Plant Biotechnology Journal

Plant Biotechnology Journal (2014) **12**, pp. 765–777 doi: 10.1111/pbi.12181

A barley PHD finger transcription factor that confers male sterility by affecting tapetal development

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Received 16 October 2013;
revised 18 January 2014;
accepted 10 February 2014.
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Keywords: pollen, plant reproduction, anther, male sterility, barley, MS1.

Summary

Controlling pollen development is of major commercial importance in generating hybrid crops and selective breeding, but characterized genes for male sterility in crops are rare, with no current examples in barley. However, translation of knowledge from model species is now providing opportunities to understand and manipulate such processes in economically important crops. We have used information from regulatory networks in *Arabidopsis* to identify and functionally characterize a barley PHD transcription factor *MALE STERTILITY1* (*MS1*), which expresses in the anther tapetum and plays a critical role during pollen development. Comparative analysis of *Arabidopsis*, rice and *Brachypodium* genomes was used to identify conserved regions in *MS1* for primer design to amplify the barley *MS1* gene; RACE-PCR was subsequently used to generate the full-length sequence. This gene shows anther-specific tapetal expression, between late tetrad stage and early microspore release. *HvMS1* silencing and overexpression in barley resulted in male sterility. Additionally, *HvMS1* cDNA, controlled by the native *Arabidopsis MS1* promoter, successfully complemented the homozygous *ms1 Arabidopsis* mutant. These results confirm the conservation of *MS1* function in higher plants and in particular in temperate cereals. This has provided the first example of a characterized male sterility gene in barley, which presents a valuable tool for the future control of male fertility in barley for hybrid development.

Introduction

Demand for food is increasing because of population growth, urbanisation and increasing affluence in the developing world. The world's population is projected to increase from 6 to 9 billion by 2050, and food needs are expected to increase by 50% by 2030 and 100% by 2050 (FAO, 2008). Providing efficient mechanisms to increase crop productivity is key to addressing these challenges, and the control of plant fertility is critical to this. Barley is of major economic importance with a total UK crop production of >5.5 million tonnes, which is used for animal and human consumption as well as in the malting industry. Yield may be reduced by stress, biotic or abiotic, as well as by inbreeding depression due to its autogamous nature.

Control of male reproduction helps facilitate plant breeding and in particular the generation of high-yielding hybrid varieties, for example, hybrid rice yields 15–20% more than most inbred varieties (Cheng *et al.*, 2007; Zhong *et al.*, 2004). There are currently limited examples of hybrid lines in barley, although Syngenta has recently released HYVIDO, a barley hybrid that guaranties 0.5 t/ha more than conventional varieties (Syngenta, 2013). One reason for this has been the lack of fundamental knowledge and genetic resources for pollen development in barley. Male sterile lines are valuable resources that greatly facilitate the production of hybrids via cross-pollination. However, there are very few examples of characterized genes involved in male fertility in cereals and a dearth of information on the molecular mechanism of pollen development in these crops.

The study of the genes involved in pollen development has principally focused on the *Arabidopsis* model (Ma, 2005) and more recently on rice (Zhang and Wilson, 2009). The transfer of knowledge from these regulatory networks to temperate cereals, for example barley and wheat, has been slow. However, recently, the characterization of important genes in cereal species has been greatly facilitated by the sequencing and annotation of a number of grass genomes. The complete genome of five grass species is available (*Oryza sativa, Sorghum bicolor, Brachypodium distachyon and Zea mays* B73) (International Brachypodium Initiative, 2010; International Rice Genome Sequencing Project, 2005; Paterson *et al.*, 2009; Schnable *et al.*, 2009) and more recently the barley genome (Mayer *et al.*, 2012). Although these grass genomes vary greatly in size, partly due to expansion of retroelement repeats, there is an underlying conserved gene order or synteny (Moore *et al.*, 1995; Wicker and Keller, 2007). This synteny can help in the translation of gene information from models to economically important species.

Much is now known about the regulatory networks in *Arabidopsis* associated with pollen wall development and the role that the anther tapetum plays in this process (Wilson and Zhang, 2009). Pollen formation in rice appears to follow a similar developmental pathway to *Arabidopsis* (Chen *et al.*, 2005; Itoh *et al.*, 2005; Wan *et al.*, 2011; Wilson and Zhang, 2009) with the development of a secretory tapetum. A number of rice male sterile mutants have now been identified and are revealing high conservation to *Arabidopsis* pollen regulatory gene networks. Key *Arabidopsis* transcription factors have been identified in the regulation of tapetal development, including *DYSFUNCTIONAL TAPETUM 1 (DYT1)* (Zhang *et al.*, 2006), *MALE STERILITY1 (MS1)* (Wilson *et al.*, 2001) and *ABORTED MICROSPORE1 (AMS1)* (Sorensen *et al.*, 2003), and many of these have also been shown to be conserved in rice (Wilson and Zhang, 2009). For instance, *OsUTD1 (UNDEVELOPED TAPETUM)* encodes a bHLH protein, which acts after tapetum initiation in an analogous manner to *AtDYT1* (Jung *et al.*, 2005). Furthermore, *OsTDR* (Li

et al., 2006; Zhang *et al.*, 2008) has been shown to play an important role during rice tapetal development and lipid transport and metabolism for pollen wall formation. Phylogenetic analysis suggests that *OsTDR* is an orthologue of the *Arabidopsis AMS* gene (Sorensen *et al.*, 2002). This conservation in function and regulation provides opportunities to identify similar genes in less-well-characterized species.

Despite the economic importance of barley, little has been established relating to the molecular regulation of its pollen development, and to date, no barley male sterile mutants have been characterized. This is partly due to the difficulty of nondestructive staging of floral material, which has now been overcome by the development of vegetative markers linked to key anther stages (Gomez and Wilson, 2012). However, additional problems of slow plant growth, difficulties in transformation and the lack, until recently (Mayer *et al.*, 2012), of full-genome information have hampered progress. In this paper, we present the first characterized male sterility gene in barley (*Hv MALE STERILITY1 (HvMS1)*), which has been identified using genomic information from *Arabidopsis,* rice and the bridging genome of *Brachypodium distachyon.*

Arabidopsis MALE STERILITY1 (*MS1*) is a plant homeodomain (PHD) finger motif transcription factor, which is critical for pollen development (Wilson *et al.*, 2001). The rice *PERSISTENT TAPETAL CELL1* (*OsPTC1*) (*OsPTC1)* gene has recently been identified as the putative orthologue of *AtMS1* (Li *et al.*, 2011), which appears to have a similar function. To date, no related gene(s) have been identified in barley. *AtMS1* shows very specific tapetal expression during microspore development and is not represented in the EST libraries. We therefore capitalized upon the close genomic relationship between *Brachypodium* and barley (Opanowicz *et al.*, 2008) to serve as a valuable link for the identification of conserved genes in temperate cereals. We used the *Brachypodium* genome alongside the rice *PTC1* gene (*AtMS1* orthologue) sequences, to identify conserved regions to amplify equivalent sequences in barley. This approach, combined with RACE-PCR and subsequent functional testing using RNAi lines in barley and complementation analysis in *Arabidopsis,* has led to characterization of the first male sterility gene in barley, *HvMALE STERILITY1 (HvMS1)*. This has demonstrated the conservation of gene function in pollen development between *Arabidopsis* and barley and has provided a valuable tool for the future manipulation of male fertility in barley.

Results

Amplification of the barley putative *MS1* orthologous gene

BLAST analysis using *AtMS1* and its rice orthologue, *OsPTC1*, did not identify any related genes in the barley database. *AtMS1* shows very specific, low-level expression only in the tapetum, from the tetrad breakdown stage to pollen mitosis I, and has not been identified in the *Arabidopsis* EST libraries. It was therefore expected that it would not be represented in barley cDNA libraries. Comparative analysis between the rice and *Arabidopsis* (*PTC1* and *MS1,* respectively) sequences showed moderate similarity (53%, Table 1); however, due to limitations of barley genome sequence availability and the relatively low levels of homology between rice, *Arabidopsis* and barley, a bridging genome of the temperate grass *Brachypodium* was used to identify regions of high conservation for primer design (Figure S1).

BLAST analysis using the *AtMS1* and *OsPTC1* sequences against the *Brachypodium* genome identified a putative orthologue, *Bradi4g31760*, with high sequence similarity to *AtMS1* and *OsPTC1* (Table 1). Alignments between these sequences were used to identify conserved regions for primer design (Figure S2; Table S1), which were used to amplify barley cDNA fragments (Figure 1a–d); sequencing of these showed high similarity to the putative *Brachypodium* orthologue and at a lower level to the *AtMS1* and *OsPTC1* sequences (Table S2). RACE-PCR, using primers based on these partial barley sequences, was used to amplify the full-length sequence (Figure 1e–g). The full-length genomic and cDNA sequences had very high similarities to the rice and *Brachypodium* orthologous nucleotide and protein sequences (Nucleotide: 87 and 91%; Protein: 83 and 83%, respectively, Table 1; Table S2). The barley gene comprised of three exons and two introns; the intron/exon borders in all four species were conserved (Figure 1h). The barley sequence comprised a CDS of 2,016 bps, encoding a predicted protein of 672 amino acids, which included a PHD finger domain at the C-terminal region (Figure 2). This conserved domain is also observed in *AtMS1* and *OsPTC1*; however, the zinc finger domain, which is seen in the *Arabidopsis* MS1 protein, was not present in the barley, rice or *Brachypodium* MS1 sequences (Figure 2). No analysis of the *HvMS1* promoter region was possible due to a lack of sequence availability.

The putative *HvMS1* gene shows expression in the anther tapetum during late meiosis and early microspore development

Wild-type barley plants were analysed by RT-PCR and qRT-PCR to determine the expression of the putative *HvMS1* gene, which was restricted to reproductive tissues (Figure 3) and was seen only in floret samples containing anthers from the tetrad to free microspore stages (Figure 3a samples E and F; staging is shown in Figure S3). No expression was observed before or after these stages, reflecting the strict temporal regulation of expression of the gene. Ears of 3–4 cm, equating to the late tetrad stage,

Table 1 Sequence similarity between MS1 genes (nucleotide and translated protein product) of *Arabidopsis (AtMS1),* rice *(OsPTC1), Brachypodium (Bradi4g31760)* and barley *(HvMS1)*

Gene	*AtMS1* 2194 bp (%)	*OsPTC1* 2040 bp (%)	*Bradi4g31760* 2082 bp (%)	*HvMS1* 2007 bp (%)
AtMS1 (NC_003076)	100	–	–	–
OsPTC1 (NC_008402)	53	100	–	–
Bradi4g31760 (XM_003578154)	54	85	100	–
HvMS1 (AK373836)	56	87	91	100
Protein	**AtMS1 672 aa (%)**	**OsPTC1 679 aa (%)**	**Bradi4g31760 693 aa (%)**	**HvMS1 668 aa (%)**
AtMS1 (NP_197618)	100	–	–	–
OsPTC1 (ADD91695)	41	100	–	–
Bradi4g31760 (XP_003578202)	41	78	100	–
HvMS1 (BAK05033)	42	83	83	100

appear to correspond to the starting point of expression of the putative *HvMS1* gene.

To define the cell-specific expression pattern of *HvMS1*, *RNA in situ* hybridization was performed on transverse sections of wild-type inflorescences containing various stages of developing florets. The *HvMS1* transcript was observed at a low level in the tapetal layer of the anther and only during a short period of time during late tetraspore/microspore release, (spike 3–4 cm) (Figure 3c), corresponding to that observed by RT-PCR analysis. No signal was observed in the sense control samples or in any other anther stages (Figure 3).

Altered expression of the putative *HvMS1* gene by silencing, or overexpression, causes reductions in fertility due to impaired pollen and anther development

HvMS1 function was analysed by RNA-mediated gene silencing in wild-type barley, variety Golden Promise. RNAi constructs were prepared using a 500-bp fragment of the *HvMS1* cDNA sequence (Figure S2), which lacked conserved motifs and did not show significant homology to other barley or *Brachypodium* sequences. Constructs were prepared by Gateway cloning into pBract207, driven by the maize *Ubi1* promoter (Rooke *et al.*, 2000) and used to transform immature barley embryos via *Agrobacterium*-mediated transformation. These RNAi plantlets were transferred to soil and grown until anthesis. Regenerated T0 plantlets were confirmed as transgenic by PCR analysis using vector-specific and *HvMS1*-specific primers (Figure S4; Table S1). Phenotypic analysis showed that several of the confirmed transgenic lines had poorly developed anthers that contained pollen with reduced viability (five lines; Figures 4 and S6). However, despite this reduction in viability, there was no overall decrease in seed set, presumably as sufficient pollen was still available for full fertilization.

Three to six plants generated from each T0 line (five independent lines) were grown until anthesis; these were further confirmed as transgenic by PCR analysis, and for three lines (Lines 14, 19 & 20), the transgene copy number was determined (IDna Genetics Ltd., JIC Norwich; Figure S5a); qRT-PCR analysis indicated that levels of *HvMS1* expression were reduced but varied slightly between tillers (Figure S5b). This was not unexpected as different levels of fertility, and presumably RNAi silencing, were observed between tillers and also individual plants. All of these T1 RNAi lines showed complete sterility in some of their spikes (Figure 4a–c; Figures S6 & S7), alongside occasional normal, fertile spikes. In comparison, wild-type plants were completely normal with full seed set in all spikes (Figure 4d–f; Figures S6 & S7).

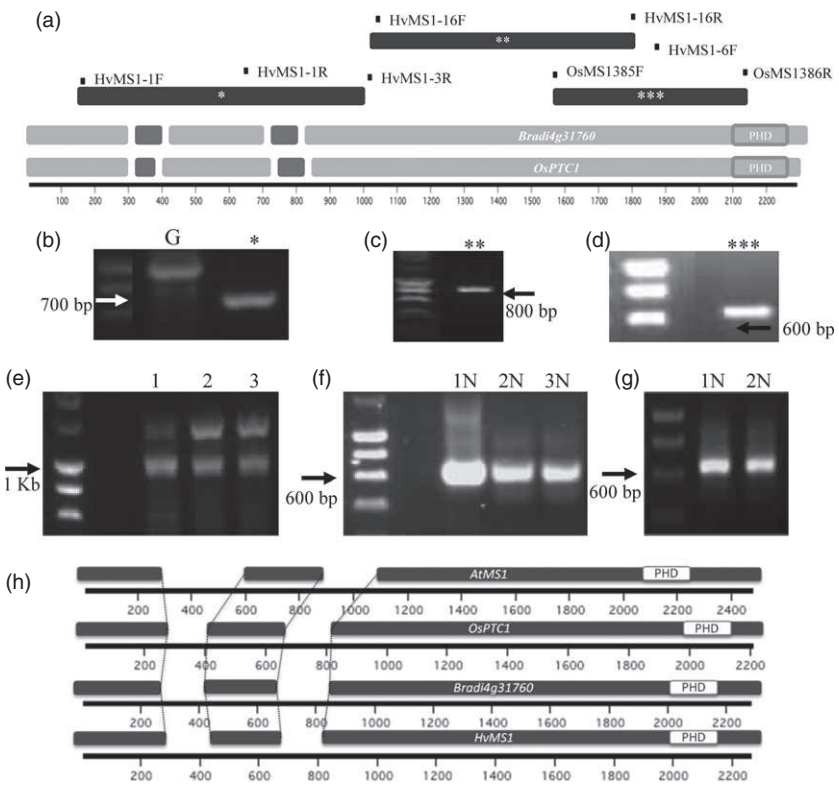

Figure 1 Identification of the putative *MALE STERILITY1* gene from barley. (a) Alignment between the rice (*OsPTC1)* and *Brachypodium* (*Bradi4g31760*) sequences and the putative *HvMS1* amplification products. Black boxes represent the barley PCR products (labels in boxes correspond to gel pictures) amplified using primers (small squares above the alignment) designed upon *OsPTC1/Bradi4g31760* conserved sequences (Figure S2). A PHD domain is indicated for *OsPTC1/Bradi4g31760* at the end of the third exon; Dark grey box: introns. Scale bar: bp. (b-d) *HvMS1* amplification products: (b) PCR product (*) amplified using HvMS1-1F and HvMS1-3R; G: genomic DNA, (c) PCR product (**) amplified using HvMS1-16F/R, (d) PCR product (***) amplified using OsMS1.385F-386R primers. Arrows show band size in marker lane. (e–g) RACE-PCR analysis. (e) Touchdown PCR performed using HvMS1-16F and GeneRacer 3′primers. Lanes 1, 2 and 3 show multiple bands including nonspecific amplifications. (f) Lanes 1N, 2N and 3N show the 3′-nested RACE-PCR using 1 μL of the 3′ RACE reaction and the GeneRacer 3′R-nested primer and the gene-specific nested forward primer. (g) Gradient-nested 5′RACE-PCR using GeneRacer5′-nested F primer and gene-specific nested HvMS1-1R. Tm (°C) = 60 (lane 1N), 61.4 (2N). Arrows show band size in marker lane. (h) Structure of the full-length putative *HvMS1* gene compared with the equivalent sequences in *Arabidopsis*, rice and *Brachypodium*. The gene shows a conserved structure of three exons and two introns in all four species.

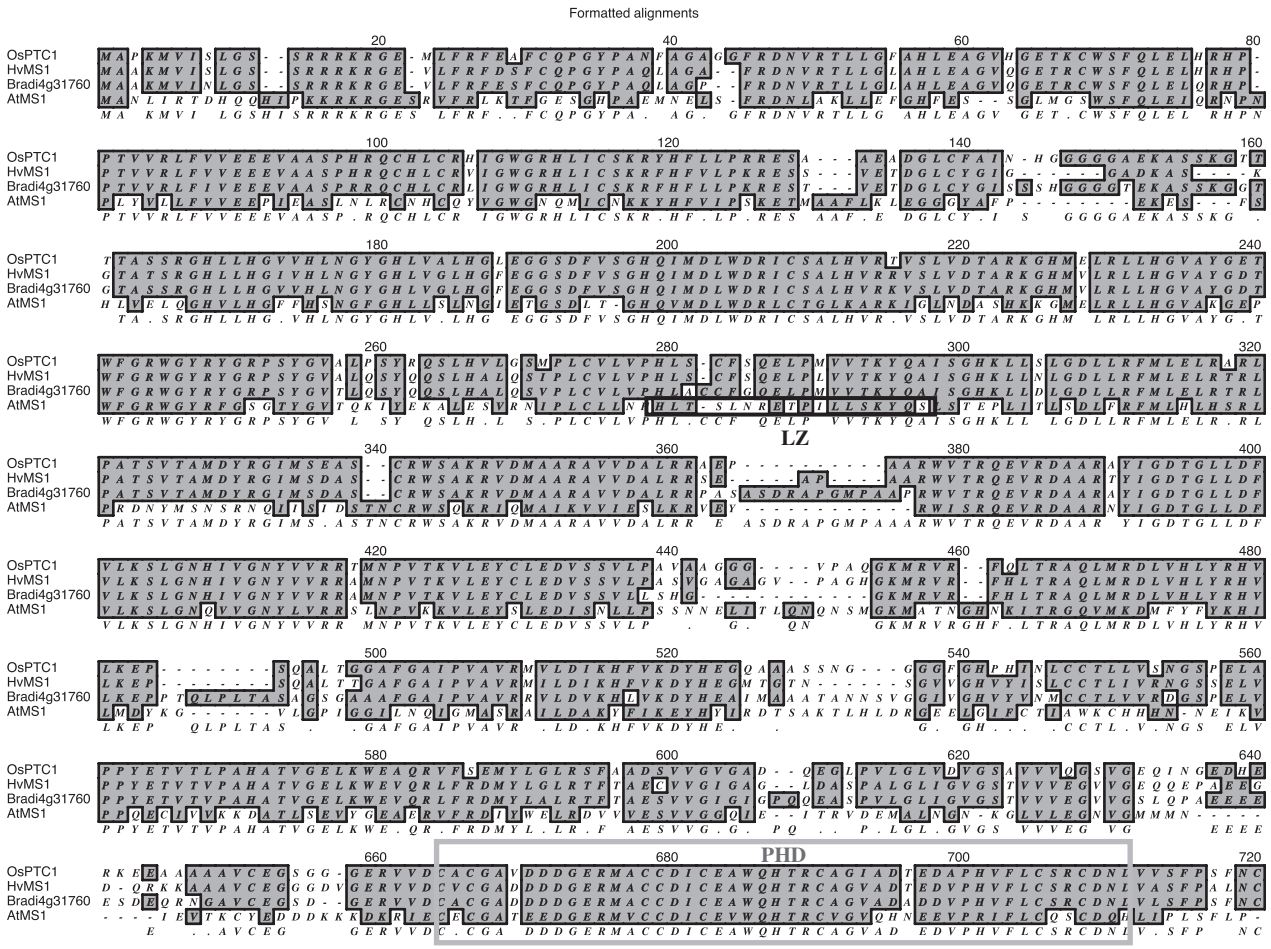

Figure 2 Alignment of the proteins encoded by *AtMS1*, *OsPTC1*, *Bradi4g31760* and *HvMS1*. Black box shows the leucine zipper (LZ) domain, only in the *Arabidopsis* protein, AtMS1. Grey box shows the PHD finger domain, common to the four proteins.

Transverse sections of anthers from sterile spikes of the T1 lines showed premature tapetal degeneration, just after tetrad release (Figure 4g,h). In contrast, the wild-type tapetum remained intact (Figure 4i) and started degenerating significantly later during microspore mitosis stages. Florets collected from the RNAi lines at late heading stage (immediately prior to anthesis) were either empty or contained inviable pollen (Figure 4j–k), in contrast to the wild type that contained abundant viable pollen (Figure 4l).

The effect of ectopic overexpression of the putative *HvMS1* cDNA was also investigated by expressing the full-length *HvMS1* cDNA regulated by the *Ubi1* promoter. This was introduced into barley by *Agrobacterium*-mediated transformation; ten independent transgenic lines were generated and confirmed as expressing *HvMS1* in leaf tissue (Figure 5a); no expression was observed in leaves from nontransgenic control plants (Figure 5a); high levels of *HvMS1* expression was also confirmed by qRT-PCR using floral tissue from these transgenic lines (Figure 5c). These overexpression lines did not display any apparent vegetative changes; however, at anthesis, all 10 lines were male sterile (Figure 5j–l). Sterility did not affect female organs, with full seed set obtained when crossed with wild-type pollen. The pollen from these overexpression lines appeared fully viable (Figure 5m,n); however, a failure of anther dehiscence and pollen release was observed in these lines (Figure 5l). The anther surface showed deposits on the outer anther wall surface of the transgenic lines, which were reduced or absent in the wild type (Figure 5i,o). Abnormal material deposition was observed on the surface of the pollen grains from the overexpression lines (Figure 5u; black arrows), although the nature of this material is unknown. The *Arabidopsis MS1* gene has been previously linked to biosynthesis and secretion of the pollen wall; it is likely that the deposits may be associated with alterations in the production and release of pollen wall materials from the tapetum. The overexpression lines also appeared to have a thicker anther endothecium (Figure 5t (black arrow)) than wild type (Figure 5q (black arrow)), and the anthers remained intact rather than opening (Figure 5s). In addition, traces of the tapetum still remained, indicating that complete tapetum degeneration did not occur (Figure 5t, white arrow). Moreover, pollen inside these anthers was sticky and failed to spread when dissected.

HvMS1 plays a conserved role in plant anther development

To establish the evolutionary relatedness of the putative *HvMS1* and the *AtMS1* gene, functional complementation of the *Arabidopsis ms1* mutant was conducted. A binary plasmid (pGkGWY) carrying the *Arabidopsis MS1* promoter and the *HvMS1* coding region fused with the YFP (yellow fluorescent protein) was introduced into *ms1MS1ttgTTG* heterozygous *Arabidopsis* plants. The resultant segregating generation was analysed for complementation of the *ms1ms1* mutation. Transgenic

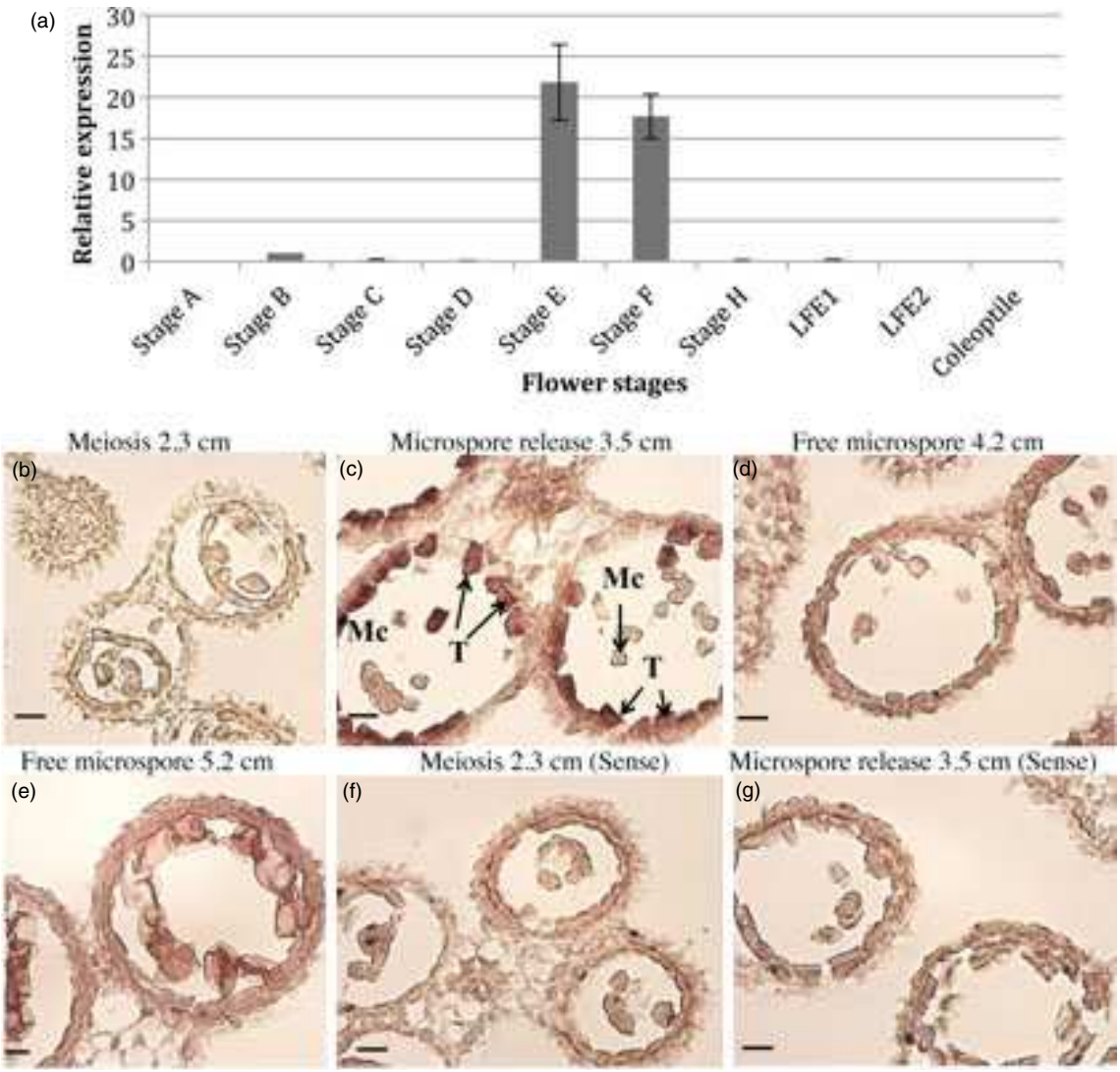

Figure 3 *HvMS1* expression analysis. (a) Quantitative RT-PCR amplification, using primers HvMS1-1F and HvMS1-2R, of barley cDNA from staged florets from sporogenous stage through to vacuolated microspores/microspore mitosis. Samples A to LFE2 were collected based on spike size (according to Gomez and Wilson (2012); Figure S3); A: (0–0.5 cm) primary sporogenous stage; B: (0.5–1 cm) primary sporogenous stage/secondary sporogenous stage; C: (1–2 cm) secondary sporogenous stage; D: (2–3 cm) PMC meiosis; E: (3–4 cm) late tetrad stage; F: (4–5 cm) free microspore stage; H: (>6 cm) tapetal degeneration/microspore mitosis); LFE1: mitosis I; LFE2: mitosis II; amplification of *HvMS1* was only seen in samples E and F. (b–g) HvMS1 RNA *in situ* hybridization to transverse sections of barley anthers. (b–e) Antisense probe; (f–g) Sense control probe. (b) PMC meiosis stage (spike = 2–3 cm, sample: 2.3 cm). (c) Microspore release stage (spike = 3–4 cm, sample: 3.5 cm). (d) Late free microspore stage (spike = 4–5 cm, sample: 4.2 cm). (e) Vacuolated microspore stage (spike = 5–6 cm, sample: 5.2 cm). (f) PMC (spike = 2–3 cm, sample 2.3 cm). (g) Early free microspore stage (spike = 3–4 cm, samples 3.5 cm). Hybridization signal (arrows) was only observed in the tapetal cells surrounding the tetrad at microspore release stage (c); no signal was detected in any of the sense control hybridizations. Bars = 30 μm. T: tapetum; Mc: microspores; PMC: pollen mother cells.

lines were initially screened to identify *ttgttg* homozygous plants; *ttg* is closely linked to the *ms1* mutation enabling early selection at the seedling stage for lack of trichomes (*ttg*) and potential homozygosity at the *ms1* locus. RT-PCR was subsequently used to genotype the segregating transgenic lines to confirm *ms1* homozygosity and *HvMS1* transgene expression. Two of ten lines were identified as *ms1* homozygous mutants, as indicated by RT-PCR expression of a larger fragment due to loss of the first splice donor site in the *ms1* mutant (Figure 6a, samples 1 and 6). All lines showed expression of the *HvMS1* transgene (Figure 6b) except for the *Arabidopsis* wild-type (Figure 6b: Wt1). No genomic DNA contamination was observed in the RT-PCR minus where RNA from samples 1 and 6 was used as template (Figure 6c). In the *ms1* mutant, no viable pollen was observed, and the siliques failed to set seeds (Figure 6g–h). However, *ms1* homozygous lines expressing *HvMS1* showed some viable pollen (Figure 6e) and partial rescue of fertility with some siliques containing viable seed (Figure 6d and f). This confirmed that *HvMS1* was able to functionally complement the *Arabidopsis ms1* mutation and rescue fertility.

Discussion

Significant progress has been made towards understanding pollen development, and much of this has come from the study of male sterile mutants in the model system *Arabidopsis*.

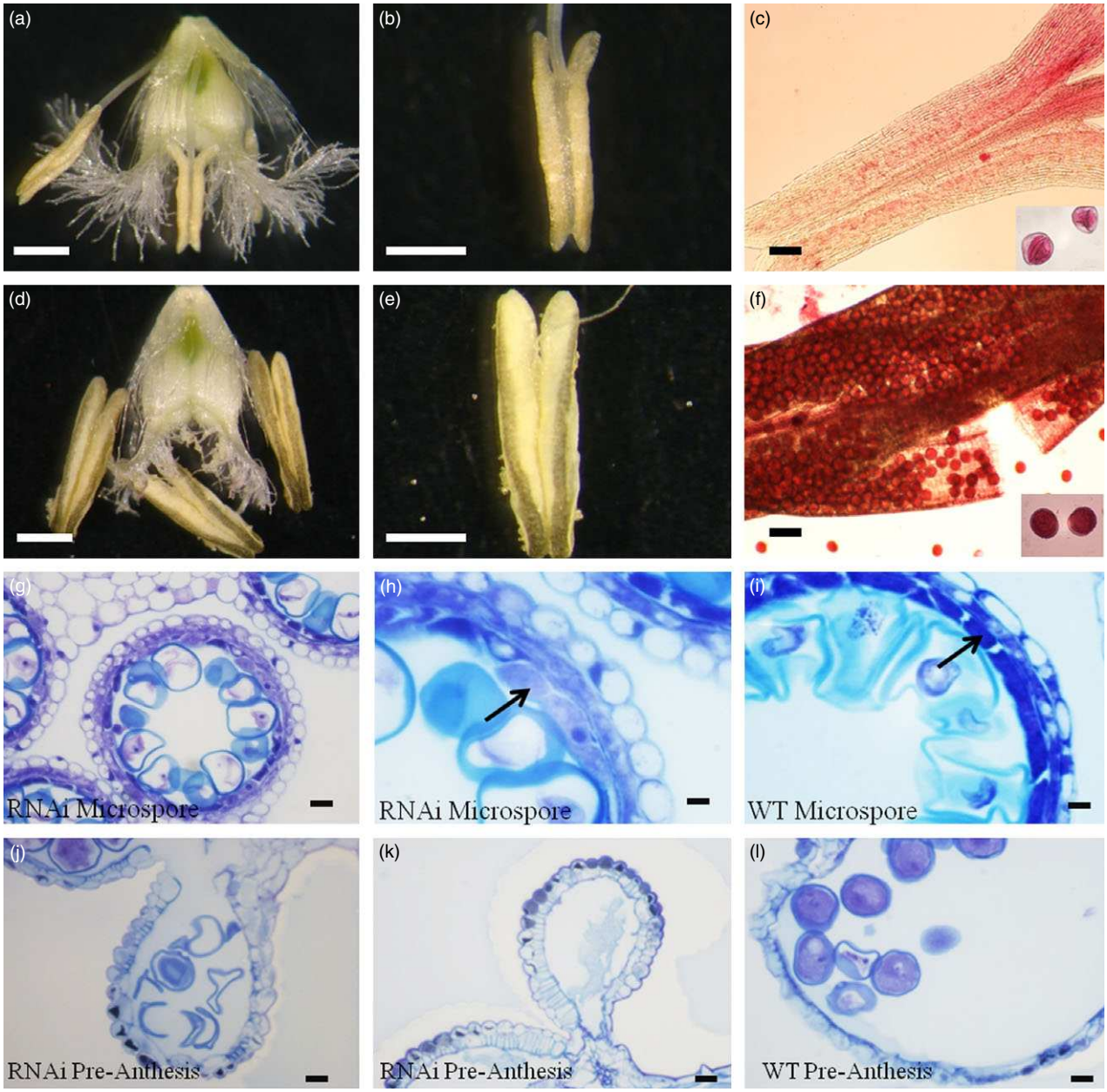

Figure 4 Comparative analysis of anther and pollen development in wild type and T1 *HvMS1RNAi* transgenic lines of barley. (a–c) HvMS1RNAi T1 transgenic line; (d–f) Golden Promise wild-type. (a) Normal pistil development in the *HvMS1RNAi* lines; the anthers were small and undeveloped (b), and contained less pollen and some unviable pollen (c), as indicated by Alexander staining. Silenced lines showed complete sterility through the whole spike, as a result of this lack of viable pollen, whereas pistils appeared normal (a). Normal pistil (d) and anther development (e) were observed in the wild-type material, with dehiscence of abundant pollen visible (e) the pollen was fully viable (Alexander staining (f)). (g–l) Transverse sections through anthers of sterile *HvMS1RNAi* silencing lines and wild type. (g-h) *HvMS1RNAi* lines and wild type (i) at free microspore stage (spikes = 4.5 cm). Silencing lines showed early tapetum degeneration (arrow) (h) when compared to the wild type (i); (j–k) *HvMS1RNAi lines* and wild-type (l) anther sections immediately prior to anthesis. No pollen was observed in the anthers of the silencing lines. Bars: a, b, d and e = 1 mm; c and f = 0.01 mm; g, j, k and l = 20 μm; h and i = 10 μm.

However, the availability of whole-genome sequence information, such as the rice (International Rice Genome Sequencing Project, I.R.G.S, 2005), *Brachypodium* (International Brachypodium Initiative, 2010), potato (Xu *et al.*, 2011), *Sorghum bicolor* (Paterson *et al.*, 2009) and *Zea mays* (Schnable *et al.*, 2009) and more recently the barley genome (Mayer *et al.*, 2012), is now making it possible to carry out these activities in crops. Nevertheless, extension of this knowledge to the temperate cereals, such as barley and wheat, has encountered difficulties, partly due to the lack of genome sequence and annotation of this sequence, but also to the scarcity of genetic resources for functional gene analysis. This is particularly apparent when trying to identify regulatory genes that may be expressed transiently, at a low level, and in a highly cell-specific manner, which are unlikely to be represented in cDNA libraries. We have shown this to be the case in the identification of the barley *MS1* gene which like its

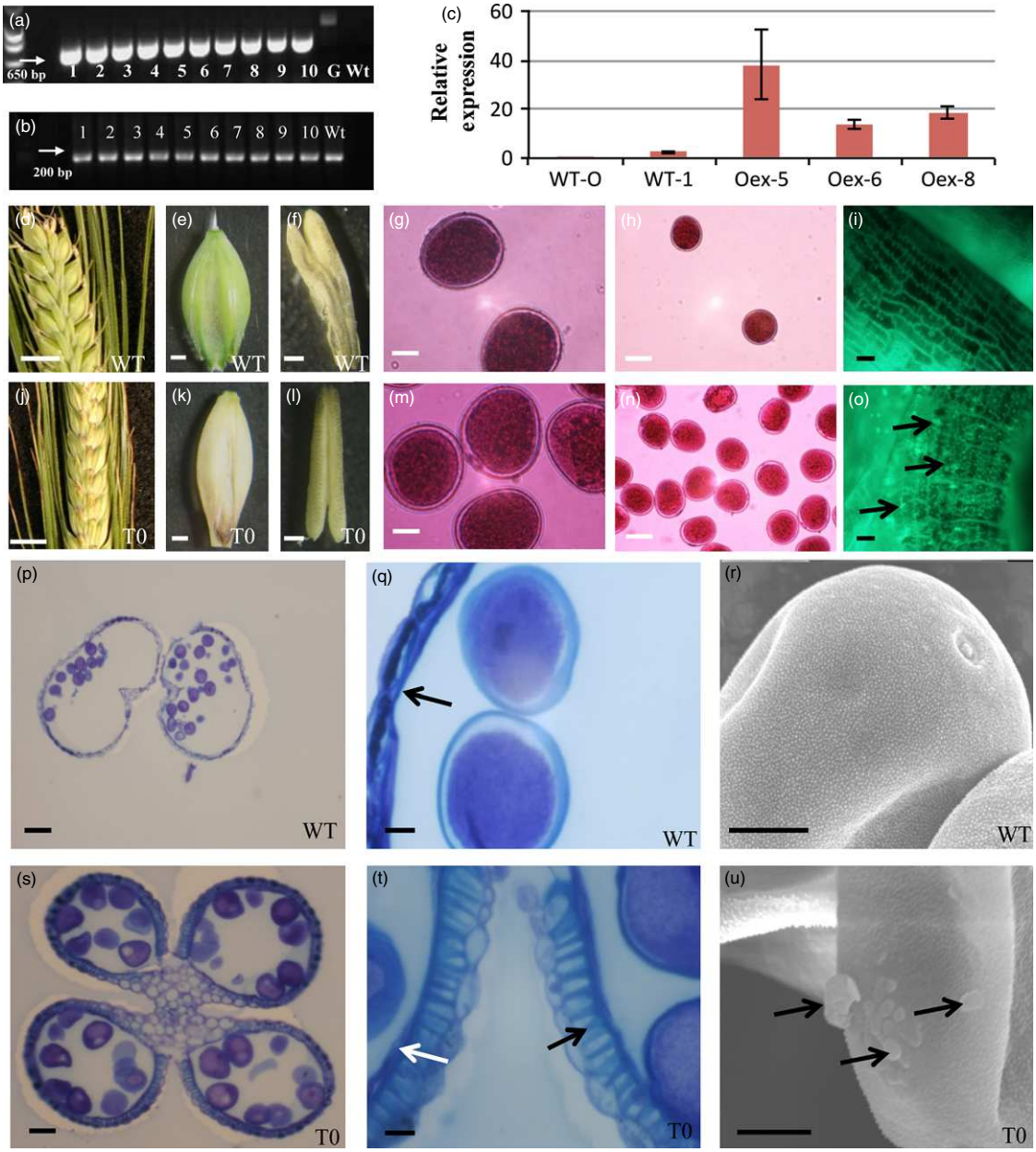

Figure 5 Analysis of *HvMS1* overexpression lines. (a) RT-PCR analysis of *HvMS1* in leaves of T0 *HvMS1* overexpression lines (1–10); no expression was seen in the wild-type barley leaves (Wt). G: Barley wild-type genomic DNA from flowers (Spike 3–4 cm) gave a larger PCR product due to the presence of an intron within the amplified region. (b) RT-PCR analysis of α-tubulin for normalization; samples as in (a). Wt: wild-type leaf cDNA. (c) Quantitative RT-PCR analysis of *HvMS1* in flower tissues; WT: wild type; Oex: Overexpression lines regulated by *Ub1* promoter (samples correspond to those in a). (d–o) Comparison between a representative T0 transgenic/*HvMS1OEx* overexpression line (8) and wild type. (d–i): wild type; (j–o) *HvMS1OEx* overexpression line (T0 generation). (d) Wild-type spike during grain fill phase. (e) Wild-type floret at grain filling stage, (f) Wild-type anther at anthesis, abundant pollen grains were observed, g-h) Wild-type pollen stained with Alexander stain, (i) Wild-type anther wall surface after ethidium bromide staining. (j) T0 transgenic spike at grain filling stage; no grain was observed inside. (k) T0 transgenic floret beyond grain filling stage. No pollen was released in the *HvMS1OEx* line, and therefore, no grain was subsequently observed. (l) T0 *HvMS1OEx* anther at anthesis. Anther dehiscence did not occur, and no pollen was released. (m-n) T0 *HvMS1OEx* pollen grains appeared viable. (o) Anther wall in overexpression line; an extra deposition of an unknown material was observed (arrows). (p, q, s, t) Transverse sections through anthers from wild type (p,q) and T0 *HvMS1OEx* lines (s,t) at anthesis. At this stage, in the wild-type anther, the septum has already broken down, and the pollen is about to be released (p), whereas in the overexpression lines, the septum and therefore the locules remained intact (s), no anther opening and pollen release was observed. In addition, at this stage, overexpression anthers showed a thicker anther endothecium (t: black arrow) and what appeared to be remains of the tapetum (t: white arrow). (r and u) SEM images of barley wild type (r) and T0 *HvMS1OEx* (u) pollen grains. *HvMS1OEx* samples showed abnormal deposits on pollen grains (u: black arrow) that might be related to the sticky appearance of the pollen in the *HvMS1OEx* anthers. Bars: d = 5 mm; e = 1 mm; f = 0.6 mm; g and m = 10 μm; h: 30 μm; n = 60 μm; i and o = 40 μm; j = 5 mm; k = 1 mm; l = 0.6 mm, p: 50 μm; q and t: 10 μm; r and u: 10 μm; s: 100 μm.

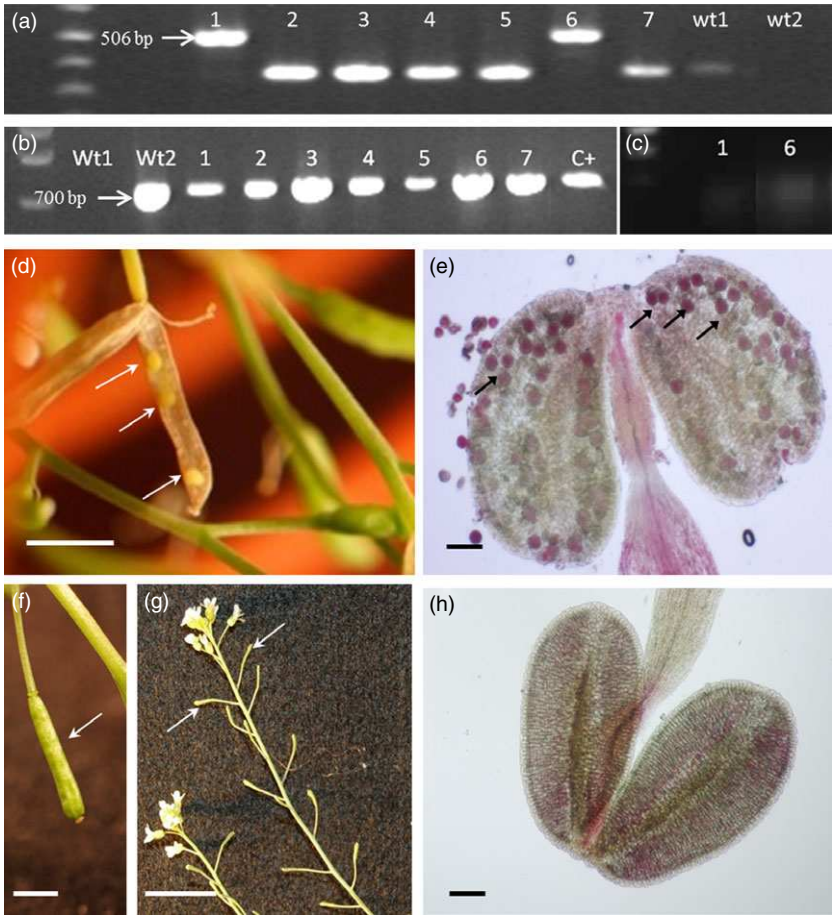

Figure 6 Functional complementation of *Arabidopsis ms1* mutation with *HvMS1*. (a) RT-PCR analysis of *AtMS1* expression in inflorescences from an F2 segregating *Arabidopsis ms1ttg x* L*er* population transformed with pGKGWY*::MS1prom::HvMS1:YFP* (7 of 10 lines shown). Using the LmRNAsp and RRT *MS1* primers (Table S1), the homozygous *ms1* splice mutant generates a 506-bp band from the mutant *ms1* gene, whereas the wild-type L*er MS1* fragment is smaller at 351 bp. The 506-bp mutant transcript was seen in lines 1 and 6, indicating that these are homozygous *ms1* mutants, the other lines all showed the 351-bp WT *MS1* band. WT_1: *Arabidopsis* L*er* inflorescence cDNA; WT_2: Barley inflorescence cDNA; (b) *HvMS1* RT-PCR expression (HvMS1-1F and HvMS1-3R primers, Table S1) was seen in cDNA from all of the putative transgenic inflorescences and the barley wild-type inflorescence material (WT_2); no expression was seen in *Arabidopsis* wild-type inflorescences (WT_1). C+; control of pGKGWY*::MS1prom::HvMS1:YFP* plasmid. (c) RT-PCR minus control. RNA was used as template, and PCR was performed using primers and conditions specified in b. (d–f) *ms1* homozygous line complemented by expression of *HvMS1* under the *AtMS1* native promoter. Siliques set seeds (d; white arrow, and f) and anthers contained viable pollen (e, arrow). (g–h) *ms1* homozygous line. No silique elongation, seed set (g) or viable pollen (h) were observed. Bars: d: 0.5 cm; f: 0.25 cm; e and h: 100 μm; g: 1 cm.

orthologues in *Arabidopsis* (Wilson *et al.*, 2001; Yang *et al.*, 2007) and rice (Li *et al.*, 2011) shows transient expression and is not represented in EST sequence databases. These difficulties have, however, been overcome by utilizing comparative analysis across a number of monocot species; this approach has facilitated identification of the barley MS1 orthologue and provided opportunities for the characterization of other orthologous genes in barley. Sequence conservation was demonstrated using the *Brachypodium* genome, which provided a valuable bridging species between *Arabidopsis*, rice and barley. Syntenic gene order is largely conserved between *Brachypodium* and the small grain cereals (Aragon-Alcaide *et al.*, 1996; Foote *et al.*, 2004; Moore *et al.*, 1993), and this has been instrumental in enabling the characterization of the wheat gene *Ph1* (Griffiths *et al.*, 2006) and the barley gene *Ppd-H1* (Turner *et al.*, 2005). BLAST analysis using *AtMS1* and *OsPTC1* against the *Brachypodium* genome (Bd21 8x release, www.Brachypodium.org) identified *Bradi4g31760*, which has 54% and 85% homology to the *Arabidopsis (MS1)* and rice (*PTC1)* sequences, respectively. The high levels of homology observed imply that this may be the putative *Brachypodium* orthologue, and given the close relationship between *Brachypodium* and barley (Opanowicz *et al.*, 2008) suggested that *MS1* function would be conserved in barley and other monocots.

Identification of the barley *MALE STERILITY1* gene

Amplification using primers to conserved regions of the three *MS1* orthologues, combined with 5′ and 3′ RACE-PCR, enabled identification of the barley putative *HvMS1* gene. This sequence had very high similarity to the rice and *Brachypodium* nucleotide and protein sequences (Table 1) with a PHD finger domain (Cys4-His-Cys3 motif) at the C-terminal region (50–80 amino acids). The PHD finger domain is conserved in animals, yeast and higher plants (Halbach *et al.*, 2000) and found in histone methyltransferase, histone acetyltransferase and chromatin binding, and DNA binding proteins (De Lucia *et al.*, 2008; Pena *et al.*, 2006; Shi

et al., 2006). In plants, their biochemical function is unknown; however, they have been associated with chromatin modification (De Lucia *et al.*, 2008). Phylogenetic analysis of the MS1 orthologues indicated that *Arabidopsis* MS1, poplar PtMS1 and rice PTC1 form a separate group within the entire family (Ito *et al.*, 2007), suggesting a conserved role in plant reproductive development. The HvMS1 protein, and its orthologues in rice and *Brachypodium*, does not contain a leucine zipper motif (LZ), which is present within the AtMS1 protein, suggesting that this may have been associated with the monocot/dicot evolutionary divergence. The *HvMS1* gene showed highly localized temporal and spatial expression in the anther tapetum from late tetrad to early microspore release (Figure 3). This localized expression was consistent with the tissue-specific expression of *AtMS1* and *OsPTC1* in *Arabidopsis* and Rice, respectively (Li *et al.*, 2011; Yang *et al.*, 2007), supporting the hypothesis that *HvMS1* is the orthologue of *AtMS1* in barley.

The *HvMS1* gene plays a critical role in pollen development

The role of the putative *HvMS1* gene was confirmed by reduced pollen viability in the T0 *HvMS1*RNAi transgenic lines (Figure S6b, c), but the presence of some viable pollen was sufficient to maintain general fertility (Figure S6b,c). However, an increase in sterility was seen in the subsequent T1 generation with complete sterility seen in some tillers (Figure 4). This effect was specific to pollen development, with no reductions in female fertility or changes in vegetative growth. The amount of sterile tillers varied between the different T1 transgenic lines but was consistent with lines showing reduced fertility in the T0 generation and correlated with presence of the RNAi construct and reduced expression of *HvMS1* as indicated by qRT-PCR (Figure S5). This complete sterility may be due to the increased transgene copy number in the T1 generation, increasing the silencing capacity beyond the required threshold level (Lindbo *et al.*, 1993). This is a common occurrence, with RNAi lines frequently showing reductions in expression 'knocked down', rather than complete silencing 'knockouts' of the target gene (Yin *et al.*, 2005). For example, (Moritoh *et al.*, 2005) reported that in rice RNAi silencing of the *OsGEN-like* gene, a member of the RAD2/XPG nuclease family involved in microspore development, caused male sterility, but plants showed variable fertility depending on the expression level of the native gene. In the current work, RNAi silencing of the *HvMS1* gene had a variable effect on different tillers within the same plant. The reason for this remains unknown, but may reflect variation in expression levels and stability between independently transformed plants and tillers (Jones *et al.*, 1985; Peach and Velten, 1991; Walters *et al.*, 1992). Alternative options for overcoming this variable silencing and obtaining full sterility could be achieved either by *HvMS1* overexpression, the use of targeted mutagenesis, (TILLING), or via the use of transgene repressor constructs.

Barley *HvMS1* RNAi silencing lines (T1) showed premature tapetum degeneration at the free microspore stage (Figure 4g–h), and by heading, anthers were empty or contained unviable pollen (Figure 4j,k). This was also observed in the *Osptc1* mutant, which showed increased tapetal cell vacuolation at microspore release, and premature cytosolic secretion into the anther locule (Li *et al.*, 2011) and in *Atms1* (Yang *et al.*, 2007). This suggests that MS1 has a conserved function in both monocots and dicots, and acts in the regulation of the breakdown of the tapetum and in the secretion of materials into the locule for pollen wall formation.

Ectopic overexpression of *HvMS1* results in abnormal anther development and functional male sterility in barley

Barley *HvMS1* overexpression lines showed some similar phenotypes to the *Arabidopsis MS1* overexpression plants, which showed occasional male sterility and abnormal growth (Yang *et al.*, 2007), although unlike the *Arabidopsis* lines, no stunted plants or vegetative abnormalities were seen in the barley lines. Closer analysis of these barley lines revealed mature, apparently viable pollen but a failure of anther dehiscence. The reason for the lack of dehiscence and resultant sterility is unclear, but may be due to altered anther endothecium development and sticky pollen. *Arabidopsis MS1* OEx lines had previously been reported to have increased indentation on the anther epidermal surface and to contain sticky pollen that suggested abnormalities in the pollen wall (Yang *et al.*, 2007). A similar sticky appearance due to excess pollen wall materials (Figure 5u), which appeared to limit pollen release, was seen in the *OExHvMS1* lines and may be equivalent to the enhanced lipid biosynthesis observed in *Arabidopsis MS1* overexpression lines (Yang *et al.*, 2007). The barley lines also showed abnormal endothecium expansion and development, with a lack of associated secondary thickening and an increased deposition of cellular materials (Figure 5). The increased expression of *HvMS1* appeared to be causing alteration in cell wall biosynthesis in these anther cell layers (Figure 5q,t). We have previously shown that the structure and thickening in the endothecium are critical for anther opening (Nelson *et al.*, 2012; Yang *et al.*, 2007); therefore, these abnormalities are likely to impair anther dehiscence. This phenotype has not been previously reported in *Arabidopsis* or rice; however, the severity of this effect may reflect increased *HvMS1* expression using the ubiquitin promoter, as compared to those induced in *Arabidopsis* and rice using the CaMV35S promoter. Previous evidence with translational fusions in *Arabidopsis* supports very tight regulation of MS1 expression. It maybe that in these barley *MS1* overexpression lines, the level of ectopic *MS1* transcript is sufficiently high in the endothecium and other anther cell layers that normal development fails to occur.

HvMS1 is a functional orthologue of AtMS1 and can rescue fertility in *Arabidopsis ms1* mutants

Precise control of *MS1/PTC1* expression appears critical for pollen development, as the *MS1* rice orthologue, *OsPTC1*, was able to restore fertility in *ms1ms1* homozygous lines when regulated using the native *Arabidopsis* promoter, but not under control of the CaMV35S promoter (Li *et al.*, 2011). Successful complementation of the *Arabidopsis ms1* mutant was observed using the native *Arabidopsis MS1* promoter to regulate the *HvMS1* gene (Figure 6), confirming that MS1 function is maintained in barley. This work has demonstrated the conservation of the regulatory network of gene expression during pollen development from dicots to monocots, and more specifically within the temperate cereals. It has also demonstrated the value of using bridging genomic information to aid in gene identification. Such approaches are vital in species where full-genome data are not available, either due to the complexities of their genomes or because the species fall into the 'orphan crop' status. This regulatory network conservation will contribute to the identification of barley genes involved in anther and pollen development, and this in turn will provide targets that have application for switchable control of fertility as part of hybrid breeding programmes in cereals.

We have therefore shown that the *HvMS1* gene is critical to pollen development and that when *HvMS1* expression is reduced a male sterile phenotype results. To our knowledge, this is the first example of a functionally characterized gene in barley that has been specifically linked to pollen development. Controlling crop fertility for hybrid development is a key breeding goal, as increased yield is frequently associated with hybrids. Understanding the molecular process of pollen development is critical to achieving this, and the characterization of such regulatory networks provides the first step in this process.

Experimental procedure

Plant material and growth conditions

Two double-rowed spring barley varieties, Optic, (kindly provided by The James Hutton Institute), Golden Promise used for transformation studies and regenerated plants, were grown under controlled conditions (15 °C/12 °C; 16 h photoperiod; 80% RH, 500 μmol/m^2/s metal halide lamps (HQI) supplemented with tungsten bulbs). Seeds were sown in 12-well pots using John Innes no. 3 compost and after 2–3 weeks transferred to 5 L pots containing Levington C2 compost (four plants each). Transgenic barley plants were generated using *Agrobacterium*-mediated transformation of immature embryos (Golden Promise) (Harwood *et al.*, 2008).

HvMS1 amplification

Basic local alignment search tool (BLAST) (Altschul *et al.*, 1990) analysis was carried out using *OsPTC1* (Os09 g0449000), the *AtMS1* orthologue in rice and *AtMS1* against *Brachypodium* (www.brachypodium.org) and barley databases such as DFCI (DFCI Barley Gene Index; http://compbio.dfci.harvard.edu/ tgi/plant.html).

Alignment between *AtMS1*, *OsPTC1* and the *Brachypodium* putative orthologous gene, *Bradi4g31760*, was performed using the Needleman–Wunsch global sequence alignment tool (Needleman and Wunsch, 1970; NCBI). This global alignment was only used on sequences that were expected to share significant similarity over most of their length. These alignments were used to identify conserved regions for primer design (Figure 2, Table S1).

Isolation of *HvMS1* full-length cDNA and cloning

Total RNA was isolated using the Qiagen RNeasy Mini Kit (Qiagen, Manchester, UK) following the manufacturer instructions from wild-type barley florets collected from 3.5 to 4.5 cm spikes (Gomez and Wilson, 2012). 1.5 μg of total RNA was used to synthesize cDNA using the SuperscriptIII cDNA synthesis kit (Invitrogen, Life Technologies, Paisley, UK). Primers designed using the most conserved regions between *OsPTC1* and *Bradi4g31760* (Table S1) were used to amplify the barley putative orthologue. RACE-PCR was conducted to obtain the full length, 5′ and 3′ ends, of the wild-type *HvMS1* mRNA.

RACE-PCR was performed using the GeneRacer kit (Invitrogen) according to the manufacturer's instructions; mRNA was reverse-transcribed using the GeneRacer TM oligo™ primer (Invitrogen). GeneRacer primers (5′ and 3′- Table S1) were combined with gene-specific 5′ (HvMS1-3R.5′) and 3′ (HvMS1-16F.3′) primers, which were designed based on the identified wild-type Optic *HvMS1* sequences. Amplification was carried out using Phusion polymerase (New England BioLabs, Ipswich, MA) and 1 μL of cDNA (200–300 ng/μL). Touchdown PCR amplification was conducted (98 °C, 2 min; five cycles of 98 °C, 30 s and 70 °C, 3 min; then five cycles of 98 °C 30 s; 70 °C, 30 s; finally thirty cycles of 98 °C, 30 s; gradient of annealing temperatures (5′ RACE: 60, 61.4, 64.4, 65 °C; 3′ RACE: 61, 62.5, 63 °C), 30 s and 72 °C, 3 min; 1×72 °C for 10 min). Nested PCR was conducted using 1 μL of the initial touchdown RACE-PCR product and the GeneRacerTM/gene-specific nested primers (5′-nested-HvMS1-1R and 3′-nested-HvMS1-6F; Table S1). Amplification was for one cycle of 98 °C, 30 s; 30 cycles of 98 °C, 30 s; gradient annealing temperature (5′ RACE: 60, 61.4, 64.4, 65C; 3′ RACE: 59 °C), 30 s; 72 °C, 90 s and one cycle of 72 °C for 10 min. The *HvMS1* full-length cDNA was amplified (HvMS1cDNAF/R primers, Table S1) and cloned into pCR8GW according to the manufacturer's instructions (Invitrogen).

HvMS1 expression analysis

Total RNA was isolated from barley florets (Optic variety) using the RNeasy Plant Kit (Qiagen). Florets were collected from the middle zone of the spike at different spike sizes (0–0.5, 0.5–1, 1–2, 2–3, 3–4, 4–5, 5–6 and 6–7 cm) (Gomez and Wilson, 2012). After treatment with DNase (Promega, Southampton, UK), RNA was purified using RNeasy spin columns (Qiagen). 1.5 μg RNA was used to synthesize the oligo (dT)-primed first-strand cDNA using the cDNA synthesis kit (Invitrogen). In parallel to RNA extraction, florets samples were fixed, resin embedded, sectioned and stained (toluidine blue 0.05% (w/v)) (Wilson, 2000).

RT-PCR amplification was conducted using the primers HvMS1-1F and HvMS1-3R (Table S1) and Phusion polymerase (NEB) for one cycle at 98 °C for 30 s, followed by 30 cycles of 98 °C for 30 s; 63 °C, 30 s; 72 °C for 30 s; 72 °C for 6 min. Control alpha-tubulin was amplified using α-Tub F/R primers (Table S1; 94 °C, 3 min; 28 × 94 °C 30 s, 61.3 °C 30 s, 72 °C 30 s; 72 °C 6 min). Quantitative RT-PCR (qRT-PCR) was performed according to Yang *et al.* (2007) using gene-specific primers (Table S1), Brilliant SYBR Green qPCR master mix (Agilent Technologies Inc., Santa Clara, CA) and the LightCycler® 480 Real-Time PCR System (Roche Applied Science, West Sussex, UK). Expression was normalized using the alpha-tubulin gene (Table S1).

In situ hybridization of *HvMS1*

A 500-bp *HvMS1* cDNA PCR product (amplified using primers HvMS1-3 F and HvMS1-3R; Table S1) was inserted into pCR4Blunt TOPO to act as a template for the hybridization probe. The fragment was amplified using M13F/R primers and purified. DIG-UTP-labelled sense and antisense probes were generated by run-off transcription with T3 and T7 RNA polymerases, respectively (Roche Applied Science).

Optic florets from various stages of development were fixed overnight with 4% (v/v) paraformaldehyde in 1xPBS at 4 °C and dehydrated in an ethanol series (30–100% (v/v)), embedded in paraffin (Paramat extra pastille, BDH Laboratory) and cut into 0.8-μm sections using a microtome HM315. Sections were hybridized with probes as previously described (Wilson, 2000) and signal detected using a DIG Nucleic Acid Detection Kit (Roche).

HvMS1 RNAi silencing

The 500-bp *HvMS1* cDNA fragment (HvMS1-3 F and HvMS1-3R; Table S1) was cloned into the pCR8GW donor vector (Invitrogen) and then into the RNAi destination vector pBract207 regulated by the *Ubi1* promoter (http://www.bract.org/constructs.htm#barley). This was then transformed into *Agrobacterium tumefaciens* strain AGL1 alongside the pSoup plasmid and selected on LB plates containing rifampicin (30 μg/mL) and kanamycin (50 μg/

mL). Transformation of immature barley embryos was performed as described by Harwood *et al.*, (2008). After regeneration on hygromycin (50 μg/mL), transformed lines were PCR checked for the transgene (UbiproFw x HvMS1-3R and Iv2Fw x HvMS1-3Fw, Table S1).

HvMS1 overexpression

The full-length *HvMS1* cDNA sequence was amplified using HvMS1cDNA Fw/Rev primers (Table S1), cloned into pCR8GW (Invitrogen) and then into the destination vector pBract214 (*Ubi1* promoter; http://www.bract.org/constructs.htm#barley), transferred into *A. tumefaciens* AGL1 and transformed into barley (Harwood *et al.*, 2008). Transgenic plants were confirmed by PCR using pBract214Fw x HvMS1-3Rv primers (Table S1).

Complementation analysis

HvMS1 cDNA amplified by primers HvMS1cDNAF/R (Table S1) was cloned into the pCR8GW-TOPO vector (Invitrogen) and into the destination vector pGKGWY::YFP (without any promoter) (Zhong *et al.*, 2008). The *Arabidopsis MS1* gene promoter (3000 bp) (Wilson *et al.*, 2001) was amplified by primers MS1promXbaI-Fw and MS1promHindIII-Rv (Table S1), digested with *XbaI* and *HindIII*, ligated into *XbaI* and *HindIII* digested pGKGWY::HvMS1cDNA::YFP, resulting in pGKGWY::MS1prom::HvMS1cDNA::YFP, which was electroporated into *A. tumefaciens* GV3101 and transformed into *A. thaliana* heterozygous *ms1-1MS1ttgTTG* plants by floral dipping (Clough and Bent, 1998). Transgenic plants were screened for the presence of the transgene on kanamycin medium (50 μg/mL).

RNA was isolated (RNeasy, Invitrogen) from *Arabidopsis* T1 line buds; 2 μg of total RNA was used to synthesize cDNA using SuperscriptIII (Invitrogen). Homozygous mutant plants were screened at the seedling stage for the *ttg* mutation, which is linked to the *ms1* mutation. The *ms1-1* mutation results in failure of the splicing of the second intron; therefore, the genetic background of the transgenic lines was confirmed by RT-PCR using the primers LmRNAspFw and RRTRv [Table S1; 94 °C, 3 min; 35 × 94 °C, 30 s, 59 °C, 30 s, 72 °C, 30 s; 72 °C, 6 min; Red-Taq polymerase (Sigma, Dorset, England)]; *ms1-1/ms1-1* homozygous lines showed a larger PCR product (506 bp) than wt *MS1* homozygous lines (351 bp) (Wilson *et al.*, 2001).

Microscopy

Alexander stain (Alexander, 1969) was used to check pollen viability. The stain contains malachite green, which stains the cellulose in pollen walls and acid fuchsin, which stains the pollen protoplasm; aborted pollen grains lack protoplasm and thus fail to stain with acid fuchsin. Anthers from flowers containing mature, nondehisced anthers, just before anthesis were placed into a droplet of stain and shaken gently to release the pollen grains. Viable grains stained dark blue or purple with a defined intact shape; dead grains stained pale turquoise-blue, often with a broken wall structure.

Acknowledgements

This work was supported by a BBSRC Targeted Priority Studentship in Crop Science to JFG. Thanks are due to The James Hutton Institute for providing the Optic seed and to The University of Nottingham, Division of Plant and Crop Sciences for Golden Promise seed. Thanks are also due to Drs Gema Vizcay Barrena, Caiyun Yang and Francisco Federico Nuñez de Caceres Gonzalez, to Mr Ben Taylor for assistance in the phylogenetic tree preparation and to Mrs Doris Klisch and Dr Guillermina Mandiondo for assistance with the qRT-PCR analysis. The authors do not have any conflict of interest associated with this work.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Phylogenetic tree of putative *MS1* orthologous cDNA sequences (the phylogenetic tree was generated using Geneious software).

Figure S2 *AtMS1/HvMS1/OsPTC1/Bradi4g31760* transcript alignment. Boxes show the conserved regions where primers were designed to amplify the barley orthologue *HvMS1*.

Figure S3 Toluidine blue stained transverse sections through wild-type barley anthers showing representative stages of pollen development equivalent to the RT-PCR samples (Figure 3).

Figure S4 Genotyping of T0 Barley RNAi-*HvMS1* lines.

Figure S5 Analysis of RNAi lines.

Figure S6 Phenotypic analysis of anther and pollen development in HvMS1RNAi T0 lines.

Figure S7 Comparison between T1 RNAi ears (Line 14). This line showed complete sterility in some tillers (s), whilst others were completely fertile (f). Bar: 0.5 cm.

Table S1 Primers used for *HvMS1* amplification, RACE-PCR, RT-PCR and genotyping.

Table S2 *HvMS1* PCR products compared with the *Arabidopsis*, rice and *Brachypodium* putative orthologues, *AtMS1, OsPTC1* and *Bradi4g31760*. The size of the barley PCR product is shown in the second column.

Table S3 Comparison between fertile and sterile tillers in T1 and T2 generation of HvMS1RNAi lines.

