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Genome reprogramming and small interfering RNA in the *Arabidopsis* germline

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Abstract

The movement of mobile small RNA signals between cells has garnered much interest over the last few years, and has recently been extended to germ cells during gamete development. Focusing on plants, we review mobile RNA signals that arise following reprogramming in the germline, and their effect on transposable element silencing on the one hand and on meiotic and apomictic germ cell fate on the other. A potential role for reprogramming and small RNA in hybrid formation and speciation is proposed.

First discovered by Barbara McClintock as “controlling elements” in maize [1], transposons are now usually thought of as ‘parasitic’ stretches of DNA that can duplicate and move from one location in the genome to another and through rapid amplification comprise a large percentage of many eukaryotic genomes. Though typically heterochromatic and transcriptionally silent, these elements play a crucial role in the structure and evolution of eukaryotic genomes [2]. When activated, transposons can have deleterious effects on the host, inserting into genic and regulatory regions or promoting chromosomal re-arrangements causing genome instability. In order to suppress this mutagenic potential, many eukaryotic species have evolved surveillance systems to target transposons to ensure they are inactivated. One such system employs double stranded RNA as substrates for the production of small interfering RNAs (siRNAs), which mediate the silencing effect by acting not only at the locus where they were derived but also at other homologous copies of the transposon in the genome [3]. This repression involves either post-transcriptional silencing, or the deposition of repressive chromatin modifications resulting in transcriptional inactivation. In plants, transcriptional silencing is coupled with DNA methylation in a process known as RNA-directed DNA methylation and is mediated by a specific class of siRNAs [4] [5]. However, DNA methylation can be maintained through cell divisions, independently of RNAi, by replication-dependent DNA methyl transferases that are also responsible for the trans-generational inheritance of silencing [6]. This heritable repression of gene expression is an example of epigenetic regulation: defined as a form of heritable variation that does not rely on changes in the primary DNA sequence. The advantage of this type of regulation is that such changes are readily reversible, making them amenable to short-term environmental modulations [7]. siRNA signals can be grouped into different classes according to size, structure or biogenesis, but they share the same general purpose –to regulate gene expression, either post-transcriptionally or by guiding epigenetic modifications. Here we

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will focus on 21nt and 24nt siRNA and examine their role in transposable element silencing in the germline.

siRNA biogenesis and mobility in the male germline

In plants, much of our current understanding of epigenetics comes from analysis of sporophytic tissues; however, recent findings point to a prominent role for epigenetic regulation through siRNAs during gametogenesis and seed development [8] [9] [10]. In contrast to animals, plant meiocytes do not differentiate during embryogenesis but instead are formed much later in development. Male gametophyte development in *Arabidopsis* starts with a diploid mother cell in the anther which undergoes meiosis forming a tetrad of haploid microspores. This is followed by the first round of pollen mitosis resulting in a bicellular grain which consists of a vegetative cell and a generative cell. The latter undergoes a subsequent mitotic division resulting in two sperm cells. The two haploid sperm cells (SC) and the vegetative cell make up the tricellular mature pollen grain. During fertilization, one sperm cell fuses with the egg cell while the other fuses with the central cell, thus initiating the development of the embryo and endosperm, respectively.

Transposons are de-repressed specifically in the pollen vegetative nucleus (VN), resulting in the reactivation and mobilization of transposable elements [8]. Though the activated transposons themselves are not passed to the sperm cell, they contribute information through pollen-specific, epigenetically activated 21nt siRNAs (easiRNAs). It has been shown using reporter genes that these small RNAs -or their precursors- move from the vegetative cell to the sperm cells, where they can influence silencing [8]. Why is epigenetic reactivation of transposons needed in the VN? One idea is that transient transposon activation occurs in the VN, where they are 'unmasked' and made apparent to the sperm cell via the production of mobile small RNA, allowing for the re-enforcement of their silencing in the sperm cell itself and/or the developing embryo after fertilization. What mediates this de-repression still remains an interesting question, as a combination of DNA demethylation and chromatin remodeling is likely involved. Examining sporophytic tissue, transposition occurs only in mutants that lose both heterochromatic histone modification and DNA methylation, such as the noted chromatin remodeler DECREASE IN DNA METHYLATION 1 (DDM1) [11] [12] [13].

To this end, *DDM1* is not expressed in the VN so it follows that transposon de-repression occurs in the pollen grain. DNA METHYLTRANSFERASE1 (MET1) also maintains repressive epigenetic states as mutants have been shown to affect imprinted loci transmitted from the pollen parent [14] and also be involved in transposon silencing as both transposons and centromeric repeats are upregulated in *met1* plants [15]. *MET1* is known to be expressed in sperm cells and is expressed early in pollen development [16]. How DNA methylation is regulated in the VN remains an interesting question and will be discussed in further detail in a following section. A similar reprogramming mechanism can be proposed for germline development in animals, as epigenetic marks (both DNA methylation and histone tail marks) are lost and reset each generation, and transient expression of transposons is induced in the germline [17] [18] [19] [20]. These transcripts are subsequently processed into piRNAs [21] that direct remethylation in sperm [22], and into endogenous siRNA that may have a similar function in oocytes [23]. Mammals have orthologs of both DDM1 (Lsh1) and MET1 (Dnmt1), but a link between reprogramming and small RNA biogenesis has yet to be established.

siRNA biogenesis and mobility in the female germline

Female gamete development begins with the differentiation of a diploid megaspore mother cell (MMC), that undergoes meiosis to form four haploid megaspores. Three of these

megaspores die, and the surviving megaspore undergoes several rounds of mitosis without cell division resulting in a multinucleate syncytium that subsequently cellularizes. The mature female gametophyte is a seven-celled structure consisting of three antipodal cells, one (diploid) central cell, two synergid cells, and one egg cell.

Arabidopsis has two unique RNA polymerases, PolIV and PolV, that have evolved an exclusive function in siRNA biogenesis and transcriptional silencing, respectively [24] [25]. Profiling of siRNA production in flowers from reciprocal crosses of PolIV mutants with wild-type plants showed that the siRNAs are 24nt in length, and that they are maternal in origin, as no change in siRNA profiles was observed in libraries made from developing seeds when the PolIV mutation was paternally inherited [9]. This indicates PolIV-dependent siRNAs come from female gametophytic cells and are likely derived specifically from maternal chromosomes. The surrounding maternal tissue also produces siRNA, as the developing integuments (seed coat) expresses the siRNA binding protein ARGONAUTE9 (AGO9) [10].

AGO9 is expressed in the ovule and in the anther, and preferentially interacts with 24-nucleotide siRNAs derived from transposable elements, as the profiling of siRNAs bound to it show the majority of reads map to transposons. These AGO9 bound 24nt siRNA then travel into the female gamete as crosses of *ago9* mutants to enhancer trap lines indicated that its activity is necessary to silence TEs in the female gametophyte [10]. These results further demonstrate the importance of siRNA production and transport to the germ cells and underline the role of epigenetic reprogramming in companion cells to produce these mobile siRNA signals. A similar AGO9 dependent 24nt based silencing pathway exists in pollen as well, where the lack of *polIV* expression may be supplemented with *polV* [26], which is a related polymerase expressed preferentially in pollen.

DNA demethylation and TE derepression in germline companion cells

DNA methylation plays a major role in silencing transposons as mutations in genes causing transposon de-repression tend to be coupled with loss of DNA methylation [15] [27]. The production of transposon-specific siRNAs in both germlines are accompanied by changes in DNA methylation of transposons in companion cells. During female gametogenesis, the development of the maternal endosperm is accompanied by genome-wide DNA demethylation, mediated by a combination of active demethylation by the DNA repair glycosylase DEMETER (DME) [28] and replication dependent passive demethylation mediated by the Retinoblastoma (Rb) pathway [29][30]. DME is an active DNA glycosylase and works through the base excision repair pathway [31], while the effects of Rb are replication coupled, with a complex involving RB-RELATED (RBR) that blocks MET1 expression. This suggests that DNA demethylation in the endosperm is accompanied by DNA remethylation during early embryo development [29] [32] [31]. These observations then can be summarized into a model whereby DNA demethylation mediated by DME and RBR in the central cell leads to transposon reactivation. Transposon mRNA is then processed into 24nt siRNAs, which presumably moves to the egg cell—either bound to AGO9 or not- and are used in the developing embryo to reinforce DNA methylation in the next generation.

The existence of 21nt siRNAs in the male germline suggests a distinct biogenesis pathway, and possibly a separate model of transposon derepression in the pollen companion cell. With regards to DNA demethylation, both DME and another DNA glycosylase REPRESSOR OF SILENCING 1 (ROS1) and its RNA binding partner ROS3 [33] are not expressed in the VN after the first mitosis. RBR is expressed in the VN, and MET1 and DDM1 proteins are absent, but both genes are expressed at the uninuclear stage [8][30]. As there is only one

cell division between the generative and vegetative lineages, passive demethylation can thus be ruled out as a mechanism for loss of methylation in the VN. Indeed, methylation profiling of specific loci in pollen and purified sperm, as well as in purified VN, has indicated that CG dinucleotides remain methylated, and CNN and CNG trinucleotides lose methylation only in specific contexts [8] [34]. Without DNA glycosylases, some other active process must exist. In animals, deamination by the activation-induced cytosine deaminase [35] [36] or the conversion of methylcytosine to hydroxymethylcytosine [37] [38] in combination with DNA repair mechanisms [39] have been proposed to contribute to active DNA demethylation. Similar mechanisms could exist in pollen.

Parental siRNAs in the developing embryo

Transposon defense is not the only role for these germline-specific siRNAs. One hypothesis is that this maternal and paternal cache of ‘transposon information’ is created to be readily accessible and equipped to encounter the other genome in the fertilized egg. In *Arabidopsis*, hybrids between species can have problems developing seed or give rise to infertile progeny [40]. Both classes of siRNA -21nt and 24nt – silence transposons with complementary sequences[41], and it has been suggested that the 21nt posttranscriptional pathway interacts with the 24nt transcriptional silencing pathway [42]. This raises the interesting possibility that both classes of parental siRNA are required in the zygote for permanent silencing[40]. In that case the load of foreign transposons could act as a hybridization barrier if they were activated in the zygote. Consistent with this idea, perturbations on the female side of the pathway through the mutation of PolIV resulted in a decrease of DNA methylation[43]. It would be interesting to see if this demethylation is coupled to the reactivation of transposons in developing embryos of offspring from mutant mothers in crosses between different accessions of *A. thaliana*.

Fortuitously, the influence of transposon derived siRNAs on interspecies crosses can be studied in *Arabidopsis*, as transposon sequences in *A. arenosa* and *A. lyrata* are notably different from *A. thaliana* [44] [45] [46] and these species are inter-fertile. In interspecific hybrids, the 21nt and 24nt siRNAs derived from the parental transposons would differ in each species germline, and would not match transposon sequences in the other parent’s genome. This could result in problems in transposon silencing in the developing embryo, especially if one class is absolutely required. Blocking these siRNA biogenesis pathways selectively in either germline through mutation could indicate whether these maternally or paternally derived siRNAs do indeed exert any influence in hybrid development, or if one of these pathways takes precedent over the other, providing some understanding as to how both these maternal and paternal caches of siRNA ensure the integrity of the parental genomes in intra-specific, but not inter-specific zygotes.

Meiosis, apomixis and germ cell fate

Remarkably, mutants in both the 21nt siRNA pathway, and the 24nt siRNA pathway, have a dramatic effect on germ cell fate[10]. In sexual plants, meiosis is initiated in the megaspore mother cell (MMC), a single large cell found in the ovule. After meiosis, one surviving haploid megaspore then enters mitosis to form the multi-nucleate embryo sac, in which the egg cell differentiates. In siRNA mutants, one or more diploid cells in the surrounding ovule differentiate as diploid functional megaspores, without meiosis. These diploid eggs are fertile and can be pollinated to give rise to triploid progeny[10].

In apomictic plant species (dandelions are one well known example) the differentiation of diploid somatic egg cells is an important step in aposporic, asexual development. Subsequent removal of the paternal chromosomes, for example by failure of karyogamy (in many species) or by selective inactivation of paternal centromeres [47], would result in fully

apomictic clonal progeny. Interestingly, species that normally undergo apospory are usually facultative apomicts, and the choice between asexual and sexual reproduction is controlled by a single locus. In many cases in which a single locus is implicated, it is embedded in heterochromatin. Thus it is possible that heterochromatin itself plays a role in suppressing apomixis [40]. Transposons are propagated much more efficiently in sexual rather than asexual species, because recombination can result in increases in copy number in the absence of selection [48]. Thus transposon siRNA, even though it silences transposons, can also promote their expansion through meiosis and recombination.

At least one gene required for RNAi, the rice argonaute MEL1, is also required for normal meiosis [49], in both micro- and mega-spore mother cells, raising the possibility that inhibition of meiosis in these mutants triggers asexual germ cell fate by signaling surrounding cells. Transposon siRNA themselves might provide such a signal, if they can silence genes required for specification of germ cell fate [50]. As these siRNA appear to move from cell to cell, both in the pollen grain [8] and in the ovule [10], they could contribute directly to this signal once the MMC is correctly specified.

In conclusion, heterochromatin reprogramming results in the production of transposon siRNA that have multiple important functions beyond silencing of the transposons themselves. These include hybrid incompatibility, the ordered progression of meiosis, and the inhibition of asexual germ cell fate, each of which relies to some extent on the ability of siRNA to move from cell to cell. Far from being inert “junk” DNA, heterochromatin is thus a central component of reproductive biology in plants, and likely in animals as well.

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Figure 1.

In the pollen grain, the two haploid sperm cells (orange circles) are supported by the larger haploid vegetative cell (green circle); in the ovule, the haploid egg cell (red) is supported by the endosperm (yellow). In pollen, the 21nt easiRNAs are synthesized in the vegetative cell and move to the sperm. In the ovule, the 24nt siRNA were found in the seed and are proposed to be produced in the endosperm (yellow).