The commitment of barley microspores into embryogenesis involves miRNA-directed regulation of members of the SPL, GRF and HD-ZIPIII transcription factor families

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SUMMARY

Microspore embryogenesis is a model for developmental plasticity and cell fate decisions. To investigate the role of miRNAs in this development, we sequenced sRNAs and the degradome of barley microspores collected prior to (day 0) and after (days 2 and 5) the application of a stress treatment known to induce embryogenesis. Microspores isolated at these timepoints were uniform in both appearance and in their complements of sRNAs. We detected 68 miRNAs in microspores. The abundance of 51 of these miRNAs differed significantly during microspore development. One group of miRNAs was induced when the stress treatment was applied, prior to being repressed when microspores transitioned to embryogenesis. Another group of miRNAs were up-regulated in day-2 microspores and their abundance remained stable or increased in day-5 microspores, a timepoint at which the first clear indications of the transition towards embryogenesis were visible. Collectively, these miRNAs might play a role in the modulation of the stress response, the repression of gametic development, and/or the gain of embryogenic potential. A degradome analysis allowed us to validate the role of miRNAs in regulating 41 specific transcripts. We showed that the transition of microspores toward the embryogenesis pathway involves miRNA-directed regulation of members of the *ARF*, *SPL*, *GRF* and *HD-ZIP/III* transcription factor families. We noted that 41.5% of these targets were shared between day-2 and day-5 microspores while 26.8% were unique to day-5 microspores. The former set may act to disrupt transcripts involved in pollen development while the latter set may drive the commitment to embryogenesis.

Key words

Hordeum vulgare, gametic embryogenesis, hc-siRNA, phasiRNA, miRNA, degradome

INTRODUCTION

Gametic embryogenesis (also called microspore embryogenesis or androgenesis) occurs when the microspore, a haploid and uninucleate precursor to the pollen grain, switches from a gametophytic to embryonic fate. This developmental transition is artificially triggered through an inductive stress treatment (Seifert et al., 2016). Microspore embryogenesis is enabled by the totipotency of plant cells and it is a model for developmental plasticity and cell fate decisions in plants (Soriano et al., 2013; Seifert et al., 2016). In the past decades, gametic embryogenesis has been exploited in research and plant breeding to obtain double haploid (DH) plants, as the initially haploid embryos can spontaneously double their chromosomal complement or be induced to do so (Seifert et al., 2016). DH technology is currently employed in many breeding programs in various crop species because it can quickly generate genetically fixed (homozygous), recombinant plants directly from an F1 hybrid (Germanà, 2011; Seifert et al., 2016). Unfortunately, the DH protocol often needs to be developed or fine-tuned on a case-by-case basis, due to a large variability in responsiveness to embryogenesis from one species to another, and even from one genotype to another within a given species (Soriano et al., 2013). One of the major bottlenecks in the process is the lack, or low efficiency, of the switch to the embryogenic pathway (Soriano et al., 2013).

It is assumed that stresses induce an interruption in transcriptional and translational activities which normally lead to pollen formation. This might then restore cell totipotency (due to cellular de-differentiation), and ultimately drive the transition to embryogenesis (Maraschin et al., 2005; Elhiti et al., 2013; Seifert et al., 2016). In other words, for the process to occur, a competent microspore has to stop the expression of genes involved in pollen development, possibly degrade the existing transcripts, and then induce expression of the genes required for commitment to embryogenesis. Although there have been investigations into epigenetic modifications that modulate transcriptional regulation, such as DNA methylation (El-Tantawy et al., 2014), histone

methylation (Berenguer et al., 2017) or acetylation (Li et al., 2014), these processes would not be expected to eliminate existing transcripts. Small regulatory RNAs (sRNAs) can accomplish this role since they can degrade and eliminate target transcripts. Furthermore, sRNAs are known to play important roles in key developmental processes such as patterning of the embryo and meristem, leaf, and flower development (D'Ario et al., 2017). However, the role(s) of sRNAs in gametic embryogenesis remains unclear.

Endogenous sRNAs are 21 to 24-nucleotide (nt) RNAs involved in transcriptional gene silencing (TGS) or post-transcriptional gene silencing (PTGS) (Borges and Martienssen, 2015; Komiya, 2017; Axtell and Meyers, 2018). The 24-nt sRNAs are known to be involved in TGS through the RNA-directed DNA methylation (RdDM) pathway, enabling the establishment and maintenance of DNA methylation (Bond and Baulcombe, 2014). Commonly named “heterochromatic small interfering RNAs” (hc-siRNAs), these sRNAs are known to suppress transposable elements (TEs) and repetitive regions (Matzke et al., 2009; Komiya, 2017). Thus, hc-siRNAs may play an important role in the early stages of development through embryogenesis via reprogramming the genomes of microspores and preserving genome integrity during chromosome doubling or the first nuclear division of microspores.

With a typical length of 21- or 22-nt, microRNAs (miRNAs) function in PTGS by facilitating the degradation or translational inhibition of mRNA molecules with complementary sites (D'Ario et al., 2017; Komiya, 2017). miRNAs have an important role in developmental phase changes since they often direct cleavage of transcripts encoding transcription factors (TFs), thus broadly affecting gene regulatory networks (Jones-Rhoades et al., 2006). Since TFs can be involved in developmental patterning or stem cell identity, miRNAs were proposed to play a role in differentiation by targeting transcripts of regulatory genes responsible for existing expression programs, thereby facilitating more rapid and robust transitions to new expression programs

(Rhoades et al., 2002; Jones-Rhoades et al., 2006). Such miRNA-assisted reprogramming could be a powerful mechanism to direct the microspore developmental transition from gametic to embryogenic cell fate during isolated microspore culture.

Most plant miRNAs perform their repressive regulation through target site cleavage; the sliced sites of target transcripts can be identified by sequencing the 3' remnants of cleavage (Shao et al., 2012). Degradome sequencing, also called parallel analysis of RNA ends (PARE; German et al., 2008; Zhai et al., 2014) or genome-wide mapping of uncapped and cleaved transcripts (GMUCT; Addo-Quaye et al., 2008), allows transcriptome-wide mapping of target cleavage sites by combining a modified 5'-RACE approach with next-generation sequencing technologies (Shao et al., 2012).

Barley (*Hordeum vulgare* ssp. *vulgare*) is considered a model species for molecular analysis of microspore embryogenesis in monocots (Soriano et al., 2013). We recently published an RNA-seq analysis of microspore embryogenesis in barley (Bélanger et al., 2018) on a cultivar (Gobernadora) known to exhibit an exceptionally good response to androgenesis (Marchand et al., 2008). In the work described here, we designed our experiments to address perceived limitations in the previous work, namely by concurrently performing both sRNA sequencing (sRNA-seq) and Parallel Analysis of RNA End sequencing (PARE-seq) for the same set of treatments on which RNA-seq alone was performed previously (Bélanger et al., 2018). This paper provides an extensive resource for functional genomics analyses on the induction of microspore embryogenesis in barley.

MATERIAL AND METHODS

Microspore production, cellular fixation and microscopy

Donor barley plants (*H. vulgare* ssp. *vulgare* cv. Gobernadora, a two-row spring barley) were grown in a greenhouse. Uniform immature spikes containing microspores at the mid-late to late-uninucleate stage were harvested as described by Esteves and Belzile (2014). We performed the analyses described below at three time points: day 0 (from freshly harvested spikes), day 2 (immediately after completion of the stress pretreatment) and day 5 (after three days in culture) as detailed in Bélanger et al. (2018). For samples at day 0, microspores were isolated from freshly harvested spikes containing haploid and uninucleate microspores. The uniformity of these microspores was improved by performing gradient centrifugation (20% maltose-mannitol; 900xg at 12°C). For samples at days 2 and 5, spikes were first subjected to a 48-h pretreatment combining thermal (26°C), osmotic (0.3M mannitol; pH at 5.34) and starvation stresses. After the pretreatment, microspores were harvested and purified via gradient centrifugation (as above). A fraction of these microspores was collected to serve as day-2 samples (i.e. immediately after completion of the pretreatment) while the rest was plated on a two-layer (solid-liquid) embryogenesis-induction medium (Li and Devaux, 2003; Esteves et al., 2014) and cultivated at 28°C for three days. Finally, to maximize the uniformity of the microspores harvested on day 5, we performed another gradient centrifugation (25% maltose-mannitol; 300xg; 12°C). The microspores were produced in four replicates and, after isolation, samples were immediately frozen in liquid nitrogen and kept at -80°C prior to RNA isolation.

Additional samples of microspores were collected, fixed and DAPI stained for microscopic analysis as described by González-Melendi et al. (2005) except that the washing of microspores was performed for 15 minutes twice. Microscopy was performed at the Plate-forme d'Imagerie Moléculaire et de Microscopie of the Institut de Biologie Intégrative et des Systèmes (Université Laval, Québec, Canada) using 10 µl of stained microspores and observed with a Zeiss Axio

Observer.Z1 (Zeiss, Gottingen, Germany) under a UV laser (excitation of 390/22 nm and emission of 460/50 nm).

RNA isolation, library construction and sequencing

Total RNA was isolated using the TriZol Reagent Solution (Applied Biosystems, Foster City, CA, USA) as per the manufacturer's instructions. RNA quality was evaluated using the Agilent RNA 6000 Nano Kit on the Bioanalyser 2100 (Agilent Technologies, Santa Clara, CA, USA). Only samples with an RNA integrity number ≥ 7.0 were kept for library construction. All samples were quantified using the DS-11 FX+ fluorometer (DeNovix, Wilmington, DE, USA). A total of 12 sRNA libraries were constructed from 5 μ g of size-selected RNA using the NEBNext® Small RNA Library Prep kit (New England Biolabs, Ipswich, MA, USA) as per the manufacturers' instructions. Then, total RNAs were combined to form an equimolar composite of all replicates corresponding to microspores at each stage of development. A total of 20 μ g of these RNA composites was used to prepare PARE-seq libraries as described by Zhai et al. (2014). Finally, 50-nt single-end sequencing was performed for these two types of RNA libraries (2 and 0.5 lanes for sRNA and PARE libraries, respectively) on an Illumina HiSeq 2000 at the University of Delaware DNA Sequencing & Genotyping Center (Newark, DE, USA).

Bioinformatics analysis of sRNA-seq data

Using cutadapt v2.9 (Martin, 2011), sRNA-seq reads were pre-processed to remove adapters (Supplementary Table 1) and discard reads shorter than 15 nt. Clean reads were mapped to the barley reference genome v2 (Mascher et al., 2017; available on Ensembl release-44) using ShortStack v3.8.5 (Johnson et al., 2016) with the following parameters: -mismatches 0, -bowtie_m 50, -mmap u, -dicermis 19, -dicermis 25 and -mincov 0.5 transcripts per million (TPM). Results generated by ShortStack were filtered to keep only clusters having a predominant RNA size observed between 20 and 24 nucleotides, inclusively. We then annotated categories of microRNA

(miRNA), phased small interfering RNA (phasiRNA) and heterochromatic small interfering RNA (hc-siRNA).

First, sRNA reads representative of each cluster were aligned to the monocot-related miRNAs listed in miRBase release 22 (Kozomara and Griffiths-Jones, 2014; Kozomara et al., 2019) using NCBI BLASTN v2.9.0+ (Comacho et al., 2009) with the following parameters: -strand both, -task blastn-short, -perc_identity 75, -no_greedy and -ungapped. Homology hits were filtered and sRNA reads were considered as known miRNA based on the following criteria: (i) no more than four mismatches and (ii) no more than 2-nt extension or reduction at the 5' end or 3' end. Known miRNAs were summarized by family and when genomes contained multiple miRNA loci per family, miRNAs were ordered per chromosomal position and renamed based on these genomic positions. Small RNA reads with no homology to known miRNAs were annotated as novel miRNAs using the *de novo* miRNA annotation performed by ShortStack. The secondary structure of new miRNA precursor sequences was drawn using the RNAfold v2.1.9 program (Lorenz et al., 2011). Candidate novel miRNAs were manually inspected and only those meeting criteria for plant miRNA annotations (Axtell and Meyers, 2018) were kept for downstream analyses. Then, the remaining sRNA clusters were analyzed to identify phasiRNAs based on ShortStack analysis reports. sRNA clusters having a "Phase Score" ≥ 30 were considered as true positive phasiRNAs. Genomic regions corresponding to these phasiRNAs were considered as *PHAS* loci and grouped in categories of 21- and 24-*PHAS* loci based on the length of phasiRNAs derived from these loci. Non-phased sRNAs having a length of 24-nt were considered as hc-siRNAs.

Finally, the read-count matrix generated by ShortStack was used to perform an expression analysis for annotated sRNAs (miRNA, phasiRNA and hc-siRNA) and to identify those that were differentially expressed over the course of microspore development. Prior to performing the expression analysis, the read-count matrix was normalized for library size using the TMM method of the edgeR program (Robinson et al., 2010; Robinson and Oshlack, 2010). To assess the

degree of uniformity among replicates of the three developmental stages, we performed an MDS analysis using edgeR. Differentially expressed sRNAs were identified using the generalized linear model (glm) test function of edgeR for developmental transitions: (i) from day 0 to day 2, (ii) from day 2 to day 5 and (iii) from day 0 to day 5. Only sRNAs having both a $\log_2FC \geq |2.0|$ and a $q\text{-value} \leq 1.0E-03$ were considered as differentially expressed. Differentially expressed sRNAs were visualized by heatmap using the R program pheatmap (<https://rdr.io/cran/pheatmap/>).

Bioinformatics analysis of PARE-seq data

To identify miRNA-target pairs in microspores, we performed a degradome analysis for miRNAs annotated in microspores through the sRNA-seq experiment. Using cutadapt v2.9, PARE-seq reads were pre-processed to remove adapters (Supplementary Table 1) and discard reads shorter than 15 nt. Then, we used PAREsnip2 (Thody et al., 2018) to predict all sRNA-target pairs and to validate the effective sRNA-guided cleavage site with PARE-seq reads. We ran PAREsnip2 with default parameters using Fahlgren & Carrington targeting rules (Fahlgren and Carrington, 2010). We considered only targets in categories 0, 1 and 2 for downstream analysis. The gene annotations available on Phytozome V13 and Ensembl Plant release-44 were used to identify *A. thaliana* and *O. sativa* orthologs. Functional annotation of orthologous genes was used to interpret the role of miRNA in microspores.

Data availability

The complete set of raw sRNA-seq and PARE-seq reads were deposited in the Sequence Read Archive under SRA accession number PRJNA634514.

RESULTS AND DISCUSSION

Microspores collected at three key stages capture the switch to embryogenesis

To study the role of sRNAs during the switch from gametophytic to embryonic development, we collected microspores prior to (day 0) or following the application of a pretreatment intended to induce embryogenesis (days 2 and 5) in the barley cultivar cv. Gobernadora. This genotype is known to be highly responsive to gametic embryogenesis (Marchand et al., 2008). We observed, with and without DAPI staining, that (i) day-0 microspores were characterized by having a single nucleus positioned close to the cell wall (Figure 1a); this corresponds to the late uninucleate stage known as the most embryogenic-responsive stage in barley (Kasha et al., 2001a); (ii) day-2 microspores primarily showed a single nucleus migrating toward the center of the cells while a few had two nuclei (Figure 1b), and (iii) day-5 microspores exhibited multiple nuclei (Figure 1c). The microspore populations were homogenous and few or no damaged or dead cells in all stages of development. The phenotypes of microspores on days 2 and 5 were very similar to those described in previous work in barley (Kasha et al., 2001b; Maraschin, 2005). Using a similar protocol, Maraschin (2005) described that a microspore was capable of releasing an embryo-like structure out of the microspore exine wall when microspores initiated nuclear divisions. Since the phenotype of most microspores indicates that nuclear divisions were initiated on day 5, we consider that, by that time, microspores have embarked on a sporophytic development leading to embryogenesis. Our observed phenotypes were highly similar (practically indistinguishable) from those described by Bélanger et al. (2018), thus suggesting an experimental reproducibility in the preparation of these materials.

Sequencing sRNAs at three early stages of gametic embryogenesis

We used sRNA-seq to obtain a comprehensive overview of sRNAs expressed in barley microspores at three pivotal time points in the course of gametic embryogenesis. In total, 412.5 million (M) sRNA reads were generated (136.6 M, 128.8 M and 147.1 M reads for microspores

on days 0, 2 and 5 respectively) over 12 sRNA libraries (three stages of development x four biological replicates). Reads were trimmed to remove adapters giving 394.8 M clean reads. Of this, a total of 150.1 M (38.0%), 133.1 M (33.7%) and 111.6 M (28.3%) reads, respectively, could be assigned as follows: reads with at least one reported alignment, reads with multiple alignments suppressed due to the -m setting (more than 50 sites), or reads that failed to align to the genome. We considered sRNAs having a read length of 20 to 24-nt as “expressed” if their abundance was equal or higher to 0.5 reads per million (TPM). To assess our experimental repeatability, we performed a multidimensional scaling (MDS) analysis for genome-mapped sRNA reads. We observed three highly distinctive, tight clusters indicating that replicates were uniform and that sRNAs expressed in microspores at each stage (days 0, 2 and 5) were similar (Figure 2). The MDS plot generated from sRNA-seq data was nearly indistinguishable from RNA-seq data previously described by Bélanger et al. (2018). Thus, both the phenotypic (above) and transcriptomic data demonstrated a high degree of experimental reproducibility, thus justifying a joint consideration of these two sources of data (sRNA-seq and previous RNA-seq), to more fully describe and interpret the developmental switch of barley microspores in gametic embryogenesis. In total, 53,189 distinct sRNAs were observed in microspores, of which 7,695, 16,066 and 29,428 sRNAs, respectively, mapped, mapped to multiple sites, or did not map to the genome at all. Until now, sRNAs have not been sequenced in barley microspores. In wheat, Seifert et al. (2016) sequenced and analyzed sRNAs for microspores at similar developmental stages. Despite the fact that we analyzed nearly four times more sequences (394.8 M vs 92.5 M clean reads), we detected fewer sRNAs than Seifert et al. (2016). The lower number of sRNAs detected in barley compared to wheat may be due to the greater genome size (16 Gb vs 5 Gb) and higher ploidy level (6x vs 2x) of the latter. For the ensuing analyses, we focused exclusively on sRNAs that mapped to unique sites in the genome. Among these, the large majority were 24-nt long (6,828; 88.7%) while most of the remaining sRNAs were 21-nt (578; 7.5%), sizes that are typically, but not exclusively, associated mainly with hc-siRNAs and miRNAs. A third category of sRNA, the

phasiRNAs, can be either 21-nt or 24-nt. As these three classes of sRNAs are distinct in their biogenesis and functions, we separately categorized, analyzed, and interpreted them as three sRNA groups corresponding to hc-siRNAs, phasiRNAs, and miRNAs.

There is strict temporal regulation of hc-siRNA throughout microspore development

Known to guide *de novo* DNA methylation of various genomic features (Borges and Martienssen, 2015; Axtell and Meyers, 2018), hc-siRNAs are of interest since they can act in reprogramming microspore genomes. Overall, we detected a total of 6,628 24-nt candidate hc-siRNAs. Of these, the abundance of 3,201 hc-siRNAs (48.3% of all) varied significantly over the course of microspore development at a $\log_2FC \geq |2.0|$. Of these, 879 (13.3%), 1,635 (24.7%) and 3,110 (46.9%) loci were identified when contrasting expression on day 0 vs 2, on day 2 vs 5 as well as on day 0 vs 5, respectively. The visualization of differentially abundant hc-siRNAs revealed three distinct groups corresponding to different expression patterns (Figure 3a). In the first group, the summed abundance of many hc-siRNA loci goes down during the stress treatment applied to microspores (Figure 3a; Cluster 1). In day 2, the abundance of these hc-siRNAs decreased prior to reaching their minimum levels in embryogenic microspores at day 5, suggesting that DNA methylation globally decreased during this developmental period via the RNA-directed DNA methylation (RdDM) pathway. In the research conducted in wheat microspores by Seifert et al. (2016), hc-siRNAs having this type of abundance pattern were not described. In the second group, the summed abundance of many hc-siRNA loci increases during the stress treatment applied to microspores (Figure 3a; Clusters 3 and 4). In a vast majority of cases, the abundance of these hc-siRNAs increased in embryogenic microspores. This suggests an activation of the RdDM pathway for specific genomic features corresponding to these hc-siRNA loci in microspores on day 2, i.e. after completion of the stress treatment. A group of hc-siRNAs having a similar abundance pattern was described by Seifert et al. (2016) in wheat microspores. In the third group, the summed abundance of hundreds of hc-siRNA loci increases markedly during the induction of

embryogenesis (Figure 3a; Cluster 2). Most of the hc-siRNAs in this group specifically accumulated in response to the induction of embryogenesis since almost no reads could be detected in microspores on days 0 and 2. Relative to the RdDM pathway, this observation suggests an increase of DNA methylation during the commitment of microspores into embryogenesis. This last group of hc-siRNAs shows an expression pattern consistent with results of Seifert et al. (2016) that described a global increase in 24-nt sRNAs in developing wheat microspores at similar stages of development. Assuming that the abundance of hc-siRNAs and DNA methylation are well correlated, the last group of hc-siRNAs is consistent with observations of El-Tantawy et al. (2014) that measured a progressive increase in DNA methylation from initial microspores to embryos in barley. To summarize, we identified three groups of hc-siRNA exhibiting a strict and distinct temporal regulation. One group drastically decreased in abundance from initial microspores to embryogenic microspores while a second group increased (either abruptly or gradually) in embryogenic microspores. It has been demonstrated that DNA hypomethylation favors microspore reprogramming, totipotency acquisition, and embryogenesis initiation, while embryo differentiation requires *de novo* DNA methylation (Solís et al., 2015; Testillano, 2018). In conclusion, the ability of barley cv. Gobernadora to engage embryonic development might be related to its robust and strict regulation of hc-siRNA in microspores.

24-nt phasiRNAs regulate protein-coding genes in embryogenic microspores

Among sRNAs expressed in plants, phasiRNAs are a distinct group of sRNAs that are easily distinguishable as a product of processive cleavage of double-stranded RNAs in regular increments (duplexes of 21-nt or 24-nt) from a well-defined terminus (Axtell and Meyers, 2018). To our knowledge, phasiRNAs have never been characterized in the gametic embryogenesis system in barley or that of any other species. Thus, we employed sRNA-seq to annotate phasiRNAs expressed in barley microspores. We detected a total of 202 phasiRNA loci (*PHAS* loci) in barley microspores, almost all of which (192; 95%) originated from 24-*PHAS* loci rather

than 21-*PHAS* (10; 5%), loci. All barley *PHAS* loci that we annotated are detailed in Supplementary Table 2. Previous studies describe pre-meiotic (21-*PHAS*) and meiotic (24-*PHAS*) phasiRNA accumulation patterns throughout the development of anthers in maize (Zhai et al., 2015) and rice (Fei et al., 2016). The near absence of 21-nt phasiRNAs that we observed in barley microspores is consistent with the fact that day-0 microspores are post-meiotic cells, and that 21-nt phasiRNAs are produced and accumulate largely in somatic cells (Zhai et al., 2015). Additionally, our observation that all 21-nt phasiRNAs identified in day-0 microspores were significantly repressed in embryogenic day-5 microspores is consistent with the meiotic development patterns reported in the above-mentioned studies in maize and rice. An expression analysis revealed that 90 of the 24-nt barley phasiRNAs that we annotated (46.9% of all 24-nt phasiRNAs) were significantly down-regulated (at $\log_2\text{FC} \geq |2.0|$) during the same period (Figure 3b). Moreover, when we relax the threshold to $\log_2\text{FC} \geq |1.5|$, the proportion of down-regulated 24-nt phasiRNAs reached 85.4% (164 of 192 24-nt phasiRNAs). When considering that microscopic analysis shows that day-5 microspores are engaged in embryogenesis, a sporophytic development, our observed down-regulation of these phasiRNAs is reasonable, given that 24-nt phasiRNAs have been described as exclusive to reproductive development (Arikrit et al., 2013; Zhai et al., 2015; Fei et al., 2016; Yu et al., 2018). Surprisingly, four 24-*PHAS* loci did not follow this general trend, as they were up-regulated in day-5 embryogenic microspores (Figure 3b). Two of these four 24-*PHAS* loci overlapped with protein-coding genes (HORVU1Hr1G006020; HORVU2Hr1G016660), which is not typical of *PHAS* loci, which are commonly derived from long, non-coding RNAs (Komiya, 2017). This observation indicates that the 24-nt phasiRNA pathway can process protein-coding genes after meiosis.

Up-regulation of miRNAs in embryogenic microspores

In barley, very few miRNAs have been deposited in miRBase and no studies have yet been conducted on microspores; thus, a *de novo* miRNA annotation of barley microspores would

provide valuable new information. We annotated a total of 68 miRNAs in microspores, including 62 known and 6 high-confidence novel miRNAs (detailed in Supplementary Tables 3 and 4), according to the criteria for plant miRNA annotation as described by Axtell and Meyers (2018). The 62 known miRNAs covered a total of 30 miRNA families, while each of the new miRNAs represents a candidate novel family. The novel miRNAs we discovered were of relatively low abundance compared to most of the known miRNAs. Thus, it seems likely that our approach to deeply sequence a population of specialized and unique cells in a previously poorly characterized species enabled their discovery. In contrast, Seifert et al. (2016) limited their investigation in wheat to miRNAs already available in miRBase (release 21), excluding known miRNAs conserved with other species, or new miRNA that hadn't yet been discovered. Additionally, Seifert et al. (2016) limited their description to just four of the 66 miRNAs they found in wheat microspores, consequently, it is not feasible to compare our findings with those of Seifert et al. (2016) in wheat. miRNAs are known to be involved in developmental transitions by targeting families of various transcription factors or other development-related genes (Jones-Rhoades et al., 2006; Chen, 2009; Fei et al., 2016). To identify miRNAs that may play a pivotal role in the developmental switch of microspores into embryogenic development, we performed an expression analysis. We found that the members of four miRNA families accounted for 85.1% of all miRNAs expressed in microspores. Multiple miRNA loci contribute to the abundance of these miRNA family members: miR156, miR166, miR167 and miR9662, whose abundance was estimated at 99 TPM, 251 TPM, 115 TPM and 786 TPM, respectively (Supplementary Table 3). We were unable to find other studies describing these miRNAs, either in gametic or somatic embryogenesis for monocot species closely related to barley. However, Wu et al. (2011) reported that miR156, miR168 and miR171 correlated with the acquisition of embryogenic competence in Valencia sweet orange (a citrus species), as non-embryogenic calli did not express these miRNAs in somatic embryogenesis. A similar finding was described in maize as Juárez-González et al. (2019) showed that miR156 and miR166 accumulate in embryogenic calli. It was further shown that

miR156 regulates the expression of *SPL2/4/5/9* thus allowing embryonic competence acquisition in Valencia callus cells (Wu et al., 2011). We detected a significant up-regulation of miR156 expression (6 loci) in microspores from days 0 to 5; thus, it is possible that miR156 may play a pivotal role in barley microspore embryogenesis via the regulation of the *SPL* transcription factor gene family.

We next determined that the abundance of 51 miRNAs (75% of the 68 we annotated) significantly differed between days 0 to 5 of microspore development (detailed in Table 1). In contrast, Seifert et al. (2016) reported that only 4 of 66 miRNAs were differentially expressed in wheat microspores at a similar stage of development. The short (five days total in barley) or long (20 days total in wheat) duration of the stressful treatments applied to microspores might explain the difference in the number of differentially expressed miRNAs between the species. We suggest that sampling wheat microspores over a longer-term treatment may mask expression changes within this interval. That being said, none of the four miRNAs shown to be differentially expressed in wheat were detected in barley microspores, thus we could not make a comparison between the species. In barley, we could distinguish four distinct miRNA expression clusters (Figure 3c; Table 1). One group of miRNAs (Figure 3c; Cluster 2) presented a specific expression pattern: their abundance was strongly induced when the stress treatment was applied to microspores, prior to being repressed when they transitioned to embryogenesis. Among miRNA members of this cluster, we retrieved miR319, miR396, miR398, miR827 and two novel miRNAs (novel.1 and novel.2). The expression pattern of these miRNAs suggests a specific response to the stress treatment applied to microspores. If we consider that stresses applied to microspores will stop pollen grain development, these miRNAs might act by triggering the degradation of key regulators involved in gametic development.

Additional miRNAs were up-regulated in microspores on day 2 after the completion of the stress treatment (Figure 3c; Table 1). The abundance of these miRNAs stayed stable (Figure 3c; Cluster 3) or continued to increase when microspores gained their embryogenic potential on day 5 (Figure 3c; Cluster 1). Among those showing the strongest up-regulation in microspores on day 5 (Table 1), we retrieved miR156, miR159, miR166, miR167, miR171, miR5071, miR1432 and a novel miRNA (novel.3). It seems likely that these miRNAs play a role in the modulation of the stress response, the repression of gametic development, and/or the gain of embryogenic potential. Since the accumulation of all these miRNAs globally increased and stayed high in microspores from day 0 to day 5, they may play a role in the dosage of target transcripts rather than eliminating them altogether. In citrus somatic embryogenesis, concordantly, it was shown that miR156 and miR171 accumulated in embryogenic calli and were correlated with the acquisition of embryogenic competence (Wu et al., 2011). Wu et al. (2011) also reported that miR159 was correlated with the formation of globular-shaped embryos, while miR166 and miR167 were required for cotyledon-shaped embryo morphogenesis. In our biological system, the latter three miRNAs were observed in microspores initiating their first mitotic divisions, a much earlier developmental stage than those described by Wu et al. (2011) in citrus. Although the three latter cases showed a distinction in gametic and somatic embryogenesis systems, more studies are needed to better describe their roles.

The commitment of microspores into embryogenesis is associated with the miRNA-directed cleavage of ARF, SPL GRF and HD-ZIPIII transcription factor families

To better investigate the role of miRNAs in the developmental switch of microspores, we performed an analysis of miRNA-target interactions. Since transcript cleavage is the principal mechanism of post-transcriptional regulation of miRNAs in plants (Jones-Rhoades et al., 2006; Voinnet, 2009; Shao et al., 2012), we sequenced the 5' ends of uncapped transcripts to validate miRNA-directed transcript cleavage sites. A total of 141.4 M reads covering one replicate of

microspores at the three stages of development (46.8 M, 41.5 M and 53.1 M reads for microspores on days 0, 2 and 5, respectively) was sequenced, mapped to reference protein-coding transcripts and used to validate putative cleavage sites for the microspore-expressed miRNAs we identified in this study (62 known and 6 novel miRNAs). Using these PARE data, we validated a total of 41 distinct miRNA targets in microspores over the three time points. Notably, we were able to validate the cleavage site for one target of the novel.3 miRNA annotated in this study (Table 2; Supplementary Table 5). This result provides strong evidence to confirm our annotation of this novel miRNA. Overall, we validated a total of 8, 25 and 31 miRNA targets in microspores on days 0, 2 and 5, respectively (Supplementary Table 5). We noted that 41.5% of these targets were shared between day-2 and day-5 microspores while 26.8% were unique to day-5 microspores (Table 2). The former set may act to disrupt transcripts involved in pollen development while the latter set may drive the commitment to embryogenesis. Although we deeply sequenced populations of unique cell types, we found the total number of validated targets to be lower than expected. This could be due, in part, to unresolved 5' UTRs, known as the principal target site of miRNAs, of barley reference transcripts. Alternatively, we don't know the kinetics of transcript degradation after miRNA-directed cleavage of targeted transcripts; thus, the turnover of cleaved targets might be so rapid that we were unable to capture the uncapped transcripts and validate these miRNA-directed transcript cleavage sites.

To better understand the roles that miRNAs may play in the interruption of pollen development, we explored their targets detected in microspores by day 2. In day-2 and day-5 microspores, PARE tags validated the cleavage of HORVU2Hr1G121110 by miR167 (Table 2). This gene is orthologous to *ARF8* in rice. Plant genomes encode several *AUXIN RESPONSE FACTORS* (*ARF*), transcription factors that activate minutes after auxin stimulation, and are involved in various biological developmental processes (Duarte-Aké and De-la-Peña, 2016). Partially redundant, *ARF6* and *ARF8* regulate the maturation of the stamen and the gynoecium by

regulating the expression of several developmental genes that coordinate the transition from immature to mature fertile flowers (Nagpal et al., 2005). Loss-of-function mutations in *ARF6* and *ARF8* result in plant sterility (Nagpal et al., 2005). Therefore, our results suggest that miR167 can indirectly interrupt the pollen developmental program via the post-transcriptional regulation of the *ARF8* gene in microspores on day 2. A recent study by Su et al. (2016) showed that over-expression of miR167 in Arabidopsis inhibited somatic embryogenesis; similarly, loss-of-function in *ARF6* and *ARF8* genes resulted in somatic embryogenesis defects. Su et al. (2016) concluded that the miR167-*ARF8* regulation module controls somatic embryogenesis in Arabidopsis. Accordingly, our results suggest that miR167 controls the accumulation of *ARF8* in barley microspores on day 5 and promotes the commitment to embryogenesis.

During the same period of development, we found that miR1130 regulates an ortholog (HORVU5Hr1G044640) of the *ZIFL1* gene (Table 2). A member of the Major Facilitator Superfamily (MFS) transporter in Arabidopsis, the *ZIFL1* gene modulates root auxin-related processes and mediates drought stress tolerance by regulating stomatal closure (Remy et al., 2013). Functional heterologous expression of this plant protein in yeast showed that *ZIFL1* confers increased resistance to auxin-derived herbicides through a reduction of the intracellular concentration of the herbicide 2,4-D (Cabrito et al., 2009). In that study, the herbicide Dicamba was added as a source of auxin to the embryogenesis induction media, suggesting that miR1130 may regulate intracellular auxin concentration through the regulation of the *ZIFL1* gene during the induction of microspore embryogenesis. Additionally, controlling auxin transport and accumulation in microspores may modulate the transcription of *ARF* genes. Thus, we suggest that together, miR1130 and miR167 may act to regulate the concentration of auxin accumulation in microspores, and the subsequent response to this hormonal stimulus.

To study the role that miRNAs can play during the commitment of microspores to embryogenesis, we explored targets exclusive to day-5 microspores. Most of the targets were orthologous to members of the *SQUAMOSA PROMOTER-BINDING-LIKE (SPL)*, *GROWTH-REGULATING FACTOR (GRF)*, and *HD-ZIP III* transcription factor families. Our results show that miR156 regulates barley orthologs of the rice *SPL2* (HORVU3Hr1G094730) and *SPL14* (HORVU0Hr1G020810) gene in embryogenic microspores (Table 2). Neither *SPL2* or *SPL14* genes have been previously shown to be involved in gametic embryogenesis. However, the miR156-*SPL* regulatory module was inferred to enhance the acquisition of the embryonic competence of citrus calli (Wu et al., 2011; Wu et al., 2015; Long et al., 2018) in somatic embryogenesis by regulating *SPL2/4/5/9*. Also, we observed that miR396 regulates several members of the *GRF* family of genes (Table 2), namely *GRF3* (HORVU2Hr1G101770), *GRF4* (HORVU0Hr1G016590; HORVU0Hr1G016610; HORVU0Hr1G026650; HORVU6Hr1G068370), *GRF5* (HORVU7Hr1G008680) and *GRF12* (HORVU2Hr1G094470). Many plant genomes encode several *GRF* proteins known to be involved in multiple developmental processes (Liebsch and Palatnik, 2020). In rice, *GRF4* has been described as a positive regulator of genes that promote cell proliferation (Hu et al., 2015; Sun et al., 2016) and the miR396-*GRF* module may regulate cell proliferation in meristematic tissues. Furthermore, rice *GRF4* activates transcription of expansin promoters in protoplasts suggesting a potential function in cell expansion (Liebsch and Palatnik, 2020). We speculate that miR396 controls the accumulation of *GRF* genes to facilitate sufficient mitotic divisions in microspores to generate a multicellular structure prior to the activation of the embryo maturation program. While none of the miR396-*GRF* interactions that we identified have previously been implicated in gametic embryogenesis, Szczygiel-Sommer and Ga (2019) showed recently that the induction of somatic embryogenesis was enhanced by the regulation of *GRF1*, *GRF4*, *GRF7*, *GRF8* and *GRF9* genes by miR396; this contributed to controlling the sensitivity of tissues to auxin treatment and enhanced embryogenesis induction in Arabidopsis. Finally, we found that miR166 targeted a barley ortholog to the Arabidopsis

REVOLUTA gene (*REV*; HORVU1Hr1G041790) (Table 2). In Arabidopsis, PHABULOSA (PHB), PHAVOLUTA (PHV) and REV are all members of the HD-ZIPIII transcription factor family, and are regulators of early zygotic embryo development (Nowak and Gaj, 2016). As proposed by Wu et al. (2015), the miR166-*REV* hub may prevent precocious activation of the embryo maturation program in citrus somatic embryogenesis. Although the roles of *SPL*, *GRF* and REV are likely distinct during induction of embryogenesis, the regulatory activity of miR156-*SPL*, miR396-*GRF* and miR166-*REV* modules in gametic or somatic embryogenesis in eudicot or monocot species suggests a functional conservation of these regulatory hubs during the acquisition of embryogenic potential for both systems.

CONCLUDING REMARKS

Our work, and that of Bélanger et al. (2018), contribute a database of gene expression (RNA, sRNA and PARE sequencing) in microspore development during embryogenesis. We chose to use the barley cultivar Gobernadora due to its high propensity for microspore conversion to embryos, despite the fact that Gobernadora is highly susceptible to albinism. Previous gametic embryogenesis studies were performed on distinct species, varieties for a given species, and using diverse experimental approaches such as various stress pretreatments and embryogenesis media formulation, thus limiting our ability to compare studies and to identify molecular determinants of microspore development. We hope that additional studies using the same barley cultivar (cv. Gobernadora), pretreatments, and culture protocols (isolated microspores) will be used for various approaches, including genomics, epigenetics, transcriptomics or proteomics. Such coordinated efforts will contribute to the development of a model for molecular changes in plant cell fate decisions.

AUTHOR CONTRIBUTIONS

S.B. and F.B designed the research; S.M. and P.E. prepared plant material; P.B. constructed sRNA and PARE libraries; S.B analyzed and interpreted the data; M.A.L. contributed to PARE analysis; P.E.J., B.C.M and F.B. contributed to data interpretation; S.B. wrote the manuscript; B.C.M and F.B. reviewed the manuscript.

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BIBLIOGRAPHY

- Addo-Quaye C, Miller W, Axtell MJ (2009). CleaveLand: a pipeline for using degradome data to find cleaved small RNA targets. *Bioinformatics*, 25:130-1.
- Arikiti S, Zhai J, Meyers, BC (2013). Biogenesis and function of rice small RNAs from non-coding RNA precursors. *Current opinion in plant biology*, 16:170-179.
- D'Ario M, Griffiths-Jones S, Kim M (2017). Small RNAs: big impact on plant development. *Trends in plant science*, 22:1056-1068
- Axtell MJ, Meyers BC (2018). Revisiting criteria for plant miRNA annotation in the era of big data. *The Plant Cell*, 30:272-284.
- Bélanger S, Marchand S, Jacques PE, Meyers B, Belzile F (2018). Differential expression profiling of barley microspore during the early stage of development in androgenesis. *G3: Genes, Genomes, Genetics*, 8:1603-1614
- Berenguer E, Bárány I, Solís MT, Pérez-Pérez Y, Risueño MC, Testillano PS (2017). Inhibition of histone H3K9 methylation by BIX-01294 promotes stress-induced microspore totipotency and enhances embryogenesis initiation. *Frontiers in plant science*, 8:1161.
- Bond DM, Baulcombe DC (2014). Small RNAs and heritable epigenetic variation in plants. *Trends Cell Biol.*, 24:100–7.
- Borges F, Martienssen RA (2015). The expanding world of small RNAs in plants. *Nature Reviews Molecular Cell Biology*, 16:727-741.
- Cabrito TR, Teixeira MC, Duarte AA, Duque P, Sá-Correia I (2009). Heterologous expression of a Tpo1 homolog from *Arabidopsis thaliana* confers resistance to the herbicide 2, 4-D and other chemical stresses in yeast. *Applied microbiology and biotechnology*, 84:927-936.
- Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, TL Madden (2009). BLAST+: architecture and applications. *BMC bioinformatics*, 10:421.
- Chen X (2009). Small RNAs and their roles in plant development. *Annual Review of Cellular and Developmental Biology*, 25:21-44.

561 Duarte-Aké F, De-la-Peña C (2016). Epigenetic advances in somatic embryogenesis in
562 sequenced genome crops. In *Somatic embryogenesis: fundamental aspects and*
563 *applications* (pp. 81-102). Springer, Cham.

564 El-Tantawy AA, Solís MT, Risueño MC, Testillano PS (2014). Changes in DNA methylation levels
565 and nuclear distribution patterns after microspore reprogramming to embryogenesis in
566 barley. *Cytogenetic and genome research*, 143:200-208.

567 Elhiti M, Stasolla C, Wang A (2013) Molecular regulation of plant somatic embryogenesis. *In Vitro*
568 *Cell. Dev. Biol. Plant*, 49:631-642

569 Esteves P, Belzile F (2014). Improving the efficiency of isolated microspore culture in six-row
570 spring barley: I-optimization of key physical factors. *Plant cell reports*, 33:993-1001.

571 Esteves P, Clermont I, Marchand S, Belzile F (2014). Improving the efficiency of isolated
572 microspore culture in six-row spring barley: II-exploring novel growth regulators to maximize
573 embryogenesis and reduce albinism. *Plant cell reports*, 33:871-879.

574 Fahlgren N, Carrington JC (2010). miRNA target prediction in plants. In *Plant MicroRNAs* (pp. 51-
575 57). Humana Press.

576 Fei, Q., Yang, L., Liang, W., Zhang, D., Meyers, B.C. (2016). Dynamic changes of small RNAs in
577 rice spikelet development reveal specialized reproductive phase RNA pathways. *J. Exp. Bot.*,
578 67: 6037-6049.

579 German M, Luo S, Schroth G, Meyers BC, Green PJ (2009) Construction of Parallel Analysis of
580 RNA Ends (PARE) libraries for the study of cleaved miRNA targets and the RNA
581 degradome. *Nature Protocols*, 4:356.

582 Germanà MA (2011). Gametic embryogenesis and haploid technology as valuable support to
583 plant breeding. *Plant cell reports*, 30:839-857.

584 González-Melendi P, Ramírez C, Testillano PS, Kumlehn J, Risuenõ MC (2005). Three
585 dimensional confocal and electron microscopy imaging define the dynamics and

mechanisms of diploidisation at early stages of barley microspore-derived embryogenesis.
Planta, 222:47-57.

Hu, J., Wang Y, Fang Y, Zeng L, Xu J, Yu H, Zhu L (2015). A rare allele of GS2 enhances grain
size and grain yield in rice. *Molecular plant*, 8:1455-1465.

Johnson NR, Yeoh JM, Coruh C, Axtell MJ (2016). Improved placement of multi-mapping small
RNAs. *G3: Genes, Genomes, Genetics*, 6:2103-2111.

Jones-Rhoades MW, Bartel DP, Bartel B (2006). MicroRNAs and their regulatory roles in plants.
Annu. Rev. Plant Biol., 57:19-53

Juárez-González VT, López-Ruiz BA, Baldrich P, Luján-Soto E, Meyers BC, Dinkova TD (2019).
The explant developmental stage profoundly impacts small RNA-mediated regulation at the
dedifferentiation step of maize somatic embryogenesis. *Scientific reports*, 9

Kasha KJ, Simion E, Oro R, Yao QA, Hu TC, Carlson AR (2001a). An improved in vitro technique
for isolated microspore culture of barley. *Euphytica*, 120:379-385

Kasha KJ, Hu TC, Oro R, Simion E, Shim YS (2001b). Nuclear fusion leads to chromosome
doubling during mannitol pretreatment of barley (*Hordeum vulgare* L.) microspores. *Journal*
of Experimental Botany, 52:1227-1238.

Komiya R (2017). Biogenesis of diverse plant phasiRNAs involves an miRNA-trigger and Dicer-
processing. *Journal of plant research*, 130:17-23.

Kozomara A, Griffiths-Jones S (2014). miRBase: annotating high confidence microRNAs using
deep sequencing data. *Nucleic acids research*, 42:68-73

Kozomara A, Birgaoanu M, Griffiths-Jones S (2019). miRBase: from microRNA sequences to
function. *Nucleic acids research*, 47:155-162.

Li H, Devaux P (2003). High frequency regeneration of barley doubled haploid plants from isolated
microspore culture. *Plant Science*, 164:379-386.

- Li H, Soriano M, Cordewener J, Muiño JM, Riksen T, Fukuoka H, Angenent GC, Boutilier K (2014). The histone deacetylase inhibitor trichostatin a promotes totipotency in the male gametophyte. *The Plant Cell*, 26:195-209.
- Liebsch D, Palatnik JF (2020). MicroRNA miR396, GRF transcription factors and GIF co-regulators: a conserved plant growth regulatory module with potential for breeding and biotechnology. *Current opinion in plant biology*, 53:31-42.
- Long JM, Liu CY, Feng MQ, Liu Y, Wu XM, Guo WW (2018). miR156-SPL modules regulate induction of somatic embryogenesis in citrus callus. *Journal of experimental botany*, 69:2979-2993.
- Lorenz R, Bernhart SH, Zu Siederdisen CH, Tafer H, Flamm C, Stadler PF, Hofacker IL (2011). ViennaRNA Package 2.0. *Algorithms for Molecular Biology*, 6:26.
- Maraschin SF (2005). Androgenic switch in barley microspores. Leiden University, Faculty of Mathematics & Natural Sciences, Institute of Biology.
- Maraschin S. F., de Priester W., Spaink H. P., Wang M. (2005). Androgenic switch: an example of plant embryogenesis from the male gametophyte perspective. *Journal of Experimental Botany*, 56:1711–1726.
- Marchand S, Fonquerne G, Clermont I, Laroche L, Huynh TT, Belzile FJ (2008). Androgenic response of barley accessions and F1s with Fusarium head blight resistance. *Plant cell report*, 27:443-451.
- Martin M (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. journal*, 17:10-12.
- Mascher M, Gundlach H, Himmelbach A, Beier S, Twardziok SO, Wicker T, ... & Bayer M (2017). A chromosome conformation capture ordered sequence of the barley genome. *Nature*, 544:427-433.
- Matzke M, Kanno T, Daxinger L, Huettel B, Matzke AJ (2009). RNA-mediated chromatin-based silencing in plants. *Current opinion in cell biology*, 21:367-376.

Nagpal P, Ellis CM, Weber H, Ploense SE, Barkawi LS, Guilfoyle TJ, Gretchen Hagen G, Alonso JM, Cohen JD, Farmer EE, Ecker JR, Reed JW (2005). Auxin response factors ARF6 and ARF8 promote jasmonic acid production and flower maturation. *Development*, 132:4107-4118.

Nowak K, Gaj M. D. (2016). Transcription factors in the regulation of somatic embryogenesis. In *Somatic embryogenesis: fundamental aspects and applications* (pp. 53-79). Springer, Cham.

Remy E, Cabrito TR, Baster P, Batista RA, Teixeira MC, Friml J, Sá-Correia I, Duque P (2013). A major facilitator superfamily transporter plays a dual role in polar auxin transport and drought stress tolerance in Arabidopsis. *The Plant Cell*, 25:901-926.

Rhoades MW, Reinhart BJ, Lim LP, Burge CB, Bartel B, Bartel DP (2002). Prediction of plant microRNA targets. *Cell*, 110:513-20

Robinson MD, McCarthy DJ, Smyth GK (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, 26:139-140.

Robinson MD, Oshlack A (2010). A scaling normalization method for differential expression analysis of RNA-Seq data. *Genome Biology*, 11, R25.

Seifert F, Bössow S, Kumlehn J, Gnad H, Scholten S (2016). Analysis of wheat microspore embryogenesis induction by transcriptome and small RNA sequencing using the highly responsive cultivar “Svilena”. *BMC plant biology*, 16:97

Shao C, Chen M, Meng Y (2012). A reversed framework for the identification of microRNA–target pairs in plants. *Briefings in bioinformatics*, 14:293-301.

Solís MT, El-Tantawy AA, Cano V, Risueño MC, Testillano PS (2015). 5-azacytidine promotes microspore embryogenesis initiation by decreasing global DNA methylation, but prevents subsequent embryo development in rapeseed and barley. *Frontiers in plant science*, 6:472.

Soriano M, Li H, Boutilier K (2013). Microspore embryogenesis: Establishment of embryo identity and pattern in culture. *Plant Reproduction*, 26:181-96.

662 Su YH, Liu YB, Zhou C, Li XM, Zhang XS (2016). The microRNA167 controls somatic
663 embryogenesis in Arabidopsis through regulating its target genes ARF6 and ARF8. *Plant*
664 *Cell, Tissue and Organ Culture (PCTOC)*, 124:405-417.

665 Sun P, Zhang W, Wang Y, He Q, Shu F, Liu H, Deng H (2016). OsGRF4 controls grain shape,
666 panicle length and seed shattering in rice. *Journal of integrative plant biology*, 58:836-847.

667 Szczygieł-Sommer A, Gaj MD (2019). The miR396–GRF Regulatory Module Controls the
668 Embryogenic Response in Arabidopsis via an Auxin-Related Pathway. *International journal*
669 *of molecular sciences*, 20:5221.

670 Testillano PS (2018). Stress-Induced Microspore Embryogenesis in Crop Plants: Cell Totipotency
671 Acquisition and Embryo Development. In: Cánovas F, Lüttge U, Leuschner C, Risueño MC
672 (eds) Progress in Botany Vol. 81. Progress in Botany, vol 81. Springer, Cham

673 Thody J, Folkes L, Medina-Calzada Z, Xu P, Dalmay T, Moulton V (2018). PAREsnip2: a tool for
674 high-throughput prediction of small RNA targets from degradome sequencing data using
675 configurable targeting rules. *Nucleic acids research*, 46:8730-8739.

676 Voinnet O. (2009). Origin, biogenesis, and activity of plant microRNAs. *Cell*, 136:669–687

677 Wu XM, Liu MY, Ge XX, Xu Q, Guo WW (2011). Stage and tissue-specific modulation of ten
678 conserved miRNAs and their targets during somatic embryogenesis of Valencia sweet
679 orange. *Planta*, 233:495-505

680 Wu XM, Kou SJ, Liu YL, Fang YN, Xu Q, Guo WW (2015). Genomewide analysis of small RNA s
681 in nonembryogenic and embryogenic tissues of citrus: microRNA- and siRNA-mediated
682 transcript cleavage involved in somatic embryogenesis. *Plant biotechnology journal*,
683 13:383-394.

684 Yu Y, Zhou Y, Zhang Y, Chen Y (2018). Grass phasiRNAs and male fertility. *Science China*,
685 61:148-154

686 Zhai J, Arikiti S, Simon SA, Kingham BF, Meyers BC (2014). Rapid construction of parallel analysis
687 of RNA end (PARE) libraries for Illumina sequencing. *Methods*, 67:84-90.

688 Zhai J, Zhang H, Arikat S, Huang K, Nan GL, Walbot V, Meyers BC (2015). Spatiotemporally
 689 dynamic, cell-type-dependent premeiotic and meiotic phasiRNAs in maize anthers.
 690 *Proceedings of the National Academy of Sciences*, 112:3146-3151.

Table 1 All differential expression of miRNAs in microspores of barley cv. Gobernadora, contrasted between all three time points (relative to the initiation of the pretreatment inducing the transition towards embryogenesis).

miRNA	Log2FC for all tested contrasts		
	Day 2 - Day 0	Day 5 - Day 2	Day 5 - Day 0
Cluster 1			
miR1130.1	1.43	0.98	2.41
miR1130.2	3.42	0.91	4.32
miR1432	4.55	0.64	5.19
miR156.2	1.73	1.19	2.92
miR156.4	1.82	0.79	2.61
miR156.5	0.79	1.81	2.60
miR167.3	1.91	2.58	4.49
miR167.4	2.23	1.17	3.40
miR167.5	1.69	1.71	3.40
miR167.6	1.15	1.74	2.88
miR171	1.90	4.81	6.71
miR5048	1.39	1.25	2.64
miR5049.1	0.43	2.22	2.65
miR5051	1.02	1.05	2.07
miR5071.1	1.95	2.21	4.15
miR5071.2	2.25	1.09	3.34
miR5071.3	1.95	1.16	3.10
miR5071.4	1.42	2.33	3.75
miR5071.5	2.18	1.16	3.33
miR5139.6	1.09	0.98	2.07
miR5144	2.12	4.26	6.39
miR8175.1	0.69	2.06	2.74
miR8175.2	1.49	1.56	3.05
miR8175.3	1.45	1.48	2.92
miR9660.1	2.21	1.07	3.28
miR9660.2	2.40	0.89	3.29
miR9662	1.95	1.21	3.15
miR9674.1	1.75	1.15	2.90
novel.3	2.75	1.75	4.50
Cluster 2			
miR319	3.73	-4.56	-0.83
miR396	1.66	-3.11	-1.45
miR398	5.85	-1.52	4.33
miR827	2.70	-1.40	1.31
novel.1	4.08	-0.73	3.35
novel.2	4.52	-1.34	3.18

Cluster 3

miR156.1	1.92	0.29	2.21
miR156.3	1.87	0.28	2.15
miR156.6	1.83	0.21	2.04
miR159	3.96	0.32	4.28
miR166.1	2.48	0.02	2.50
miR166.2	2.31	1.69	4.01
miR166.3	2.52	0.00	2.52
miR166.4	2.65	-0.18	2.47
miR166.5	2.47	0.05	2.52
miR393	1.82	0.73	2.55
miR394	3.20	0.47	3.67
miR5049.2	2.18	0.04	2.22
miR5083.1	1.99	0.13	2.11
miR9773.1	2.12	-0.12	2.00
novel.4	3.11	-0.40	2.71

Cluster 4

miR5139.1	-0.74	-1.65	-2.39
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Table 2 Summary of miRNA targets that were validated via PARE-Seq. The described miRNAs were captured in barley microspores (cv. Gobernadora) at different time points of gametic embryogenesis.

miRNA	Target gene	Orthologous gene	Name	Target function
Microspores on day 0				
miR168	HORVU7Hr1G007000	AT1G48410	AGO1	RNA-mediated gene silencing, Translation regulation
miR396	HORVU6Hr1G084590	AT1G79690	NUDT3	Hydrolase
miR1127	HORVU4Hr1G042610	AT5G19390	ROPGAP7	Signal transduction, Cell wall biogenesis
Microspores on day 2				
miR1127	HORVU2Hr1G070360	AT5G50600	HSD1	Lipid biosynthesis, Lipid metabolism, Steroid biosynthesis
miR1127	HORVU3Hr1G078090	AT3G58830	T20N10_180	
miR1130	HORVU4Hr1G064990	AT2G32840		Negative regulation of transcription by RNA polymerase II
miR1130	HORVU6Hr1G057140	AT2G23360	FPP7	Unknown
miR1130	HORVU7Hr1G122690	AT3G09920	PIP5K9	Carbohydrate metabolic process, Cellular amino acid metabolic process
Microspores on day 5				
miR166	HORVU1Hr1G041790	AT5G60690	REV	Differentiation, Transcription, Transcription regulation
miR156	HORVU3Hr1G094730	Os01g0922600	SPL2	Transcription regulation
miR156	HORVU0Hr1G020810	Os08g0509600	SPL14	Transcription regulation
miR396	HORVU2Hr1G101770	Os04g0600900	GRF3	Transcription regulation
miR396	HORVU0Hr1G016590	Os02g0701300	GRF4	Transcription regulation
miR396	HORVU0Hr1G016610	Os02g0701300	GRF4	Transcription regulation
miR396	HORVU0Hr1G026650	Os02g0701300	GRF4	Transcription regulation
miR396	HORVU6Hr1G068370	Os02g0701300	GRF4	Transcription regulation
miR396	HORVU2Hr1G094470	Os04g0574500	GRF12	Transcription regulation
miR1127	HORVU1Hr1G060530	AT5G18860	NSH3	Response to jasmonic acid
miR1130	HORVU4Hr1G045760	AT1G18660		mRNA processing
Common to day 0 and day 2				
miR5049	HORVU7Hr1G085310	AT4G13360		Hydrolase, mitochondrial
miR1127	HORVU2Hr1G086380	AT5G48930	HCT	Cell wall biogenesis/degradation, Auxin homeostasis
Common to day 0 and day 5				
miR1127	HORVU7Hr1G059130	AT5G03880	MER24.18	Cell redox homeostasis
miR1127	HORVU7Hr1G056530	AT1G66950	ABCG39	Transport
Common to day 2 and day 5				
miR167	HORVU2Hr1G121110	Os04g0671900	ARF8	Auxin signaling pathway, Transcription regulation

miR396	HORVU7Hr1G008680	Os06g0116200	GRF5	Transcription regulation
miR1127	HORVU3Hr1G051000	AT5G20970	HSP20	
miR1127	HORVU7Hr1G097520	AT4G28470	RPN1B	Protein catabolic process
miR1130	HORVU5Hr1G044640	AT5G13750	ZIFL1	Transport
miR5048	HORVU7Hr1G065130	AT5G15730	CRLK2	Protein phosphorylation
miR5048	HORVU7Hr1G043150	AT5G15730	CRLK2	Protein phosphorylation
miR9674	HORVU0Hr1G031920	Os04g0350000	Rf1	Restorer of fertility
miR9674	HORVU0Hr1G035310	Os04g0350000	Rf1	Restorer of fertility
miR9674	HORVU1Hr1G010890	Os04g0351333	Rf1	Restorer of fertility
miR9674	HORVU1Hr1G010970	Os10g0497300	Rf1	Restorer of fertility
miR9674	HORVU1Hr1G010990	Os10g0497300	Rf1	Restorer of fertility
miR9674	HORVU1Hr1G011020	Os04g0350000	Rf1	Restorer of fertility
miR9674	HORVU1Hr1G011150	Os04g0350000	Rf1	Restorer of fertility
miR9674	HORVU1Hr1G011160	Os04g0351333	Rf1	Restorer of fertility
miR9674	HORVU1Hr1G011250	Os04g0350000	Rf1	Restorer of fertility
miR9674	HORVU1Hr1G011300	Os04g0351333	Rf1	Restorer of fertility
Common to day 0, day 2 and day 5				
novel.3	HORVU3Hr1G070880	AT3G16360	AHP4	Cytokinin signaling pathway, Two-component regulatory system

Figure 1 Samples of microspores obtained from the barley cv. Gobernadora at the three stages of early gametic embryogenesis corresponding to (a) freshly harvested microspores without stress (day 0), (b) microspores just after the completion of the stress treatment (day 2) and (c) embryogenesis-induced microspores (day 5). The phenotype of these microspores was captured without (left) and with DAPI staining (right) for microspores on days 0, 2 and 5 using a Zeiss Apoptome microscope under UV laser illumination (excitation of 390/22 nm and emission of 460/50 nm) at a 40x magnification.

Figure 2 Variation in sRNA expression profiles within and between microspore samples. A MDS plot shows the high degree of uniformity among replicates of the same stage as well as the distinctness between stages.

713 **Figure 3** Dynamics of variation in sRNA abundance through barley microspore development.
 714 Microspores were analyzed in the early stages of development in androgenesis for differentially
 715 expressed (at $\log_{2}FC \geq |2.0|$ and $q\text{-value} \leq 1.0E-03$) hc-siRNAs (a) 24-nt phasiRNAs (b) and
 716 miRNAs (c). Heatmaps were generated from a read-count matrix normalized for library size and
 717 transformed in reads per million. Heatmaps were row normalized.

718 **Supplementary Table 1** The 5' and 3' adapters used to construct libraries.

	5' adapter	3' adapter
sRNA-seq	5'-GTTTCAGAGTTCTACAGTCCGAC GATC-3'	5'-AGATCGGAAGAGCACACGTCTG AACTCCAGTCAC-3'
PARE-seq	5'-GTTTCAGAGTTCTACAGTCCGAC GATC-3'	5'-TGGAATTCTCGGGTGCCAAGGA ACTCCAGTCAC-3'

719

720 **Supplementary Table 2** Coordinates and abundance of the 202 PHAS loci detected in barley
 721 microspores (cv. Gobernadora) undergoing gametic embryogenesis.

<i>PHAS</i> loci ID	Chr	Start	End	Total abundance captured over 12 microspore libraries (rpm)
21PHAS_14322	3H	11468203	11468794	21.3
21PHAS_20868	3H	666495064	666495851	3.8
21PHAS_20987	3H	671295658	671296326	2.7
21PHAS_33492	5H	584990830	584991582	1.9
21PHAS_34003	5H	610464867	610465221	1.5
21PHAS_35755	6H	15137173	15137811	2.4
21PHAS_42227	7H	21074671	21075109	3.6
21PHAS_45781	7H	414260885	414264622	8.1
21PHAS_47766	7H	614055096	614055820	1.8
21PHAS_41814	7H	7355310	7355944	4.6
24PHAS_263	1H	13054359	13054962	25.6
24PHAS_268	1H	13068606	13070067	107.3
24PHAS_272	1H	13081265	13081853	39.6
24PHAS_46	1H	2112051	2112791	10.0
24PHAS_452	1H	21184712	21185479	20.8
24PHAS_472	1H	21635818	21636352	6.5
24PHAS_478	1H	21906855	21907639	5.6
24PHAS_480	1H	21912182	21912444	2.9
24PHAS_484	1H	22024537	22025274	12.0
24PHAS_492	1H	22164473	22165032	10.8
24PHAS_3397	1H	364128661	364132480	36.7
24PHAS_104	1H	4694003	4694589	9.4
24PHAS_4720	1H	487210157	487210328	58.9
24PHAS_4723	1H	487322711	487323442	253.0
24PHAS_112	1H	5141100	5142006	14.0
24PHAS_5591	1H	531451194	531452501	1.7
24PHAS_6022	1H	544721420	544721891	5.0
24PHAS_1120	1H	74270142	74270793	15.2
24PHAS_6878	2H	16876358	16877406	8.2
24PHAS_6891	2H	17054345	17055506	4.5
24PHAS_6895	2H	17089892	17090399	5.7
24PHAS_6901	2H	17158984	17159895	3.0
24PHAS_9080	2H	188226877	188228839	331.8
24PHAS_6992	2H	19237294	19238107	11.4
24PHAS_7004	2H	19326424	19327410	3.4
24PHAS_7009	2H	19367354	19368148	5.6
24PHAS_7013	2H	19463512	19464618	8.7

24PHAS_7025	2H	19689880	19690467	7.1
24PHAS_7152	2H	24564837	24565433	18.6
24PHAS_7162	2H	24899805	24900585	21.0
24PHAS_7265	2H	29567069	29568231	4.0
24PHAS_7474	2H	38376025	38378231	182.6
24PHAS_11697	2H	586819680	586822269	248.9
24PHAS_12394	2H	663464067	663466596	53.4
24PHAS_6621	2H	6708667	6709785	2.8
24PHAS_12564	2H	679346270	679347678	2.4
24PHAS_6627	2H	6991738	6992043	5.8
24PHAS_13189	2H	715835306	715835812	72.0
24PHAS_13281	2H	717666992	717667913	5.5
24PHAS_13462	2H	728781631	728782617	3.8
24PHAS_13482	2H	729376598	729377279	5.5
24PHAS_13483	2H	729400706	729401490	42.0
24PHAS_13486	2H	729497742	729498130	8.7
24PHAS_13489	2H	729558100	729558949	7.4
24PHAS_13685	2H	744773597	744774368	6.5
24PHAS_13804	2H	751202233	751203753	20.0
24PHAS_13852	2H	753195658	753196020	3.9
24PHAS_13853	2H	753200440	753201076	1.6
24PHAS_13895	2H	755104957	755106046	7.1
24PHAS_13986	2H	760732391	760733419	1.7
24PHAS_14019	2H	762094431	762095616	1.7
24PHAS_14020	2H	762116991	762118168	1.8
24PHAS_14093	2H	764844581	764845875	13.5
24PHAS_14108	2H	765730662	765732083	251.3
24PHAS_14110	2H	765919784	765920827	4.8
24PHAS_14353	3H	11740486	11741320	15.4
24PHAS_14383	3H	12085831	12086703	4.2
24PHAS_14384	3H	12120475	12121345	14.1
24PHAS_14385	3H	12123138	12123852	7.4
24PHAS_14391	3H	12294741	12295148	2.3
24PHAS_14392	3H	12296082	12297280	3.5
24PHAS_14474	3H	16256234	16257111	2.8
24PHAS_16117	3H	185045546	185047175	102.2
24PHAS_14534	3H	18905936	18908130	3.6
24PHAS_14698	3H	26975711	26976984	3.7
24PHAS_17125	3H	314356585	314358472	27.8
24PHAS_14205	3H	3661188	3662229	5.8
24PHAS_18743	3H	525824091	525824462	42.1
24PHAS_15179	3H	63340143	63340569	74.9
24PHAS_20715	3H	664150651	664151323	4.3

24PHAS_20725	3H	664327397	664327804	4.5
24PHAS_15208	3H	66434284	66436103	3.0
24PHAS_20728	3H	664359557	664360104	3.7
24PHAS_20911	3H	667876276	667877972	6.7
24PHAS_21215	3H	683875004	683877450	2.3
24PHAS_21219	3H	683899084	683899910	21.1
24PHAS_21221	3H	683902316	683903381	8.1
24PHAS_21233	3H	684159299	684160072	58.9
24PHAS_21235	3H	684171527	684173251	23.6
24PHAS_21240	3H	684233486	684234316	3.2
24PHAS_21242	3H	684242678	684243510	6.1
24PHAS_21248	3H	684372625	684373460	17.5
24PHAS_21251	3H	684381656	684382642	9.8
24PHAS_21254	3H	684389081	684390158	9.8
24PHAS_21574	3H	698405608	698406032	19.1
24PHAS_14254	3H	7693336	7695103	2.4
24PHAS_21614	4H	1232114	1233156	5.8
24PHAS_21886	4H	12435409	12438601	70.7
24PHAS_23512	4H	169628030	169631418	95.3
24PHAS_22324	4H	37361049	37362224	15.0
24PHAS_22326	4H	37379486	37380118	9.8
24PHAS_22338	4H	37681972	37682964	40.4
24PHAS_22339	4H	37695386	37696271	10.1
24PHAS_22388	4H	43171499	43172430	43.0
24PHAS_22389	4H	43181935	43182742	30.1
24PHAS_22392	4H	43493799	43494612	32.7
24PHAS_27704	4H	622935559	622936823	15.8
24PHAS_27912	4H	633812349	633814825	24.9
24PHAS_29466	5H	109616707	109620398	39.5
24PHAS_31814	5H	453066126	453072802	228.6
24PHAS_32291	5H	507349064	507349976	23.9
24PHAS_32774	5H	548843429	548845358	2.4
24PHAS_32879	5H	553897889	553898723	12.4
24PHAS_32880	5H	553971675	553972443	3.2
24PHAS_32970	5H	556833630	556834202	10.4
24PHAS_28273	5H	5587310	5588002	39.9
24PHAS_28274	5H	5588957	5589947	6.0
24PHAS_33457	5H	582637654	582639849	7.3
24PHAS_34293	5H	623928857	623930166	2.9
24PHAS_34365	5H	626025368	626027216	182.2
24PHAS_28314	5H	6319371	6320356	13.4
24PHAS_34521	5H	632530990	632531931	25.6
24PHAS_28317	5H	6341068	6342316	22.5

24PHAS_28320	5H	6352728	6353329	14.7
24PHAS_28323	5H	6372769	6373300	3.8
24PHAS_28331	5H	6499566	6500489	8.4
24PHAS_34940	5H	652041234	652043511	2.8
24PHAS_28334	5H	6551607	6553132	17.5
24PHAS_35108	5H	659336027	659337316	7.3
24PHAS_35149	5H	660944471	660945690	5.2
24PHAS_35160	5H	661060785	661063176	4.0
24PHAS_29363	5H	94270838	94271647	45.9
24PHAS_35606	6H	10954184	10954730	5.6
24PHAS_35608	6H	10956100	10956941	21.0
24PHAS_35610	6H	10960288	10960790	18.2
24PHAS_35614	6H	11071879	11072528	15.1
24PHAS_35615	6H	11073419	11074092	29.8
24PHAS_35618	6H	11088920	11089614	44.1
24PHAS_35622	6H	11123413	11124257	23.4
24PHAS_35628	6H	11301466	11302319	8.8
24PHAS_35806	6H	16645266	16646341	2.4
24PHAS_36128	6H	36173676	36174384	6.6
24PHAS_36266	6H	44042823	44047172	97.6
24PHAS_40746	6H	543603160	543603685	4.8
24PHAS_40748	6H	543700616	543701081	14.7
24PHAS_40749	6H	543734455	543735267	21.3
24PHAS_40750	6H	543737752	543738859	6.4
24PHAS_40751	6H	543856342	543857069	7.2
24PHAS_40752	6H	543858757	543859066	4.7
24PHAS_40957	6H	555942455	555943569	2.5
24PHAS_41280	6H	570939408	570942261	6.7
24PHAS_41285	6H	571024040	571025511	18.7
24PHAS_41286	6H	571027635	571028320	2.7
24PHAS_41289	6H	571041270	571041644	84.6
24PHAS_36607	6H	70476310	70477145	19.1
24PHAS_41963	7H	11341442	11342513	3.9
24PHAS_41964	7H	11380125	11381193	3.8
24PHAS_42015	7H	14297407	14297945	13.7
24PHAS_42017	7H	14419777	14419974	10.7
24PHAS_42019	7H	14435981	14436570	12.5
24PHAS_42021	7H	14447177	14448617	5.3
24PHAS_42023	7H	14484536	14485400	4.0
24PHAS_42030	7H	14735397	14736383	11.8
24PHAS_42041	7H	14949791	14950623	35.7
24PHAS_42052	7H	15502548	15502916	2.3
24PHAS_42055	7H	15527552	15528404	4.0

24PHAS_42057	7H	15543784	15544259	9.6
24PHAS_42059	7H	15574368	15574914	11.2
24PHAS_42063	7H	15617409	15617717	12.4
24PHAS_42075	7H	15667079	15667886	9.5
24PHAS_42078	7H	15673681	15674595	71.8
24PHAS_42092	7H	15934967	15935551	4.1
24PHAS_42099	7H	16188506	16188795	6.0
24PHAS_42100	7H	16188973	16189373	4.9
24PHAS_42470	7H	32268202	32269098	4.4
24PHAS_42617	7H	41001346	41002354	23.0
24PHAS_42619	7H	41005367	41006409	8.7
24PHAS_42653	7H	43887144	43888996	17.3
24PHAS_42711	7H	46466089	46467935	4.7
24PHAS_47112	7H	566467182	566467765	9.0
24PHAS_47139	7H	568548901	568549595	11.3
24PHAS_47146	7H	568689134	568690021	3.7
24PHAS_47187	7H	571782434	571782975	5.5
24PHAS_47188	7H	571792628	571793469	30.9
24PHAS_47889	7H	619383864	619384720	130.7
24PHAS_48004	7H	625274352	625274927	1.8
24PHAS_48144	7H	632308011	632308901	3.2
24PHAS_48157	7H	633013157	633014228	3.7
24PHAS_48305	7H	636391193	636392080	2.6
24PHAS_48306	7H	636408765	636409414	3.9
24PHAS_48312	7H	636582711	636584183	5.1
24PHAS_48337	7H	637600962	637601806	9.5
24PHAS_48343	7H	637666684	637667402	21.3
24PHAS_48345	7H	637729899	637730788	11.8
24PHAS_48551	7H	643000930	643002647	5.5
24PHAS_48650	7H	645403322	645404928	4.0
24PHAS_48780	7H	649918353	649919340	4.3
24PHAS_48870	7H	652224124	652224805	7.2
24PHAS_49727	Un	101568653	101572067	14.6
24PHAS_49094	Un	9394321	9394968	2.3
24PHAS_49097	Un	9401319	9402253	4.5
24PHAS_49099	Un	9418578	9419171	5.9

723 **Supplementary Table 3** Coordinates and abundance of the 68 annotated miRNAs in barley
724 microspores (cv. Gobernadora) undergoing gametic embryogenesis.

miRNA	Chromosome	Start	End	Total abundance captured over 12 microspore libraries (rpm)
miR1127	2H	156304849	156306165	1.4
miR1130.1	1H	517537524	517539284	10.0
miR1130.2	5H	558876253	558877962	0.7
miR1432	2H	166771786	166771953	0.9
miR156.1	2H	623451730	623451867	14.8
miR156.2	3H	59655592	59655682	0.9
miR156.3	3H	59655786	59655875	7.0
miR156.4	5H	499573181	499573663	5.1
miR156.5	5H	499573813	499574078	2.4
miR156.6	6H	362942108	362943254	68.4
miR159	3H	14332438	14332747	24.6
miR166.1	1H	353507033	353507137	92.1
miR166.2	5H	513807917	513808017	2.5
miR166.3	5H	593550048	593550127	84.7
miR166.4	6H	539379081	539379103	1.6
miR166.5	6H	539379309	539379352	29.5
miR166.6	7H	558749092	558749512	40.6
miR167.1	4H	590940939	590941052	1.3
miR167.2	4H	591009157	591009266	1.4
miR167.3	5H	595203284	595203376	1.9
miR167.4	6H	136921506	136921623	5.9
miR167.5	Un	58632427	58632921	103.9
miR167.6	Un	58635472	58635548	0.5
miR168	6H	34582524	34582666	27.4
miR171	4H	617878928	617879096	1.5
miR1878	5H	479890906	479890931	2.0
miR319	3H	140433531	140433705	3.0
miR393	2H	756463338	756463486	0.6
miR394	3H	649738003	649739841	0.6
miR396	6H	564855150	564855213	1.1
miR398	3H	637805239	637806060	1.7
miR408	7H	622016838	622016929	0.6
miR444	2H	583382959	583383500	2.3
miR5048	7H	550741190	550742460	28.9
miR5049.1	3H	95702904	95703500	1.0
miR5049.2	1H	534280976	534281606	1.3
miR5051	4H	588904352	588906553	7.0
miR5071.1	3H	667572215	667572472	6.8
miR5071.2	3H	667603611	667603714	1.2
miR5071.3	4H	387684886	387685014	1.7
miR5071.4	5H	289162901	289163053	0.6
miR5071.5	6H	572661234	572661255	1.9

miR5083.1	1H	554520362	554520768	1.2
miR5083.2	1H	554613391	554613666	0.6
miR5139.1	2H	457339091	457339814	0.6
miR5139.2	3H	267694291	267694389	2.7
miR5139.3	3H	546431702	546431746	0.9
miR5139.4	5H	325277882	325277997	1.5
miR5139.5	7H	566782938	566783769	1.0
miR5139.6	Un	126464383	126464540	6.0
miR5144	2H	23937585	23939427	1.3
miR8175.1	4H	285327304	285327355	0.6
miR8175.2	4H	611607026	611607134	2.9
miR8175.3	7H	164139781	164139848	1.3
miR827	2H	620316184	620316763	3.9
miR9660.1	6H	558257705	558257876	4.3
miR9660.2	6H	558398762	558398935	4.5
miR9662	6H	104007160	104007445	735.9
miR9674.1	3H	638444020	638444191	3.0
miR9674.2	6H	15557530	15557700	13.4
miR9773.1	3H	673282151	673282417	16.6
miR9773.2	7H	329970778	329971374	0.7
novel.1	1H	6577445	6577556	1.0
novel.2	4H	192456595	192456819	1.9
novel.3	5H	471378581	471378848	0.7
novel.4	7H	532917020	532917319	4.7
novel.5	7H	575649579	575649830	5.7
novel.6	7H	593828495	593828636	0.9

725

726 **Supplementary Table 4** Sequences of mature miRNA, miRNA* and their putative precursors as well as their abundance detected in microspores
 727 of barley cv. Gobernadora undergoing gametic embryogenesis.

	miRNA	Reads	miRNA*	Reads	Precursor sequence and structure
Novel.1	AGCACUUUGAAUUUAGGUUGAACU	239	UAGCACUUUGAAUUUAGGUUGAAC	8	GUACGAAUUUAGAGACAAAAUUCACCUGAAUUUCAA ((.((((((((((((((((((((((((((((((((((((
Novel.2	CUAGGACUUUGAAUUUGGGUUGAA	281	UAGGACUUUGAAUUUGGGUUGAAU	26	UCGAAUUUAAAAGACAAAAUUCACCUGAAUUUAAAAGU)(((((((((.(((.((((((((((((((((((((((((((((
Novel.3	AUUUUAAUUGACGCUCAAACG	109	UUUGAGCGUCAAUUAAUACGA	9	ACUAUUACUCCCUCCGUCCCAUUUUAAUUGACGCUCU)((((((((((((((((((((((((((((((((((((
Novel.4	AAGGCCUGAAGCUAAGCAACGUGC	1069	ACGUUGC UUAGCUUCAAGCCUUGA	17	GAGGCGCAGUGCUCACCUGCACGUUGC UUAGCUUCA .)).)))))))))((((((((((((((((((((((((((((
Novel.5	UCGAGGAGCUCUGUACAAAAAAG	623	UUUUUGUACGAAGCUCCUCGAAU	7	GUUUUGGUAGUUUAUUAACAUCGAGGAGCUCUGUAC (((((((.(((.((((((((((((((((((((((((((((
Novel.6	UUGCGUCAUUUAAUUCGGAUC	152	UCGGAAUUAAUUGACGCAGCC	60	UAGUUAGUUACUCCCUCCGAUCGGAAUUAAUUGACG ...)))).((((((((((((((((((((((((((((

729 **Supplementary Table 5** Detail of miRNA targets validated by PARE tags in barley microspores (cv. Gobernadora) undergoing gametic
730 embryogenesis.

miRNA	Target gene	Degradome					Orthologous gene	Orthologous gene symbol	
		Cleavage Position	Fragment Abundance	Category	P-value	Duplex			
Microspore on day 0									
miR1127	HORVU2Hr1G086380	1429	16	2	0.0047	5' AACAAAGTATTTCCGGACGGAG 3' 3' AAGATCCTGTTTCATAAAGGGCTGCCTCCCTCA 5'	AT5G48930	HCT	
miR1127	HORVU4Hr1G042610	3267	7	2	0.0446	5' AACAAAGTATTTCC-GGACGGAG 3' 3' AGGAGGCTGTTTCATAAAGGTTCTGCCTCCCTC 5'	AT5G19390	ROPGAP7	
miR1127	HORVU7Hr1G056530	64	5	2	0.0022	5' AACAAAGTATTTCCGGACGGAG 3' 3' AGGATACTGTTTCATAAAGCCTGCCTCCCTCA 5'	AT1G66950	ABCG39	
miR1127	HORVU7Hr1G059130	957	5	2	0.0051	5' AACAAAGTATTTCCGGACGGAG 3' 3' AGGATACTGTTTCATAAAGCGTACCTCCCTCA 5'	AT5G03880	MER24.18	
miR168	HORVU7Hr1G007000	4986	12	2	0.0161	5' TCGCTTGGTGCAGATCGGGAC 3' 3' GTAGACCA-CGAACCACGTCGAGCCCTTGACAA 5'	AT1G48410	AGO1	
miR396	HORVU6Hr1G084590	2275	8	2	0.0316	5' TTCCACAGCTTTCTTGAACCTT 3' 3' TTCCGAAAGGTGTCGAAGGAGCTGGAAACATT 5'	AT1G79690	NUDT3	
miR5049.1	HORVU7Hr1G085310	3579	24	2	0.0090	5' ATTATTTAGGTACAGAGGAAG 3' 3' TGATGTTT-ATAAATCCATGTCTCCCTCATGCT 5'	AT4G13360		
novel.3	HORVU3Hr1G070880	2332	30	2	0.0029	5' ATTTTAATTGACGCTCAAACG 3' 3' GCTAGGTATAATTAACAGCGAGTTTGCTTACA 5'	AT3G16360	AHP4	

Microspore on day 2

miR167.3	HORVU2Hr1G121110	2360	3	0	0.0004	5' TGAAGCTGCCAGCATGATCTA '3 o 3' TGCTTATG-TTCGACGGTCGGACTAGATAGCCC '5	Os04g0671900	ARF8
miR396	HORVU7Hr1G008680	174	45	2	0.0101	5' TTCCACA-GCTTTCTTGAACCTT '3 3' AACCTAAGGTGTCCGAAAGAACTTGCCAACGC '5	Os06g0116200	GRF5
miR1127	HORVU2Hr1G086380	1429	48	0	0.0015	5' AACAAAGTATTTCCGGACGGAG '3 AAGATCCTGTTTCATAAAGGGCTGCCTCCCTCA '5	AT5G48930	HCT
miR1127	HORVU2Hr1G070360	1224	31	0	0.0060	5' AACAAAGTATTTCCGGACGGAG '3 3' AAGATCCTGTTTCATAAAGCCCTGCCTCCCTCA '5	AT5G50600	HSD1
miR1127	HORVU3Hr1G051000	2868	21	2	0.0099	5' AACAAAGTATTTCCGGACGGAG '3 3' AAGATCCTGTTTCATAAAGCCCTGCCTCCCTCA '5	AT5G20970	HSP20
miR1127	HORVU7Hr1G097520	3060	13	2	0.0169	5' AACAAAGTATTTCCGGACGGAG '3 3' AAGATACTGTTTCATAAAGGACCGCCTCCCTCA '5	AT4G28470	RPN1B
miR1127	HORVU3Hr1G078090	1532	48	0	0.0019	5' AACAAAGTATTTCCGGACGGAG '3 3' AAGATCCTGTTTCATAAAGCCCTGCCTCCCTCA '5	AT3G58830	T20N10_180
miR1130.1	HORVU6Hr1G057140	314	6	0	0.0010	5' TCTTATGTTGTGGGACGGAG-G '3 o o 3' TTTGCGAGAATATAATACCCTGCCTCACTCAT '5	AT2G23360	FPP7
miR1130.1	HORVU7Hr1G122690	130	6	2	0.0014	5' TCTTATGTTGTGGGACGGAGG '3 o o 3' TTTGCGAGAATATAATACCCTGCCTCCCTCAT '5	AT3G09920	PIP5K9
miR1130.1	HORVU5Hr1G044640	2241	7	2	0.0026	5' TCTTATGTTGTGGGACGGAGG '3 o o 3' TTTGCGAGAATATAATACCCTGGCTCCTTCAT '5	AT5G13750	ZIFL1

miR1130.2	HORVU4Hr1G064990	1255	51	2	0.0366	5' CTCTGTAACCTTAATATAAGAC '3 o 3' ATGTCTAAGACATTGAATTGTACACTGTTGTA '5	AT2G32840	
miR5048	HORVU7Hr1G043150	576	29	0	0.0018	5' TATATTTGCAGGTTTtaggtct '3 o 3' GTTCTCATATAATCGTCTGAAATCCAGAGCCA '5	AT5G15730	CRLK2
miR5048	HORVU7Hr1G065130	516	29	0	0.0019	5' TATATTTGCAGGTTTtaggtct '3 o 3' GTTCTCATATAATCGTCTGAAATCCAGAGCCA '5	AT5G15730	CRLK2
miR5049.1	HORVU7Hr1G085310	3579	25	2	0.0066	5' ATTATTTAGGTACAGAGGAAG '3 3' TGATGTTT-ATAAATCCATGTCTCCCTCATGCT '5	AT4G13360	
miR9674.1	HORVU0Hr1G031920	1487	11	0	0.0039	5' TTAAATTTCTCCATAGCATCA '3 TAGACTAATTTAAAGAGGTACCGTAGCCAGTT '5	Os04g0350000	Rf1
miR9674.1	HORVU0Hr1G035310	653	11	2	0.0250	5' TTAAATTTCTCCATAGCATCA '3 3' TAGACTAATTTAAAGAGGTACCGTAGCCAGTT '5	Os04g0350000	Rf1
miR9674.1	HORVU1Hr1G010890	1602	11	0	0.0026	5' TTAAATTTCTCCATAGCATCA '3 3' TAGACTAATTTAAAGAGGTACCGTAGCCAGTT '5	Os04g0351333	Rf1
miR9674.1	HORVU1Hr1G010970	1545	11	1	0.0058	5' TTAAATTTCTCCATAGCATCA '3 3' TAGACTAATTTAAAGAGGTACCGTAGCCAGTT '5	Os10g0497300	Rf1
miR9674.1	HORVU1Hr1G010990	824	11	0	0.0114	5' TTAAATTTCTCCATAGCATCA '3 3' TAGACTAATTTAAAGAGGTACCGTAGCCAGTT '5	Os10g0497300	Rf1
miR9674.1	HORVU1Hr1G011020	824	11	1	0.0072	5' TTAAATTTCTCCATAGCATCA '3 3' TAGACTAATTTAAAGAGGTACCGTAGCCAGTT '5	Os04g0350000	Rf1
miR9674.1	HORVU1Hr1G011150	1558	11	0	0.0026	5' TTAAATTTCTCCATAGCATCA '3 3' TAGACTAATTTAAAGAGGTACCGTAGCCAGTT '5	Os04g0350000	Rf1

miR9674.1	HORVU1Hr1G011160	1593	11	0	0.0029	5' TTA A A T T T C T C C A T A G C A T C A 3' 	Os04g0351333	Rf1
						3' T A G A C T A A T T T A A A G A G G T A C C G T A G C C A G T T 5'		
miR9674.1	HORVU1Hr1G011250	888	11	0	0.0088	5' TTA A A T T T C T C C A T A G C A T C A 3' 	Os04g0350000	Rf1
						3' T A G A C T A A T T T A A A G A G G T A C C G T A G C C A G T T 5'		
miR9674.1	HORVU1Hr1G011300	1667	11	1	0.0048	5' TTA A A T T T C T C C A T A G C A T C A 3' 	Os04g0351333	Rf1
						3' T A G A C T A A T T T A A A G A G G T A C C G T A G C C A G T T 5'		
novel.3	HORVU3Hr1G070880	2332	46	2	0.0031	5' A T T T T A A T T G A C G C T C A A A C G 3' 	AT3G16360	AHP4
						3' G C T A G G T A T A A T T A A C A G C G A G T T G C C T A C A 5'		
Microspore on day 5								
miR156.1	HORVU3Hr1G094730	897	7	1	0.0037	5' T G A C A G A A G A G A G T G A G C A C 3' 	Os01g0922600	SPL2
						3' C A A C A G A C T G T C T T C T C T C T C G T G T C A A C A 5'		
miR156.1	HORVU0Hr1G020810	845	4	2	0.0010	5' T G A C A G A A G A G A G T G A G C A C 3' 	Os08g0509600	SPL14
						G A C T C A A C T G T C T T C T C T C T C G T G T C G A C C 5'		
miR166.1	HORVU1Hr1G041790	1063	9	2	0.0171	5' T C G G A C C A G G C T T C A T T C C C C 3' o	AT5G60690	REV
						3' T G C T T A G G C C T G G T C C G A A G T A G G G T C C G T A G 5'		
miR167.3	HORVU2Hr1G121110	2360	4	2	0.0012	5' T G A A G C T G C C A G C A T G A T C T A 3' o	Os04g0671900	ARF8
						3' T G C T T A T G - T T C G A C G G T C G G A C T A G A T A G C C C 5'		
miR396	HORVU2Hr1G101770	666	10	2	0.0326	5' T T C C A C A - G C T T T C T T G A A C T T 3' 	Os04g0600900	GRF3
						3' A C G C A A A G G T G T C C G A A A G A A C T T G C C A A C G C 5'		
miR396	HORVU0Hr1G016590	324	10	2	0.0233	5' T T C C A C A - G C T T T C T T G A A C T T 3' 	Os02g0701300	GRF4
						3' A C G C A A A G G T G T C C G A A A G A A C T T G C C A A C G C 5'		

miR396	HORVU0Hr1G016610	571	10	0	0.0057	5' TTCCACA-GCTTTCTTGAAC TT 3' 3' ACGCAAAGGTGTCCGAAAGA ACTTGCCAACGC 5'	Os02g0701300	GRF4
miR396	HORVU0Hr1G026650	851	10	2	0.0203	5' TTCCACA-GCTTTCTTGAAC TT 3' 3' ACGCAAAGGTGTCCGAAAGA ACTTGCCAACGC 5'	Os02g0701300	GRF4
miR396	HORVU6Hr1G068370	840	10	2	0.0160	5' TTCCACA-GCTTTCTTGAAC TT 3' 3' ACGCAAAGGTGTCCGAAAGA ACTTGCCAACGC 5'	Os02g0701300	GRF4
miR396	HORVU7Hr1G008680	174	324	0	0.0058	5' TTCCACA-GCTTTCTTGAAC TT 3' 3' AACCTAAGGTGTCCGAAAGA ACTTGCCAACGC 5'	Os06g0116200	GRF5
miR396	HORVU2Hr1G094470	597	4	0	0.0078	5' TTCCACA-GCTTTCTTGAAC TT 3' 3' CGCCGAAGGTGTCCGAAAGA ACTTGCCGCGC 5'	Os04g0574500	GRF12
miR5048	HORVU7Hr1G043150	576	6	2	0.0308	5' TATATTTGCAGGTTT TAGGTCT 3' oo 3' GTTCTCATATAATCGTCTGAAATCCAGAGCCA 5'	AT5G15730	CRLK2
miR5048	HORVU7Hr1G065130	516	6	2	0.0325	5' TATATTTGCAGGTTT TAGGTCT 3' oo 3' GTTCTCATATAATCGTCTGAAATCCAGAGCCA 5'	AT5G15730	CRLK2
miR9674.1	HORVU0Hr1G031920	1487	18	0	0.0039	5' TTAAATTTCTCCATAGCATCA 3' TAGACTAATTTAAAGAGGTACCGTAGCCAGTT 5'	Os04g0350000	Rf1
miR9674.1	HORVU0Hr1G035310	653	18	0	0.0050	5' TTAAATTTCTCCATAGCATCA 3' 3' TAGACTAATTTAAAGAGGTACCGTAGCCAGTT 5'	Os04g0350000	Rf1
miR9674.1	HORVU1Hr1G010890	1602	18	0	0.0026	5' TTAAATTTCTCCATAGCATCA 3' 3' TAGACTAATTTAAAGAGGTACCGTAGCCAGTT 5'	Os04g0351333	Rf1
miR9674.1	HORVU1Hr1G010970	1545	18	0	0.0029	5' TTAAATTTCTCCATAGCATCA 3' 3' TAGACTAATTTAAAGAGGTACCGTAGCCAGTT 5'	Os10g0497300	Rf1

miR9674.1	HORVU1Hr1G010990	824	18	0	0.0114	5' TTA AATTCTCCATAGCATCA '3 3' TAGACTAATTTAAAGAGGTACCGTAGCCAGTT '5	Os10g0497300	Rf1
miR9674.1	HORVU1Hr1G011020	824	18	0	0.0036	5' TTA AATTCTCCATAGCATCA '3 3' TAGACTAATTTAAAGAGGTACCGTAGCCAGTT '5	Os04g0350000	Rf1
miR9674.1	HORVU1Hr1G011150	1558	18	0	0.0026	5' TTA AATTCTCCATAGCATCA '3 3' TAGACTAATTTAAAGAGGTACCGTAGCCAGTT '5	Os04g0350000	Rf1
miR9674.1	HORVU1Hr1G011160	1593	18	0	0.0029	5' TTA AATTCTCCATAGCATCA '3 3' TAGACTAATTTAAAGAGGTACCGTAGCCAGTT '5	Os04g0351333	Rf1
miR9674.1	HORVU1Hr1G011250	888	18	0	0.0088	5' TTA AATTCTCCATAGCATCA '3 3' TAGACTAATTTAAAGAGGTACCGTAGCCAGTT '5	Os04g0350000	Rf1
miR9674.1	HORVU1Hr1G011300	1667	18	0	0.0024	5' TTA AATTCTCCATAGCATCA '3 3' TAGACTAATTTAAAGAGGTACCGTAGCCAGTT '5	Os04g0351333	Rf1
miR1127	HORVU7Hr1G056530	64	6	2	0.0037	5' AAC AAGTATTTCCGGACGGAG '3 3' AGGATACTGTTTCATAAAGCCTGCCTCCCTCA '5	AT1G66950	ABCG39
miR1127	HORVU3Hr1G051000	2606	6	2	0.0054	5' AAC AAGTATTTCCGGACGGAG '3 3' AGGATACTGTTTCATAAAGCCTACCTCCCTCA '5	AT5G20970	HSP20
miR1127	HORVU7Hr1G059130	957	6	0	0.0058	5' AAC AAGTATTTCCGGACGGAG '3 AGGATACTGTTTCATAAAGCGTACCTCCCTCA '5	AT5G03880	MER24.18
miR1127	HORVU1Hr1G060530	3168	8	2	0.0031	5' AAC AAGTATTTCCGGACGGAG '3 3' AGGATGCTGTTTCATAAAGCCTGCCTCCCTCA '5	AT5G18860	NSH3
miR1127	HORVU7Hr1G097520	3060	14	2	0.0215	5' AAC AAGTATTTCCGGACGGAG '3 3' AAGATACTGTTTCATAAAGACCGCCTCCCTCA '5	AT4G28470	RPN1B

miR1130.1	HORVU5Hr1G044640	2241	7	2	0.0017	5' TCTTATGTTGTGGGACGGAGG '3	AT5G13750	ZIFL1
						o o		
						3' TTGCGAGAATATAATACCCTGGCTCCTTCAT '5		
miR1130.1	HORVU4Hr1G045760	1719	5	2	0.0240	5' TCTTATGTTGTGGGACGGAGG '3	AT1G18660	
						o o o		
						3' TTAGCAAGAATATAATACCTTGCTCCCTCAT '5		
novel.3	HORVU3Hr1G070880	2332	35	2	0.0031	5' ATTTTAATTGACGCTCAAACG '3	AT3G16360	AHP4
						3' GCTAGGTATAATTAACAGCGAGTTGCCTACA '5		