is introduced during the transformation with *Agrobacterium*, plants are analyzed in heterozygous condition (T1), and transgenes have been introduced in different regions (with a different epigenetic regulation) of the genome.

The way of presenting the images in Figure 5 is quite confusing. In addition to taking into account the cell type, images should be grouped considering the genetic backgrounds to which they belong.

Regarding the strategy based on VirD5 expression, the “unexpected” result for *pSMC1* is actually logical, since SMC1 is indispensable for sister chromatid cohesion, chromosome condensation and DNA repair, not only in meiotic cells (Lam et al., 2005³).

Seed quantification in siliques of transformants expressing VirD5 under control of *pZYP1A* and *pSPO11* is necessary. In addition, a cytological analysis would be useful to determine the effect of the presence of VirD5 on meiotic chromosome segregation. As the authors state “interference with meiotic chromosome segregation by VirD5 could still allow for the generation of a small fraction of balanced gametes with 5 chromosomes”. This could easily be confirmed with a simple cytological analysis. Aneuploidies (which are tolerated in Arabidopsis) could be the cause of the different size of the seeds in transformants expressing VirD5 under control of *pSPO11*. Was this difference in seed size observed in the case of *pZYP1A*?

In conclusion, the results of the two strategies are only coincident for only one of the 14 promoters analyzed (*pSPO11-1*). Using the 1803F-GFP system, the activity of *pSPO11-1* resulted in GFP foci in male meiocytes and VirD5 expression under control of this promoter did not affect vegetative development, but produced a reduction in the seed set. In summary, the strategies presented in this paper could be interesting in determining the meiotic activity of the promoters. However, it would be necessary to improve the experimentation associated with more detailed cytological observations, proper analyses and quantitative analyses of the different observed parameters (GFP foci, seed set content) in several plants. Nevertheless, these methodologies should be combined with information about tissue-specific expression levels and functional analyses of the genes tested.

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Is the rationale for developing the new method (or application) clearly explained?

Yes

Is the description of the method technically sound?

Partly

Are sufficient details provided to allow replication of the method development and its use by others?

Partly

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Meiosis, Arabidopsis, cytogenetics, Homologous Recombination

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 11 June 2019

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In this paper, van Tol and co-authors present a method to test the specificity of promoters to be expressed during meiosis with two different methods. Both are based on published transcriptomic data. Promoters supposed to be expressed in meiosis are used in two different experimental systems. One aims to address GFP at centromeres of meiotic cells/. The other is

based on the expression of an Agrobacterium toxic protein VirD5 that should kill plant cells if expressed during plant development and renders plant sterile if it is only expressed during gametogenesis. In principle, both strategies are very interesting. However, experiments need more controls to obtain any conclusions.

Is the rationale for developing the new method (or application) clearly explained?

The authors could also mention that the need of meiotic specific promoters is a prerequisite to (i) study the function of genes that are essential to cell cycle genes (ii) to inactivate some genes by RNAi or other methods that are ubiquitously expressed but essential to meiosis (iii) to overexpress genes selectively during meiosis....

Is the description of the method technically sound?

There is just one detail. The cloning is supposed to remove the FLAG based on Figure 2 because the Sal1 and Not 1 sites are on each side of the FLAG. However, on Figure 1 the FLAG is kept after the cloning of the promoters

Are sufficient details provided to allow replication of the method development and its use by others? If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

In general, cytological experiments suffer of a lack of appropriate controls.

As mentioned by the other reviewer, RFP cannot be used as a control in Figure 4B. Where are the scale bars in all the figures? It is known that GFP loses its fluorescence in meiotic columns after a few minutes. Details are needed to be sure that control cells and tested cells are visualized in the same conditions. The absence of fluorescence does not mean that the construct is not expressed. It could be expressed for a short period of time. It is very important to visualize meiotic stages by adding either DAPI or propidium iodide. Another method would be to fix the cells and make spreads to be sure of the meiotic stage. In Figure 4C, the absence of DNA staining makes impossible to determine the stage of meiosis. And the assumption of having 5 dots in a meiocyte is wrong. There are stages in which meiocytes have 10 dots, others where they have 5 dots and they are not haploid (Armstrong et al, JCS, 2001¹). In Figure 4D, it is difficult to know which cells are shown. They are no meiotic spreads for sure. Moreover, with the pictures provided it is almost impossible to count the dots. Again in Figure 5, it is always very difficult to determine the stages of meiotic cells.

For the Vir5 experiments, we fully agree with the first reviewer, the sterility should be quantified by counting seed sets.

For the conclusions, when expression is not detected in any cell types, the absence of expression cannot be relied to the weakness of the promoters. The first control would be to be sure that the fragment containing the promoter is functional. For that, complementation would be the best way to be sure of the functionality of the promoter. One of the best example is DMC1 promoter which has been used several times to express different constructs (example in Yang et al, Plant Physiol, 2008²). The lack of expression could mean that the authors have missed the stage of expression and the same explanation could be given for other promoters. As the meiotic stages are not mentioned in this study, it is hard to know if meiosis has been fully explored.

Finally, as mentioned by the other reviewer, SMC1 is known to be expressed outside meiosis (Schubert et al., 2009³). This way that transcriptomic data on meiocytes should be used with precautions.

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Is the rationale for developing the new method (or application) clearly explained?

Partly

Is the description of the method technically sound?

Partly

Are sufficient details provided to allow replication of the method development and its use by others?

No

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

No

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Meiosis, Recombination, molecular genetics

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

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The work from Niels van Tol et al. presents two strategies to assess promoter activity in Arabidopsis meiosis. The identification of promoters with expression specific to meiosis is of interest to the field. However, some limitations in the techniques used and in the experimental designs impaired the conclusions drawn by the authors.

Specific comments:

Page 7, Figure 4B: What is the rationale to use RFP signal as control of autofluorescence for the GFP signal? These two signals have different excitation and emission wavelengths and the RFP signal will not provide any information on how much autofluorescence passing through the GFP filter is detected. A wild type plant without GFP protein is the best control for GFP autofluorescence.

Page 7, Figure 4A and 4B: It is unclear to me whether the cells labelled with GFP are meiocytes or not. It is also unclear at what stage of meiosis the cells are. It is important to use a chromatin dye here, e.g. DAPI. – see below for more comments on this

Page 7, Figure 4C: It is not apparent that we have cells with 10 and 5 chromocenters. It seems to me that there is a range of chromocenters from 3 to 10 – this would support the idea that the number of chromocenters doesn't necessarily reflect the ploidy content but rather reflects specific stages in the cell cycle.

Page 7, Figure 4D: On the 2 zoom-in images ($2n=10$ and $n=5$), it seems to me that none of the two cells presented are meiocytes.

Page 8 - "this dropped to approximately five GFP foci in meiocytes" - I don't understand the idea that cells with less chromocenters are meiocytes. The interest of using this technique is to test for promoter activity in meiocytes. However, the authors have not fully demonstrated that they can identify meiocytes in their assay.

The authors seem to suggest that the number of chromocenters could help differentiate between meiocytes and somatic cells but the authors are not referring to any paper to support their claim. The authors then suggest that a cell with fewer than 10 chromocenters is either "haploid or that chromocenters were in close proximity". The authors didn't comment on the latter possibility and its implication with meiosis, which makes their explanation unclear. The former possibility is that cells are haploid. On the images in figure 4B, the cells seem widely spaced out, ruling out that cells are at dyad-tetrad stage and so if the cells are haploids, there are post-meiosis and the technique is no longer suitable to assess for promoter activity in meiocytes.

More worrying, the authors show two cells on the the 2 zoom-in images ($2n=10$ and $n=5$) in figure 4D. In figure 4D, both cells are somatic cells – the change in chromocenter number likely reflects a change in the somatic cell cycles but there is no meiocyte and no post-meiotic cell on the 2 zoom-in images provided here. There is also no evidence that one of the two cell has reduced ploidy content.

Since the technique described by the authors is intended to test promoter activity in meiocytes, it is thus essential to accurately differentiate meiocytes from somatic cells. Unfortunately, Figure 4

shows no clear evidence for presence of meiocytes. I suggest the authors to include a chromatin dye - meiotic chromatin is very distinctive and more structured (with formation of the meiotic chromosome axis) than somatic chromatin.

Page 10 and Table 2: It is surprising that most promoters from known meiotic genes give no signal. What meiotic stages were used for the analysis? How are the data comparable and how can we accurately assess the sensitivity of the technique if we cannot clearly state the stage of meiosis? This can easily be resolved by adding a chromatin dye to the tissue preparation. If some of the preparation had immature germ cells then the data presented in Table 2 would be inaccurate and not showing promoter activity in meiosis. It is important that the authors address this major point of concern and demonstrate that the analysis was done on meiotic cells at the same stage (ideally prophase I since the authors used promoters from protein-coding genes important in prophase I of meiosis). Chromatin-stained prophase I of meiosis are very distinctive from later stages of meiosis or from somatic cells and the analysis will be more straightforward.

Figure 6: I don't see the rationale for not adding a scale bar for each image. The entire figure is unclear and there is no explanation in the text to help me understand the figure. The data presented here seems to have limited interest and doesn't add more support to the conclusions drawn by the authors and therefore the figure could be removed.

Page 11. The VirD5 expression system is supposed to test promoter activity in meiosis – as suggested throughout the text and in the title. However, in my opinion, this system tests promoter activity during plant life cycle (reproductive) but it will not give indication on cell specificity. The authors acknowledged this limitation in the discussion (p14). The system is based on the reduction of plant fertility, but many reasons could cause this phenotype other than defects in meiosis: defect in the tapetum, post meiosis defects, pollen or ovule viability defects...and therefore, I would describe the system as a method to test promoter activity in floral tissues, irrespective of the cell type.

Page 11 “unexpected for pSMC1, which is more or less a meiosis specific promoter based on available gene expression data”. This is not entirely accurate. SMC1 is a well characterised core component of the cohesin ring complex and is expressed in every cells. There are substantial published data studying smc1 mutant phenotype showing pleiotropic effects. This raises some concerns on the validity to use the expression data for the work presented here.

Table 3: Comparing ratio of expression is not informative – the authors should report values of expression for meiocytes and somatic cells separately. What is the definition of reduced fertility? The authors need more clarity here. Is it a quantitative or a qualitative assessment of plant fertility?

Page 12 “quality of seeds” - what does this mean? The authors should use more technical words.

Page 12 “all positions in the silique ... seeds”. The authors should use a more quantitative methodology. The observations should be better described for the all section on “VirD5 expression under control of meiosis specific promoters pZYP1A and pSPO11-1” and the claims made by the authors lack scientific support.

I suggest the authors to count the number of seed-set per silique

"As the flowers did not show any obvious..." The authors cannot exclude an effect downstream meiosis. A lot of meiotic promoters are active in pollen and because the analyses of pZYP1 and pSPO11-1 specificity in Figure 5 and Table 2 are inconclusive, we cannot rule out this possibility "pSPO11-1 is likely to be a very meiosis-specific promoter with at most ..." This is a vague statement with no scientific support. What about the implication of other cell types in the reproductive system?

"Pollen production as well as viability were indistinguishable from wild type plants...". This data suggests that male meiosis is normal, meaning that this technique is not suited to specifically assess gene expression in meiocytes.

"It might have been that female gametogenesis was partially distorted but further experimental ..." This is quite elusive. The authors are claiming to develop a technique to test for promoter activity in meiosis specifically. Unfortunately, it seems that the VirD5 system isn't specific to male meiosis. The authors should investigate female meiosis if they want to demonstrate that their technique can actually be used to test for meiotic promoter activity.

Figure 8 is vague. The technique used for Figure 8A is arbitrary. The authors could analyse the data using a quantitative methodology, e.g. seed set per silique, silique length, variation between samples and plants, statistical test... Figure 8B: I don't understand what the interest is to present this figure. If the authors want to show that the seeds have different sizes, it is not apparent on the photos. Figure 8C: It seems that the plants on the left panel with virD5 have slightly retarded growth compared to the plants on the right with the GFP system. The authors should comment on this observation, which may suggest that pSPO11-1 isn't meiotic specific.

Page 13 "We have described two novel strategies to ... expression in meiocytes" The authors need to provide more scientific evidence to support this claim for the reasons mentioned above, or rephrase their claim - e.g. virD5 system could be used to assess floral expression specific while the GFP-based method could be used in complement to assess for cell type specificity

Page 14 "GFP foci localized to the pericentromeric ... ploidy determination" – This statement is not entirely accurate. Centromeric clustering can reduce GFP centromeric foci without changing ploidy level.

Page 14 "GFP signals in meiocytes could ... progenitor cells" – This sentence is not clear and need some clarifications.

Is the rationale for developing the new method (or application) clearly explained?

Yes

Is the description of the method technically sound?

No

Are sufficient details provided to allow replication of the method development and its use by others?

Partly

If any results are presented, are all the source data underlying the results available to

ensure full reproducibility?

No source data required

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: meiosis, plant reproduction, cytogenetics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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