Investigation of sister kinetochore orientation during meiosis I and implications in engineered apomixis

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Abstract

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Despite tremendous strides in our understanding of the specialized cell division that occurs in developing sex cells, the rules that govern step-wise chromosome segregation in the course of meiosis remain incomplete. Protein complexes known as kinetochores position themselves at defined regions of chromosomes to direct their segregation during cell division. During meiosis, kinetochores of replicated chromosomes work in tandem to ensure co-segregation only at the first division and not at the second. While comprehensive studies in a variety of model systems have helped to clarify its actors and mechanism, differing models persist that explain how sister kinetochores mono-orient prior to the first division. Here we present the results of a screening method that uncover novel factors that direct sister co-orientation, and functional study that aims to define their role. The findings provide strong support for a cohesion-based mechanism of sister kinetochore co-orientation. Functional understanding of chromosome segregation is further applied in the pursuit of clonal seed production in cultivated barley. Apomixis is a reproduction strategy that yields clonal progeny through seeds, achieved by skipping meiosis and fertilization. The implementation of apomixis in modern agriculture holds promise for reducing breeding cycles and fixing hybrids. Identification and functional characterization of the genes governing recombination, monopolar orientation, and the second meiotic division during barley meiosis are presented as a means to engineer apomeiosis in a new crop species. Further, we show that misexpression of a dominant embryogenesis factor

can trigger parthenogenesis and induce haploids in barley. Together, we show that apomeiosis and parthenogenesis can be engineered in a close relative of wheat, showing promise for a broad application of synthetic apomixis.

Dedication

This work is dedicated to Bill G. Mahlandt, for being a scientific inspiration and a much-missed figure in my life; to my parents, for supporting me in every decision I've made; and to C, for being a guiding light that made this possible in many ways.

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Chapter 1: Introduction

Meiotic chromosome segregation

Mitosis and meiosis

To properly transmit genetic information equally and faithfully, cells orchestrate a complex and strictly regulated program to equally divide, known as mitosis. Mitosis ensures that each of the chromosomes are first replicated, and then segregated to two daughter cells containing the same genetic material in an equational division. Replicated chromosomes, or sister chromatids, are joined together by ring-like protein complexes called cohesins that act to tether sister DNA. This "cohesion" keeps sister chromatids joined together until their removal initiates segregation to opposing daughter cells (Nasmyth & Haering, 2009). Certain regions of the chromosomes carry specific sequences termed centromeres that are characterized by repetitive DNA and are often enriched for cohesins (Plohl et al., 2014). Centromeres in most organisms consist of a single defined location on each chromosome, although many species also display what are known as holocentromeres, where multiple centromeric regions are dispersed along the chromosome (Marques & Pedrosa-Harand, 2016). A defining feature of centromeres is the presence of specialized nucleosomes associated with DNA, where the histone H3 is replaced with a specific H3 variant called CENH3, or CENP-A (McKinley & Cheeseman, 2016). The presence of CENH3, rather than certain sequences, is the major determinant for centromere identity and function, thus making the position of centromeres epigenetically defined. CENH3 in turn recruits a large multi-subunit protein complex known as the kinetochore, which acts as a microtubule binding interface and thus coordinates the proper segregation of chromosomes (McKinley & Cheeseman, 2016). The activities of cohesins, the composition of the kinetochore, and mechanisms of microtubule attachment will be described in detail in later sections. Importantly, these machineries also direct chromosome segregation during meiosis.

In contrast to mitosis where a single round of replication and division give rise to identical daughter cells, meiosis employs a single round of replication followed by two rounds of division, executing reductional rather than equational chromosome segregation. Following replication, homologous chromosomes pair and exchange DNA during recombination, and in

the process form structures that physically connect replicated chromosomes. Two divisions without an intervening replication segregate first the homologous chromosomes and second the chromatids, ensuring that daughter cells possess half the DNA content of the progenitor cells. These products of meiosis are the precursors of gametes that will later fuse during fertilization to complete the process of sexual reproduction. Meiosis is thus a modified cell division that alters key aspects of chromosome segregation to give rise to sex cells. Accordingly, many aspects of mitotic chromosome segregation are conserved during meiosis: cohesins maintain activities in ensuring sister chromatid cohesion and kinetochores assemble at centromeres to direct chromosome segregation. However, meiosis employs dramatic innovations within both machineries to accommodate differences in chromosome dynamics and cell division. Protein subunits that are present only during meiosis and not mitosis create unique flavors of cohesin and kinetochore complexes that are necessary for carrying out the specialized functions of meiotic chromosome segregation. The following sections will aim to describe in greater detail the mechanisms of this process.

SMC complexes

Cohesins are one configuration of a protein complex family known as Structural Maintenance of Chromosomes, or SMC complexes. As their name suggests, SMC complexes were initially identified as proteins important for chromosome segregation and packaging. While cohesin has important roles in tethering sister chromosomes by joining DNA, other SMC complexes called condensins and SMC5-6 act to package or condense chromosomes and assist in DNA repair, though each show functional overlap (Uhlmann, 2016). All SMC complexes consist of a core ring-like structure composed of three different protein subunits that topologically capture DNA to carry out their activities (Hoencamp & Rowland, 2023). The ring structure of cohesin consists of SMC1, SMC3, and a kleisin subunit SCC1 (also known as RAD21) that connects both SMC subunits. Condensins and SMC5-SMC6 contain alternate proteins in place of each subunit. In addition to the core ring structure, additional accessory subunits associate with the kleisin subunit of the ring. Important accessory subunits of cohesin complexes include SCC3, SCC2, and PDS5. A common mode of action shared among SMC complexes is a process termed loop extrusion, during which their ATPase activity has been shown to push or “extrude” chromatin through their ring structure to actively extend and maintain loops of DNA (Davidson & Peters, 2021; Higashi & Uhlmann, 2022). Loop extrusion facilitates the compaction of chromosomes and also introduces contacts between

distant DNA sequences to form what are known as topologically associating domains (TADs), physical structures of chromosomes that in turn can influence transcriptional regulation (Hoencamp & Rowland, 2023). *In vitro* experiments have shown that both condensins (Ganji et al., 2018) and cohesins (Davidson et al., 2019) actively perform loop extrusion. Despite shared activities among SMC complexes and the importance of both condensin and cohesin during chromosome segregation, only cohesin is able to link sister DNAs and promote sister chromatid cohesion.

Cohesion establishment

The loading of cohesin to entrap sister DNA occurs during the replication phase of the cell cycle in both mitotic and meiotic cells. Two mechanisms establish cohesion between sister chromatids, the 'conversion' and 'de novo' pathways (Srinivasan et al., 2020). The conversion pathway involves four proteins known in yeast as Tof1/Csm3, Ctf4, and Chl1 (TCCC), whereas the de novo pathway involves the Ctf18-RFC complex and the Scc2 accessory subunit of cohesin. The conversion pathway relies on cohesin complexes already associated with unreplicated DNA (non-cohesive cohesin, such as those involved in forming loops) passing over the replication fork to link sister DNA. The de novo pathway instead loads cohesin directly onto replicated DNA in a mechanism that involves Ctf18-RFC's role as a loader of proliferating cell nuclear antigen (PCNA) (Bermudez et al., 2003; H. W. Liu et al., 2020). Ctf18-RFC regulates levels of PCNA at replication forks, and PCNA in turn recruits the Scc2 subunit of cohesin to DNA to establish cohesion (Psakhye et al., 2023). Ctf18-RFC consists of three core protein subunits, namely CTF18, DCC1, and Ctf8 (Mayer et al., 2001). Mutations in individual genes involved in both conversion and de novo pathways have been observed to show defective sister chromatid cohesion, although not complete loss (Mayer et al., 2004). Simultaneous abrogation of both pathways however leads to severe loss of cohesion and synthetic lethality, and accordingly both have been suggested to contribute nearly equally to the establishment of cohesion (Srinivasan et al., 2020). Beyond yeasts, Ctf18-RFC possess conserved roles in cohesion establishment in vertebrates and plants (Kawasumi et al., 2021; Takahashi et al., 2010). Studies investigating Ctf18-RFC during meiosis have been limited, but have highlighted its importance for sister chromatid cohesion in budding yeast meiosis II (Petronczki et al., 2004) and mouse meiosis I (Berkowitz et al., 2012). Notably, mutations in *CTF18* and *DCC1* were recovered from a screen in fission yeast to identify drivers of the monopolar orientation of kinetochores during meiosis I

(Yokobayashi & Watanabe, 2005), drawing a connection between cohesin loading and kinetochore orientation. Following cohesion establishment, cohesin is stabilized by the activities of Eco1, which acetylates the SMC3 subunit of cohesin to prevent its removal from chromatin (Ben-Shahar et al., 2008; Ivanov et al., 2002). However, the later removal of 'cohesive' cohesin complexes from chromatin is crucial for proper chromosome segregation during both mitosis and meiosis, and the two main pathways that direct cohesin removal will be described in the following section.

Cohesin protection and removal

Cohesin is removed from chromosomes in a stepwise manner during both mitosis and meiosis via two temporally separated and distinct mechanisms (Waizenegger et al., 2000). The first mechanism, the prophase pathway, works against the Eco1-mediated stabilization of cohesin through acetylation (Rowland et al., 2009; Sutani et al., 2009). WAPL, a protein first identified in Drosophila, associates with the Pds5 accessory subunit of cohesin (Gandhi et al., 2006; Kueng et al., 2006) and opens the cohesin ring at the SCC1-SMC3 interface (Buheitel & Stemmann, 2013; Eichinger et al., 2013). WAPL-Pds5 interaction is blocked by the acetylation-driven recruitment of a vertebrate protein known as Sororin that occupies the WAPL binding site and thereby prevents cohesin removal (Nishiyama et al., 2010; Rankin et al., 2005). Absence of WAPL prevents the resolution of sister chromatids and causes chromosome segregation errors, whereas overexpression of WAPL leads to early loss of sister chromatid cohesion (Gandhi et al., 2006; Haarhuis et al., 2013). Phosphorylation of cohesin by the kinases PLK (Polo-like kinase), Aurora B, and Cdk1 has also been shown to be important for cohesin removal by disrupting the activities of Sororin (Hauf et al., 2005; Nishiyama et al., 2013). The prophase pathway also has crucial roles in meiosis-specific processes by regulating cohesin removal and cohesin-mediated loop formation (Barton et al., 2022; Challa et al., 2016). There is also support for the existence of a meiosis-specific prophase pathway involving WAPL and PLK that drives removal of REC8-containing cohesin to regulate chromosome compaction (Challa et al., 2019). Functional homologs of Eco1, WAPL, and Sororin have been characterized in plants (Bolaños-Villegas et al., 2013; De et al., 2014; Prusén Mota et al., 2024; Singh et al., 2013), suggesting conservation of the prophase pathway across kingdoms.

The second pathway of cohesin removal is carried out by Separase, a protease that cleaves the kleisin SCC1 subunit of cohesin (Uhlmann et al., 2000). During mitosis, this proteolytic cleavage results in the loss of all remaining cohesin and triggers the separation of chromatids (Nakajima et al., 2007); during meiosis, two rounds of separase activity trigger the segregation of homologous chromosomes at meiosis I and the segregation of chromatids at meiosis II (Kitajima et al., 2003; Siomos et al., 2001). The activity of Separase is regulated by the anaphase promoting complex/cyclosome (APC/C), which destroys an inhibitor of Separase known as Securin when the spindle assembly checkpoint (SAC) is satisfied (Hornig et al., 2002; Musacchio & Salmon, 2007). To maintain sister chromatid cohesion after WAPL-driven cohesin removal during mitotic prophase and Separase-driven cohesin removal during meiosis I, a family of proteins called Shugoshins (Sgo) localize to the centromere of chromosomes to protect cohesin from removal (Kitajima et al., 2004). Both metazoans and plants often possess two copies of shugoshins originating from independent duplications that in some systems show specialization between mitosis and meiosis. During mitosis, vertebrate SGO1 localizes to the kinetochore and forms a complex with protein phosphatase 2A (PP2A) to dephosphorylate centromeric cohesin and thereby prevent its removal by WAPL (H. Liu et al., 2013). During meiosis, a different SGO (SGO2 in vertebrates and SGO1 in fission yeast) also recruits PP2A to the kinetochore to dephosphorylate centromeric REC8-containing cohesin and prevent its removal by Separase at anaphase I (Lee et al., 2008; Riedel et al., 2006). This protection activity by SGO confers a stepwise loss of meiotic cohesin by first removing cohesin from arms at anaphase I and later from centromeres at anaphase II. As a result, homologous chromosomes segregate at the first division, and sister chromatids segregate only at the second division. Two shugoshin proteins have been identified in plants that both function in cohesin protection during meiosis, but lack essentiality during mitosis in contrast to yeast and vertebrate shugoshins (Cromer et al., 2013). A securin homolog, Patronus, has also been identified in plants that specifically protects cohesin removal from separase during meiotic interkinesis (Cromer et al., 2013, 2019). Shugoshins also possess a tension-sensing ability that triggers the relocalization of proteins involved in microtubule attachment such as Aurora kinase, and thus have a role in proper chromosome segregation beyond cohesin protection (Kawashima et al., 2007; Nerusheva et al., 2014). Shugoshin proteins also cooperate with Aurora kinase to promote sister chromatid cohesion at centromeres (Resnick et al., 2006) and to favor monopolar orientation of kinetochores during meiosis (Hauf et al., 2007), the mechanics of which will be discussed in later sections. In

summary, shugoshins are kinetochore-localizing proteins crucial for accurate chromosome segregation during meiosis and mitosis.

The kinetochore

The kinetochore is a large multi-subunit protein complex that assembles at certain regions of chromosomes and acts as an interface between chromatin spindle microtubule fibers, thus having a crucial role in chromosome segregation. The kinetochore is composed of over 100 proteins (Cheeseman, 2014) that coordinate to assemble on chromosomes, facilitate attachment of microtubules, and withstand tension and immense pulling forces (Ye et al., 2016) during mitosis and meiosis. The kinetochore is often divided into two functional parts, namely the inner and outer kinetochore. A key function of the inner kinetochore is the coordinated localization to centromeres, and a major component of the inner kinetochore is known as the constitutive centromere associated network (CCAN), which in vertebrates is comprised of 16 different subunits (Ariyoshi & Fukagawa, 2023). As stated previously, centromeres are epigenetically defined regions of the chromosome, marked by the presence of H3 histone variant CENH3, also known as CENP-A (centromeric protein A). CENP-A acts as the interface between chromatin and the kinetochore and has been shown to be essential for assembling the kinetochore (Gascoigne & Cheeseman, 2011). CENP-A directly interacts with the CCAN through two inner kinetochore proteins, CENP-C and CENP-N (Musacchio & Desai, 2017). CENP-C in turn acts as a scaffold for the outer kinetochore by binding with a the KMN subcomplex (Knl1, Mis12, Ndc80), a group of proteins that define the outer kinetochore and are directly involved in the binding of microtubules (Cheeseman, 2014; Musacchio & Desai, 2017). In short, the inner kinetochore associates most closely with chromatin, while the outer kinetochore acts as the microtubule binding interface. In between, many components of the kinetochore are considered to variously function as “linkers” or “scaffolds” that coordinate kinetochore assembly (Gascoigne & Cheeseman, 2011). Accordingly, scaffold proteins such as CENP-C act as important coordinators of the mitotic and meiotic kinetochore, linking the constitutive parts of the kinetochore while also attracting other proteins that modulate kinetochore function (Tanaka et al., 2009). CENP-C specifically acts as a docking site for kinases such as Aurora B and PLK that regulate the attachment of microtubules during both mitosis and meiosis (Taylor et al., 2023; Zhou et al., 2017). Aurora B positioning at CENP-C has also been shown to be important for kinetochore assembly by facilitating its interaction with the outer kinetochore (Bonner et al., 2019). CENP-C also

localizes meiosis-specific proteins to the kinetochore needed for kinetochore mono-orientation (Kim et al., 2015). The mechanisms of microtubule attachment and kinetochore orientation are both highly regulated and will be discussed in the following sections.

Microtubule attachment and the spindle assembly checkpoint

After kinetochore assembly at the centromeres of chromosomes, microtubules must attach to each kinetochore at the right time and in the right configuration. During mitotic metaphase, the kinetochore from each sister chromatid must undergo bipolar attachment, where sister kinetochores face opposing spindle poles. Microtubules then attach to sister kinetochores and generate stable tension that causes alignment of chromosomes at the metaphase plate. A central pathway regulating this process is the spindle assembly checkpoint, or the SAC, which is activated when all microtubules have not been properly attached to each kinetochore, and negatively regulates the APC/C to prevent anaphase onset until stable attachments are made. A key component of the SAC is the mitotic checkpoint complex (MCC) that consists of four proteins: MAD2, Mad3, Bub3, and CDC20, a subunit of the APC/C involved in destruction of securing (Musacchio & Salmon, 2007). MCC proteins localize to kinetochores, where high levels of MAD2 persist at unattached kinetochores. MAD2 levels reduce as attachment occurs, and thus the MCC acts as a signal for attachment status. Attachments can however occur in the wrong orientation, for example when microtubules from one spindle pole attach to a kinetochore facing the opposite pole, a scenario known as merotelic attachment. Another component of the SAC is the chromosomal passenger complex (CPC), which works to destabilize these incorrect attachments and encourage the re-attachment of new ones (G. Cairo & Lacefield, 2020). The Aurora B kinase component of the CPC specifically drives attachment error correction, and also acts as a tension-sensor that recognizes stably attached bi-orienting chromosomes (Musacchio, 2015). After correct attachment and alignment, the SAC turns off and signals a green light that allows the APC/C to trigger the progression of the cell cycle. During meiosis I, monopolar attachment of kinetochores occurs rather than bipolar attachment, a requirement to segregate homologous chromosomes rather than sister chromatids. The SAC must therefore recognize attachments that would be incorrect during mitosis, as correct ones. Further, the tension between sister kinetochores required for SAC satisfaction in mitosis is absent in meiosis. Work in mouse oocytes has shown that mono-orientation of kinetochores and tension conferred by chiasmata help to overcome these differences, and have also suggested that the meiotic SAC is less sensitive to incorrect

attachments (Touati & Wassmann, 2016). Importantly, Aurora B also promotes correct attachments during meiosis and promotes bi-orientation of homologous chromosomes (G. Cairo & Lacefield, 2020). The next section will focus of the orientation of kinetochores and its role in proper meiotic chromosome segregation.

Mono-orientation of sister kinetochores at meiosis I

Meiosis ensures that one round of replication and two rounds of division without an intervening replication step produce haploid recombined spores. Meiosis is thus considered a reductional process, compared to equational mitosis that yields diploid non-recombined spores. A key innovation necessary for reductional division and meiotic success is the monopolar orientation of sister kinetochores at meiosis I. During mitosis, kinetochores on each chromosome orient towards opposing spindle poles to facilitate proper microtubule attachment and segregation, in a process known as bi-orientation. As discussed in the previous section, Aurora B kinase of the chromosomal passenger complex helps to promote kinetochore bi-orientation and sense tension on the spindle when kinetochores are attached and aligned at metaphase. Sufficient tension satisfies the spindle assembly checkpoint (SAC) and promotes the APC/C to trigger anaphase, leading to equational segregation of chromosomes. During meiosis I, homologous chromosomes connected by chiasmata align at metaphase. These structures are termed bivalents and are composed of two homologous sets of replicated sister chromatids that each possess so-called sister kinetochores. In contrast to mitosis, homologous chromosomes segregate apart and sister chromatids co-segregate during meiosis I. In order to ensure sister chromatids segregate together, sister kinetochores orient towards the same spindle pole rather than the opposing one, in a process termed monopolar orientation or sister kinetochore co-orientation (Figure 1). Monopolar orientation also ensures tension is transmitted across the bivalent to allow anaphase progression. The modified orientation of sister kinetochores at the first meiotic division has long been appreciated (Östergren, 1951), yet its precise mechanism remains enigmatic. Work carried out in the last three decades in model yeasts has established two functional mechanisms driving monopolar orientation depending on the species considered. In budding yeast (*S. cerevisae*), a protein complex called Monopolin harbors a meiosis specific subunit, Mam1, required for monopolar orientation (Toth et al., 2000). Another subunit of the monopolin complex, Csm1, promotes Monopolin's localization to kinetochores and is also required for monopolar orientation (Sarkar et al., 2013). Csm1's interaction with the outer kinetochore protein Dsn1 physically

crosslinks sister kinetochores to promote mono-orientation (Corbett et al., 2010; Sarangapani et al., 2014). Although meiotic cohesin REC8 is required for maintaining sister chromatid cohesion during meiosis I, REC8 is notably not required for monopolar orientation in budding yeast (Toth et al., 2000). Conversely, studies in fission yeast (*S. pombe*) established that cohesin REC8 is required for monopolar orientation (Watanabe & Nurse, 1999) and that a meiosis-specific protein called Moa1 promotes REC8 cohesion in the vicinity of the kinetochore (Yokobayashi & Watanabe, 2005). Removal of REC8 cohesin in the vicinity of kinetochores but not at peri-centromeres abolishes monopolar orientation in mouse (Ogushi et al., 2021), and *rec8* mutants reverse monopolar orientation in Arabidopsis meiocytes lacking chiasmata (Chelysheva et al., 2005), broadly supporting a cohesion-dependent model in metazoans and plants. A meiosis-specific kinetochore protein was identified in mouse called Meikin perturbs sister kinetochore association and reverses monopolar orientation in achiasmatic meiocytes, proposed to be a Moa1 functional homolog (Kim et al., 2015). Thus, in budding yeast sister kinetochores are physically fused to drive co-orientation, whereas fission yeast, plants, and metazoan sister kinetochores are brought together by meiotic cohesin (Nasmyth, 2015). Both Moa1 and Meikin localize to the kinetochore by interacting with the inner kinetochore protein CENP-C, and both interact with Polo kinase (PLK1) to promote

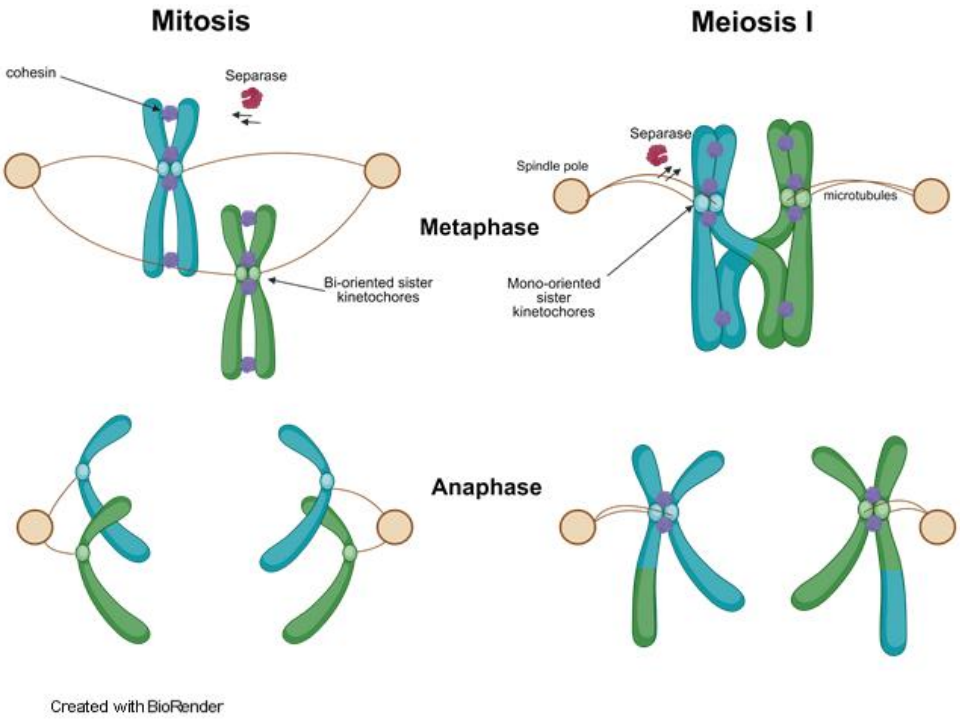

Figure 1. Monopolar orientation of sister kinetochores at meiosis I.

mono-orientation. Moa1 and Meikin, together with a functionally similar protein in budding yeast (Spo13) have been proposed to be functionally conserved regulators of kinases at the meiotic kinetochore (Galander & Marston, 2020). Polo-kinase phosphorylation of REC8 promoted by Moa1/Meikin/Spo13 is thus thought to underly mono-orientation by modulating REC8 cleavage by separase (Galander & Marston, 2020; Maier et al., 2021). Spo13/Meikin also appears to have roles beyond kinase regulation at meiosis I, by coordinating meiosis II chromosome alignment (Maier et al., 2021) and repressing APC/C activity (Rojas et al., 2023). Beyond meiosis-specific kinetochore proteins, error correction has also been implicated in imposing monopolar orientation. Aurora B positioned at centromeres by Sgo2 prevents merotelic attachments and favors monopolar attachments in fission yeast, leading to Aurora B and Sgo2 mutants displaying defective monopolar orientation (Hauf et al., 2007). Similarly, chiasmata favor monopolar attachment by repositioning Aurora B in fission yeast (Sakuno et al., 2011), supporting the requirement for chiasmata in mono-orientation in mouse and plants (Chelysheva et al., 2005; Kim et al., 2015). The outer kinetochore has also been implicated in monopolar orientation, as knockdown of the outer kinetochore component Mis12 shows evidence of bi-orienting kinetochores in maize (Li & Dawe, 2009). The current model for monopolar orientation thus suggests that cohesion at the core centromere promotes monopolar orientation by closely linking sister kinetochores, and that proper kinetochore-microtubule attachments driven by bivalent structure support this configuration. Organisms with point centromeres such a budding yeast employ an alternative mechanism to directly fuse sister kinetochores. What remains to be clarified is the precise post-translational modification activities of the identified meiosis-specific kinetochore proteins and how specifically cohesin is regulated at the core centromere. A further question is whether this mechanism is broadly conserved across kingdoms, as Spo13/Meikin homologs have not been identified in plants.

Synthetic apomixis

The aims of synthetic apomixis

Modern agriculture relies upon the use of a long-observed phenomenon known as heterosis, or hybrid vigor. Two breeding lines of a given crop can display distinct values of agronomic traits of interest such as biomass or yield. When crossed, the F1 hybrid progeny of these two

lines can display significant improvements in biomass or yield in addition to other pleiotropic traits. Hybrid vigor has contributed to significant yield gains in the past century in maize, and remains the default strategy in maize seed production (Hochholdinger & Baldauf, 2018). Due to meiotic recombination, the F2 progeny of hybrids segregate for the traits of interest, obviating the need for continuous production of hybrid seed. Hybrid seed production requires the separation of male and female flowers in most crops, most often by the disruption or removal of the male component. Physical separation of male tassels and female ears in maize facilitates this process, but crops such as wheat have both male and female flowers tightly packed into florets. One proposal to bypass the need for repeated hybrid seed production involves the introduction of apomixis into modern crops (Bicknell & Koltunow, 2004). Apomixis is a reproductive strategy observed in many wild grasses that produces clonal seeds, skipping meiosis and fertilization to yield identical progeny through generations. Apomixis introduced in F1 hybrids could thus fix genome-wide heterozygosity and transmit hybrid vigor across generations through seeds. Work in the past two decades has shown that engineering key aspects of the meiotic program and the fertilization checkpoint can trigger the production of clonal seeds in model systems and selected crop species. The next sections will aim to describe means to synthetically induce apomixis in sexually reproducing species.

Strategies to engineer apomeiosis

During seed development in most sexual flowering plants, a haploid egg cell and a haploid pollen fuse to yield a diploid embryo following fertilization. Key to this process is the reductional process of meiosis, which reduces a diploid megaspore mother cell to a haploid megaspore (Hand & Koltunow, 2014). A widely observed mechanism of natural apomicts involves a pathway known as diplospory, in which meiosis is completely avoided to yield a diploid megaspore that develops into an unreduced diploid egg cell (Hand & Koltunow, 2014). This egg cell later undergoes parthenogenesis to develop into a diploid embryo without fertilization. This strategy of skipping meiosis is termed apomeiosis, and ensures not only that the egg cell is unreduced, but also that the transmitted genome is identical to the mother cell as recombination is avoided. Apomeiosis is thus one half of diplosporous apomixis, the other being development of the embryo through parthenogenesis. Studies in the natural apomict dandelion have identified a genetic locus responsible for diplospory (Vijverberg et al., 2010), but the precise mechanism remains elusive and thus does not yet offer a strategy for transfer to crops. Significant work in the mechanisms of plant meiosis have however identified an

alternative strategy to engineer apomeiosis. Three key characteristics of meiosis are recombination, a unique first division, and the occurrence of a second division. The genetic determinants of each process have been clarified over the past two decades in plants, and simultaneous mutation of three meiotic genes that govern these processes can effectively trigger an avoidance of meiosis. This genotype is termed *MiMe*, or Mitosis-instead-of-Meiosis, as the elimination of the key meiotic hallmarks turns meiosis into a mitotic-like division (d'Erfurth et al., 2009). *MiMe* mimics naturally occurring apomeiosis, and thereby can be used as an effective tool to engineer one part of apomixis. The first meiotic hallmark affected in *MiMe* is recombination, a process that physically links homologous chromosomes and promotes the exchange of DNA that drives genetic diversity. Recombination can be ablated in *MiMe* by mutating *SPO11*, a DNA topoisomerase complex encoded by the genes *SPO11-1* and *SPO11-2* in plants (Benyahya et al., 2020; Grelon et al., 2001). SPO11 is required for double strand break (DSB) formation during meiosis, a critical initial step in recombination initiation. Loss of function mutations in *SPO11-1* or *SPO11-2* prevent the formation of DSBs and recombination subsequently fails to take place, resulting in disrupted meiotic chromosome segregation and a failed meiosis (Grelon et al., 2001). *spo11* mutants thus completely remove the first key characteristic of meiosis in *MiMe*. Deletion of additional genes involved in DSB formation, *PRD1*, *PRD2* or *PRD3*, also effectively ablate recombination and can be used instead of *spo11* (De Muyt et al., 2007, 2009). The second meiotic hallmark affected in *MiMe* is a modified first division that promotes the co-segregation of sister chromatids. During mitotic division, sister chromatids are segregated to opposing daughter cells in part due to the orientation of kinetochores, large protein structures that link spindle microtubules to chromosomes. Kinetochores on each sister chromatid separate and 'bi-orient' during mitosis, facing opposing spindle poles to facilitate the equal division of chromatids. Sister kinetochores during meiosis 'mono-orient', closely associating to face a single spindle pole and promoting the segregation of homologous chromosomes but not sister chromatids. Mono-orientation, or monopolar orientation, relies on the meiosis-specific cohesin subunit REC8 in plants (Chelysheva et al., 2005). REC8 is thought to promote the close association of sister kinetochores, and also ensures sister chromatid cohesion until the second meiotic division. REC8 is also important for meiotic recombination, essential for stabilizing recombination intermediates. Deletion of *rec8* in the model plant Arabidopsis thus results in a disrupted meiotic prophase that triggers chromosome fragmentation at the first division (Chelysheva et al., 2005). Combining *rec8* with *spo11*

however results in the alignment of free homologous chromosomes prior to the first meiotic division due to an absence of recombination. Due to loss of monopolar orientation, sister kinetochores 'bi-orient' as during mitosis, triggering the equal segregation of sister chromatids and mimicking a mitotic division (d'Erfurth et al., 2009). Mutations in REC8 thus remove the second key characteristic of meiosis within *MiMe*. Lastly, the third key meiotic process is the occurrence of a second division. In plants, the cell cycle regulator OSD1 regulates the meiosis I-meiosis II transition (d'Erfurth et al., 2010). OSD1 likely promotes the entry in meiosis II by repressing the activities of the anaphase promoting complex/cyclosome (APC/C) (Cromer et al., 2012), a large multi-subunit complex that coordinates cell cycle progression during mitosis and meiosis. Accordingly, *osd1* mutants fail to enter meiosis II and exit meiosis after the first division (d'Erfurth et al., 2010). Mutations in OSD1 are thus effective in ablating the third key meiotic characteristic in *MiMe*. OSD1 acts together with the cyclin TAM and the APC/C component TDM1, and deletion of *TAM* or a *TAM*-phosphorylation site within *TDM1* also triggers meiotic exit after meiosis I (Cromer et al., 2012). All three mutants yield unreduced diploid spores rather than reduced haploid spores at the conclusion of meiosis. Combining *rec8spo11* with *osd1*, *tam*, or *TDM-P17L* simultaneously removes all three key meiotic processes and yields the production of non-recombined, unreduced diploid spores that develop into viable gametes. *MiMe* has been proven beyond the model Arabidopsis, and has to date been successfully implemented for clonal gametes in rice and tomato (Mieulet et al., 2016; Y. Wang et al., 2024). Thus, apomeiosis can be engineered in plants by the disruption of three meiotic genes.

Strategies to engineer parthenogenesis

In most flowering plants, the formation of viable gametes and the sperm and egg cell is followed by a double fertilization event. One haploid sperm cell fuses with a haploid egg cell to give rise to a diploid embryo, while another haploid sperm cell fuses with the central cell to give rise to triploid endosperm needed for embryo development. In naturally occurring diplosporous apomicts, the diploid egg cell produced from apomeiosis skips fertilization and develops into a diploid embryo independent of the sperm cell. This strategy to skip the fertilization process is termed parthenogenesis, and represents the second key process in apomixis following apomeiosis (Hand & Koltunow, 2014). Parthenogenesis ensures that only the maternal genome is transmitted into the embryo by omitting the paternal contribution. In two different natural apomicts, two independent genes have been found to control

parthenogenesis in a dominant manner. In the apomictic wild grass *Pennisetum squamulatum*, a *BABY BOOM-like* gene (*PsASGR-BBML)* located within a known parthenogenesis locus and expressed in the egg cell was found to be sufficient for triggering parthenogenesis when transformed in sexual plants (Conner et al., 2015). *BABY BOOM* (*BBM*) genes are AP2/ERF transcription factors involved in embryo development and expressed in early embryos and seeds (Boutilier et al., 2002). In apomictic dandelion, a *PARTHENOGENESIS* (*PAR*) gene was recently identified within a known parthenogenesis locus; *PAR* is also expressed preferentially in the egg cell in apomictic plants (Underwood et al., 2022). A transposable element inserted in the promoter of *PAR* is exclusively observed in apomictic plants and is thought to promote its egg cell expression; sexual dandelion plants possess a recessive *par* allele that lack the insertion and are expressed highly in pollen (Underwood et al., 2022). Both egg-cell expressed *BBM* and *PAR* are capable of triggering parthenogenesis when transformed into crop species of interest. Rice *BBM1* under the control of an egg-cell specific promoter can initiate embryogenesis and induce haploids, and can further induce apomixis to yield clonal seeds when combined with rice *MiMe* (Khanday et al., 2019; Vernet et al., 2022). Dandelion *PAR* expressed under a similar egg-cell promoter can trigger parthenogenesis in lettuce (Underwood et al., 2022). Preferential expression of *BBM1* and *par* genes in the male genome but not in the female prior to fertilization support a model that suggest both genes similarly act as initiators of embryogenesis, and can be mis-expressed in the egg cell to engineer parthenogenesis.

As the central goal of synthetic parthenogenesis is the exclusion of the male genome contribution, an alternative strategy to *BBM*/*PAR* is the induction of haploids. Haploid induction relies upon the elimination of one of the parent genomes, generally the male, prior to fertilization to prevent the fusion of sperm and egg. One mechanism identified in the model Arabidopsis involves the mutation of the H3-histone variant CENH3, a key component of the centromere required for proper chromosome segregation during meiosis and mitosis. While null *cenh3* mutants compromise mitosis and cause lethality, modification of the tail domain of CENH3 (*GFP-tailswap*) is viable but meiosis is affected (Ravi & Chan, 2010). Crossing of *GFP-tailswap* to wild-type Arabidopsis plants results in the mis-segregation of chromosomes from the modified parent and induces haploids. This strategy can effectively induce haploids and trigger parthenogenesis in maize and wheat (Kelliher et al., 2016; Lv et al., 2020). Another mechanism to eliminate one parental genome involves null mutations in

MATRILINEAL (*MTL/PLA1/NLD*), a gene underlying a long-used method of haploid induction in maize (Kelliher et al., 2017). *MTL/PLA1/NLD* encodes a phospholipase important for pollen development (Gilles et al., 2017). *mtl/pla1/nld* mutants trigger the spontaneous formation of haploids likely owing to aneuploidy within pollen (Jiang et al., 2022), and provide a loss-of-function allele for haploid induction. *mtl/pla1/nld* has also been proven to be effective in rice and wheat (H. Liu et al., 2020; Yao et al., 2018). Compared to *BBM*/*PAR* approaches to engineered parthenogenesis, genome elimination is hindered by lower haploid induction rates. CENH3 variants used to induce parthenogenesis also still require a crossing step that presents a possible limitation. Mis-expression of embryogenesis factors thus may be a favorable approach to engineered parthenogenesis, although genome elimination offers feasible alternatives.

Synthetic apomixis in crops

Strategies to engineer apomeiosis and parthenogenesis have to date been successfully combined to produce clonal seeds in Arabidopsis and rice. In Arabidopsis, *MiMe* paired with modified CENH3 provided the first proof-of-concept of synthetic apomixis and showed that maternal heterozygosity can be maintained in clonal progeny (Marimuthu et al., 2011). In rice, *mtl/pla1/nld*, egg-cell *BBM1*, and egg-cell *PAR* have each been paired with *MiMe* to yield clonal seeds and engineer apomixis (Khanday et al., 2019; Song et al., 2024; Vernet et al., 2022; C. Wang et al., 2019). Early evidence exists that combining *BBM1/PAR* and *MiMe* in a unified expression cassette may be most effective in high rates of clonal seed production, reaching over 60% induction rates rice using *PAR*, and up to 95% using *BBM1* (Song et al., 2024; Vernet et al., 2022). Comparatively lower clonal seed induction rates are obtained using *mtl/pla1/nld* genome elimination, possibly owing to fertility losses (C. Liu et al., 2022). Embryogenesis factors *BBM* and *PAR* mimic the likely pathway employed by natural apomicts, and this may underly greater induction rates and fewer pleiotropic effects on development. What remains to be clarified is whether the described strategies for synthetic apomixis can be broadly applied to other economically important crops; conservation of key meiotic genes and embryogenesis factors seem to offer promise in this direction (Chen et al., 2022; Y. Wang et al., 2024).

Aims of this work

The following chapters will describe efforts to clarify the mechanism of monopolar orientation in plants and to assess whether synthetic apomixis can be broadly applied to new species of interest, using the widely cultivated barley as a test case. In Chapter 2, genetic screening, molecular genetics, and cytological techniques are employed in the model Arabidopsis to identify factors and postulate a mechanism behind a crucial process of meiotic chromosome segregation. In Chapter 3, phylogenetic analysis, genome editing, and cytometry techniques are used in the crop species *Hordeum vulgare* (barley) to modify key aspects of sexual reproduction with the goal of introducing apomixis into a sexually reproducing crop.

Chapter 2: Monopolar orientation of kinetochores at meiosis is enforced by cohesin, CENP-C, and the desumoylase ASP2

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Abstract

The first division of meiosis is unique in its capacity to halve the ploidy of the future gametes. To this end, one key innovation compared to a mitotic division is the monopolar orientation of the pairs of sister kinetochores required for the proper separation of homologs at meiosis I. How monopolar orientation is imposed is unclear and seems to vary in eukaryotes. Here we performed a forward genetic screen in *Arabidopsis thaliana*, specifically designed to identify the molecular components imposing monopolar orientation of sister kinetochores, based on their ability to restore fertility in haploid plants. We show that all four cohesin subunits (REC8, SCC3, SMC1, SMC3), cohesion establishment factors CTF18 and DCC1, and the cohesin protectors SGO1/2 and PANS1 promote monopolar orientation. Further, we show that monopolar orientation involves the inner kinetochore protein CENP-C and the desumoylase ASP2. We show that weakened monopolar orientation is associated with the spitting of sister kinetochores at metaphase I and reduced levels of cohesin. Taken together, the findings demonstrate that cohesion establishment and protection, kinetochore function, and SUMOylation thus enforce monopolar orientation in plants and support a cohesion-driven model of kinetochore orientation at meiosis I that is conserved across kingdoms.

Introduction

Meiosis is a cell division that promotes chromosomes to exchange genetic information and reduce their number by pairing homologous chromosomes and executing two rounds of division. Despite major strides in recent decades, our understanding of how exactly meiosis

ensures replicated chromosomes are made to segregate together at one division and divide only at the next remains incomplete (McAinsh & Marston, 2022; Nasmyth, 2015). A central player in the process is the kinetochore, large protein complexes that enable the attachment of microtubules to chromosomes. During meiosis I, the kinetochores of replicated sister chromatids act as a unified microtubule-binding face that 'mono-orient' towards one spindle pole, as opposed to 'bi-orienting' during mitosis. This process is known as monopolar orientation, or sister kinetochore co-orientation. The outcome of this co-orientation after the onset of the first division is the segregation of homologous chromosomes to opposing poles rather than chromatids, representing a reductional rather than equational division that allows for the later separation of sisters in pursuit of haploid spores. Essential for chromosome segregation are the activities of cohesin, a member of the SMC complex family, ring-shaped complexes that are involved in the packaging and organization of chromosomes during mitosis and meiosis (Uhlmann, 2016). Cohesin is responsible for creating cohesion between replicated sister chromatids during S phase (Gruber et al., 2003). During meiosis, cohesin rings containing a meiosis-specific 'kleisin' subunit Rec8 entrap sister chromatids. In contrast to mitosis, Rec8-containing cohesin in the vicinity of peri-centromeres is protected from removal by the protease Separase during meiosis I (Blattner et al., 2016). A conserved family of proteins called shugoshins drive this protection by recruiting the phosphatase PP2A to peri-centromeric Rec8 (Cromer et al., 2013; Kitajima et al., 2004; Sun et al., 2023; Zamariola et al., 2013). While clear that this protected cohesin is necessary to maintain sister chromatid cohesion after the first division, the absence of Rec8 also abolishes sister kinetochore co-orientation in fission yeast, plants, and vertebrates (Chelysheva et al., 2005; Ogushi et al., 2021; Shao et al., 2011; Watanabe & Nurse, 1999). A common mechanism has been proposed that suggests differences in cohesion between sister chromatids along their arms and at their core- and peri-centromeres during meiosis are fundamental to establishing the monopolar orientation of sister kinetochores prior to the first meiotic division (Nasmyth, 2015; Sakuno et al., 2009; Watanabe, 2004). This presents a model in which Rec8 at the core centromere but not the peri-centromeres is crucial for the co-orientation of sister kinetochores, supported by findings that the depletion of core-centromeric Rec8 causes bi-orientation in mouse (Ogushi et al., 2021). The proper attachment of microtubules to kinetochores during meiosis has also been implicated in co-orientation. Aurora kinase is involved correcting microtubule-kinetochore attachments during mitosis, and similarly works to promote correct attachments to mono-orienting kinetochores during meiosis I (Hauf et al., 2007). The role of cohesin in not however universal, as point-centromere

containing budding yeast harbors a protein complex called Monopolin containing a meiosis-specific subunit Mam1, required for monopolar orientation in *S. cerevisae* (Toth et al., 2000). Monopolin directly cross-links sister kinetochores and drives co-orientation independent of cohesion; functional homologs of Mam1 have not however been identified outside of *S. cerevisae* (Petronczki et al., 2006; Rabitsch et al., 2003; Sarangapani et al., 2014; Toth et al., 2000).

Notably, meiosis-specific proteins that localize to kinetochores have been identified in yeast and vertebrates that drive co-orientation in a cohesion-dependent manner (Kim et al., 2015; Yokobayashi & Watanabe, 2005); mouse Meikin and fission yeast Moa1 function in localizing Rec8-phosphorylating kinases directly to the kinetochore to promote core centromere cohesion (Maier et al., 2021; Yokobayashi & Watanabe, 2005). Functional homologs of Meikin/Moa1 nor any additional factors beyond cohesin subunits Rec8 and SCC3 that have a role in co-orientation have not been identified in plants, raising the question of whether such a mechanism is broadly conserved.

Here, a screening strategy in Arabidopsis was undertaken to identify factors involved in monopolar orientation. Mutant alleles identified in cohesin. kinetochore, and SUMOylation factors abolish co-orientation and implicate cohesion as a central mode of action in Arabidopsis monopolar orientation. We further show that sister kinetochores physically separate prior to the first meiotic division in cohesin mutants and *asp2*. Reduced levels of meiosis-specific cohesin in cohesin mutants and *asp2* suggests a weakening of cohesion that may underly a predisposition for sister kinetochores to bi-orient. Mutations in the inner kinetochore component CENP-C and the desumoylase ASP2/ULP2 also abolish co-orientation, likely through cohesion regulation. The findings strengthen the model that diverse pools of cohesins occupy chromatin and direct stepwise segregation of chromosomes.

Results

A forward genetic screen for monopolar orientation

We designed a specific genetic screen to identify factors promoting the monopolar orientation of kinetochores at meiosis I, based on the restoration of the fertility of haploid plants. In haploid Arabidopsis plants, meiosis still occurs but chromosomes cannot recombine because of the

absence of homologs (Figure 2A) (Cifuentes et al., 2013; Ravi & Chan, 2010). At anaphase I, the resulting five univalents do not separate into chromatids like at mitosis (i.e. 5:5) but rather segregate as single units at anaphase I (e.g. 3:2), demonstrating that the monopolar orientation of the sister chromatid kinetochores is maintained in haploid meiosis. The resulting unbalanced first division is followed by a second division that distributes sister chromatids. As a consequence, haploid plants have extremely low fertility. However, turning meiosis into mitosis in haploids with the *MiMe* mutations bypasses the meiotic defects and restores fertility (Cifuentes et al., 2013). *MiMe* is composed of three mutations - *spo11, rec8* and *osd1*- that abolish the three key differences between meiosis and mitosis, recombination, the monopolar orientation of kinetochores, and the occurrence of the second division, respectively (d'Erfurth et al., 2009). Of particular interest here, the double mutant *spo11 osd1* is sterile because the monopolar orientation of kinetochores prevents balanced segregation of sister chromatids. We thus performed a forward genetic screen in haploid *spo11 osd1* mutants with the rationale that any mutation that would affect monopolar orientation of kinetochores would restore fertility, as the *rec8* mutation does. Fertility is easy to assess visually by simply looking at the size of the fruits, making a large forward genetic screen feasible.

In practice, we took advantage of the TailSwap haploid inducer line (Marimuthu et al., 2011), whose genome is eliminated in a proportion of embryos following crosses, generating haploid progeny containing only the genome of the other parent. We crossed the haploid inducer as female with plants heterozygous for *spo11-1* and *osd1* mutations and homozygous for the *glabra1* mutation, which confers a glabrous appearance to the leaves (Figure 2B). We applied moderate EMS mutagenesis on the seeds and selected the ~30% of haploid plants based on the *glabra1* phenotype (plants with trichomes were eliminated at an early stage of development), as haploid results from the elimination of the genome of the haploid inducer line, which is wild type for *GLABRA1*. One additional advantage of the screen in a haploid context is the phenotypic expression of recessive mutations in the first generation. As EMS mutagenesis is applied on seeds that contain several pluripotent cells, the resulting plants are chimeric for the induced mutations, as strikingly exemplified by chlorotic mutations observed in sectors of some M1 plants (Figure 2C). We screened ~9000 *glabra* plants, looking for branches with longer fruits, as candidates for carrying a mutation affecting the monopolar orientation of kinetochores (Figure 2D). Branches with longer fruits were genotyped for *osd1* and *spo11* and ploidy was tested through chromosome spreads on terminal buds. In many cases, these were diploid,

despite having the *glabra* phenotype. This noise could result either from the spontaneous doubling of somatic cells (Ravi & Chan, 2010), or more complex genetic events associated with genome elimination (Tan et al., 2015). In total, we retained thirteen lines derived from haploid branches with enhanced fertility. A combination of whole genome sequencing, genetic mapping, and candidate testing with independent alleles led to the identification of the causal mutations in these thirteen lines, corresponding to ten genes (Table 1).

We further tested and quantified the effect of the identified mutations on monopolar orientation, through their ability to modify chromosome segregation and fertility in diploid plants in the absence of recombination (Figure 3 and 4). In diploid *spo11-1 or spo11-2*, the absence of recombination leads to the presence of ten univalents at metaphase I (figure 4D), which because of monopolar orientation, mostly segregate as single units at anaphase I, without separation of the sister chromatids, resulting mostly in the random distribution of the ten univalents (e.g. 7:3) (Figure 4E-F) (Grelon et al., 2001). Occasionally, we observed the separation of one or two pairs of sister chromatids, indicating that univalents can infrequently orient in a bipolar manner at metaphase I in *spo11-1 or spo11-2* (Figure 4J). This unbalanced segregation at meiosis I causes a quasi-sterility of the *spo11-1* and *spo11-2* mutants, alone or in combination with *osd1* mutants that skip the second division (Figure 3A). A mutation that abolishes monopolar orientation, is thus expected to provoke the biorientation of univalents, separation of sisters, and restored fertility of *spo11 osd1* mutants. We tested these predictions in the candidate mutations identified in the screen.

The four subunits of the Cohesin complex promote monopolar orientation

The cohesin subunits *REC8* and SCC3 were previously shown to be involved in monopolar orientation of kinetochores in Arabidopsis (Chelysheva et al., 2005). Consistently, the screen described above identified two mutations in *REC8*, one introducing a STOP codon at position 20 and one changing the proline codon at position 60 into a serine. Another line contained a missense mutation in *SCC3* (R326>G). This confirms the efficiency of the screen and the importance of these two cohesin subunits in imposing monopolar orientation at meiosis I. In addition, causal mutations were found in the two other cohesin subunits, SMC1 and SCC3 (*smc1-C624Y, smc3-G129E,* Table 1). The causality of the *smc3*-G129E mutation was confirmed by genetic mapping, being the sole EMS-induced mutation in the *mp226* line that co-segregates with fertility of *spo11 osd1*. The causality of the *smc1-C624Y* mutation was confirmed through allelic testing with the previously characterized *smc1* null mutation

SALK_017437 (Schubert et al., 2009). SMC1-C624 falls within the conserved hinge domain required for cohesin's stable association with chromosomes (Mishra et al., 2010). As SCC3, SMC1, and SMC3 are each encoded by a single gene and essential for plant development (Chelysheva et al., 2005; C. Liu et al., 2002; Schubert et al., 2009), the mutations identified here must represent partial or separation-of-function alleles affecting meiosis without impairing plant viability.

The *spo11 osd1* double mutant has very low fertility, yielding less than one seed per fruit on average (Figure 3A). In contrast, the *spo11 osd1 rec8-3* mutant shows a high level of fertility with 44 seeds per fruit, similar to the single *osd1* mutant (t-test p=0.53). The *smc1-C624Y* mutation also increased the fertility of *spo11 osd1*, but to a lesser extent than *rec8-3* (Figure 3A, 28 seeds). The *rec8-P60S* and *smc3-G129E* mutations had a milder, but significant, effect on *spo11 osd1* fertility (Figure 3A). This suggests different penetrance in monopolar orientation, with *rec8-3* having the strongest effect, *smc1-C624Y* being intermediate, *and rec8-P60S* and *smc3-G129E* having the mildest effect. We then observed chromosome behavior on male meiotic chromosome spreads. In *spo11-1*, the ten univalents mostly segregate at meiosis as a single unit in an erratic manner (e.g. 7:3), with occasional mixed segregation (Figure 4E, 4J). In contrast, in *spo11-1 rec8-3*, *spo11-1 smc3*-G129E, and *spo11-1 smc1-C624Y* we observed metaphase I with aligned univalents followed by balanced segregation of sister chromatids, resulting in a 10:10 segregation (Figure 4G-I, J). This modification of chromosome segregation compared to *spo11* was almost fully penetrant in *spo11-1 rec8-3 and spo11-1 smc1-C624Y*, but less frequent in *spo11-1 rec8-P60S* and *spo11-1 smc3-G129E* (Figure 4J), which is consistent with the lower restoration of fertility observed in *spo11 osd1 rec8-P60S and spo11 osd1 smc3-G129E* (Figure 3A). Altogether, this shows that all core Cohesin subunits promote the monopolar orientation of sister kinetochores at meiosis I. The central role of REC8 suggests that Cohesin complexes containing alternative α-Kleisins subunit (SYN2-4)(Schubert et al., 2009)) do not sustain monopolar orientation at meiosis. SMC1, SMC3, and SCC3, which are single-copy and essential Cohesin subunits, may be more crucial than suggested by the effects of the inevitable partial alleles analyzed here. The *smc1-C624Y* mutation which has a strong effect on monopolar orientation, also affects plant development with reduced plant height. Altogether, this strongly reinforces the conclusion that the entire cohesin complex is crucial in imposing monopolar orientation at meiosis I in plants.

Cohesin, cohesion establishment, and desumoylation mutants show separated kinetochores and weakened centromeric cohesion

REC8 is essential for double-strand break repair at meiosis, leading to chromosome fragmentation and full sterility of the single *rec8* null mutant (i.e. in the presence of DNA double-strand breaks generated by SPO11-1/SPO11-2) (Bai et al., 1999; Chelysheva et al., 2005). While null mutation of *REC8* results in meiotic failure and subsequent sterility, *rec8-P60S* undergoes largely normal meiosis and shows no effect on fertility. The single *smc3*-G129E was fully fertile, and meiosis was indistinguishable from the wild type (Figure 3), notably normal alignment of bivalents and segregation of homologs at meiosis I. This parallels the mild effect of this allele on the monopolar orientation of univalents (Figures 3 and 4). The observation that bi-orientation is only observed in univalents confirms that chiasmata are required for monopolar orientation.

In contrast, the single *smc1-C624Y* single mutant showed strongly reduced fertility and meiotic defects (Figures 3 and 5). In most cells, five bivalents aligned at metaphase I with an arrangement similar to the wild type, suggesting normal mono-orientation of sisters in the presence of chiasmata. Univalents were occasionally observed, suggesting an incomplete implementation of crossovers (Figure 5D). These univalents aligned at metaphase I in a bipolar manner, consistent with a defect in maintaining monopolar orientation of univalents, although not in bivalents.

In chromosome spreads of metaphase I cells, DAPI-stained bivalents resemble stretched diamonds, with centromeric tips observable at the edges of bivalents (Figure 5A). In the wildtype, centromeric tips appear as sharp points that likely represent the close association of sister centromeres (Figure 5G). Centromeres in *smc1-C624Y* bivalents however appear split at metaphase I in 53% of centromere pairs (Figure 5H-I). Immunostaining of CENH3 on *smc1-C624Y* bivalents confirmed this separation and revealed an average distance of 500nm between the center of the sister kinetochores, compared to an average of 340nm in the wildtype (Figure 6 A-C). Telophase I and metaphase II showed two sets of 10 chromatids suggesting a complete failure of protection of peri-centromeric sister chromatid cohesion at anaphase I (Figure 5E, F). This suggests that sister chromatid cohesion is weakened in *smc1-C624Y*, notably in the vicinity of centromeres, which in turn could affect the robustness of monopolar orientation.

SHUGOSHINs promote monopolar orientation, with SGO2 playing a predominant role

Two SHUGOSIN homologs, SGO1 and SGO2, were shown to protect centromeric cohesion at meiotic anaphase I in *Arabidopsis* (Cromer et al., 2013; Zamariola et al., 2013, 2014). SGO1 and SGO2 protect centromeric cohesion partially redundantly, with SGO1 having a more important role than SGO2. In the screen described here, mutations affecting a splicing site in both *SGO1* and *SGO2* were identified (Table 1). The role of SGO1 and SGO2 in monopolar orientation was confirmed by introducing the independent T-DNA alleles *sgo1-2* and *sgo2-1* (Cromer et al., 2013) into *spo11-2 osd1*. Interestingly, *sgo2* provoked a stronger restoration of fertility than *sgo1* (Figure 3A). Consistently, chromosome spreads of male meiocytes in *spo11-2 sgo1* and *spo11-2 sgo2* showed that *sgo2* provoked a stronger reduction of monopolar orientation (Figure 4J). This shows that SGO2 has a more crucial role than SGO1 in imposing monopolar orientation, while it is the opposite for centromeric cohesion protection. Combining *sgo1* and *sgo2* mutations further restored the fertility of *spo11-2 osd1*, and bipolar orientation (Figures 3 and 4). Thus, both SGO1 and SGO2 promote monopolar orientation in a partially redundant manner, with SGO2 playing a more important role. The observation that SGO1 and SGO2 have opposite relative importance for monopolar orientation and cohesin protection suggests that these two proteins have a specific function and may suggest that different pools of cohesins promote monopolar orientation and centromeric cohesion.

As cohesins and cohesin protectors appear to promote monopolar orientation, we reasoned that PATRONUS, an Arabidopsis Securin homolog which protects cohesins by antagonizing Separase (Cromer et al., 2013, 2019), could also promote monopolar orientation. Indeed, the *pans1* mutation was able to promote the bipolar distribution of sister chromatids in *spo11-1* (Figure 4). Note that the *pans1* mutation cannot be found in our forward screen as *pans1* is synthetic lethal in combination with *osd1* (Singh et al., 2015).

Cohesion establishment factors CTF18 and DCC1 promote monopolar orientation

The forward screen for monopolar function yielded two additional genes that interact with cohesin, CTF18 and DCC1, which are known to work together to facilitate the establishment of sister chromatid cohesion during replication as members of the RFC^{Ctf18} complex (Mayer et al., 2001; Petronczki et al., 2004; Takahashi et al., 2010). Two alleles were isolated for DCC1 and CTF18, both affecting splicing sites and thus presumably strong or null alleles (Table 1).

Two CRISPR deletion alleles was generated in DCC1 for independent confirmation, *dcc1-1* and *dcc1-2*, that both carry an 1163bp deletion in the coding sequence of DCC1, predicted to retain only the first 29 of 388 amino acids. *dcc1-2* and *ctf18-mp193* restored fertility of *spo11-1 osd1* to intermediate levels, similar to *smc1-C624Y* or *sgo2-1*, but less than *rec8-3* (Figure 3). We next observed male meiotic behavior by performing chromosome spreads. In *spo11*, univalents randomly segregate due to monopolar orientation or occasionally show a mixed segregation at meiosis I (Figure 4E). In *dcc1-2 spo11-1* and *ctf18 spo11-1* segregation patterns corresponded to an intermediate penetrance of both mutations compared to weak cohesin mutants or *rec8* (Figure 4J). This suggests a greater importance of *CTF18* and *DCC1* than *SMC3-G129E* and *SGO1* in imposing monopolar orientation, though below that of *REC8.*

Despite the importance of both CTF18 and DCC1 for monopolar orientation, the *dcc1* and *ctf18* single mutants did not affect develop or fertility (Figure 3B), suggesting that neither factors are essential at mitosis or meiosis. This reinforces the requirement for chiasmata in promoting monopolar orientation, as only *dcc1-2 spo11-1* and *ctf18 spo11-1* show defective monopolar orientation. The meiotic behavior of *dcc1-1* however showed splitting of centromeres tips at metaphase I in 17% of centromere pairs in a manner similar to *smc1-C624Y* (Figure 5I). Accordingly, immunostaining of CENH3 in *dcc1-1* revealed a separation of on average 460nm between sister CENH3 signals relative to 340nm in the wildtype (Figure 6A-C), signifying a premature separation of sister kinetochores prior to anaphase onset.

Immunostaining of REC8 showed a significant reduction of REC8 cohesin at centromeres and broadly on chromosomes in *dcc1-1* relative to the wild type (Figure 7 C, D). Thus, less meiotic cohesin persists along chromosome arms and at centromeres during meiosis I in the absence of *DCC1*. This suggests that cohesion establishment factors, whose activities take place long before meiosis I, may promote the robustness of monopolar orientation-relevant cohesin pools by ensuring sufficient cohesin is loaded during replication. Meiotic cohesin levels important for kinetochore orientation are reduced in the absence of cohesion promoted by the Ctf18-RFC complex, resulting in the premature dissociation of sister kinetochores and a preference for bi-orientation rather than mono-orientation in the absence of chiasmata.

The inner kinetochore protein CENP-C promotes monopolar orientation and cohesion protection

Among the mutants recovered in the screen, one had an insertion of one base pair in the coding sequence of the kinetochore protein CENP-C. Genetic mapping to a single mutation confirmed that this mutation was causal in restoring fertility of *spo11-1 osd1* (Figure 3A). The *cenpc-T29** mutation induces a frameshift at exon T29. Intriguingly, the *cenpc-T29** mutant is viable, while the CENP-C protein is presumably essential in *Arabidopsis* as in other eukaryotes (Talbert et al., 2004). However, an ATG codon at position 56 may serve as an alternative translation start codon that would generate a CENP-C protein truncated of its N terminus. The *cenpc-T29** mutation restores fertility of *spo11-1osd1*, at levels less than *rec8-3* but greater than *smc3-G129E* and *sgo1-2*, yielding 18 seeds (Figure 3A). We next looked at male meiosis by performing chromosome spreads. In *spo11-1* mutants, univalents randomly segregate following meiosis I due to monopolar orientation. The *cenpc-T29** allele is able to invoke a 10:10 balanced segregation of sister chromatids in the *spo11-1* mutant and show a moderate level of mixed segregation (Figure 4J). The frequency of biorientation is reduced compared to *rec8-3* and *smc1-C624Y* (Figure 4J), corresponding to intermediate penetrance observed in restored fertility in *cenpc-T29*spo11-1osd1* (Figure 3A). These findings demonstrate that the kinetochore itself plays a role in monopolar orientation. The fertility of the single *cenpc-T29** mutant is reduced to 50 seeds per silique, compared to greater than 60 seeds in the wild type (Figure 3B). Despite slightly reduced fertility, the meiotic behavior of *cenpc-T29** is largely normal, and separated centromeres are only observed in 2% of centromere pairs at metaphase I, comparable to the wild type and in contrast to *dcc1* or *smc1-C624Y* (Figure 5E). Immunostaining of REC8 showed a slight reduction in the signal of meiotic cohesin at centromeres and broadly on chromosomes in *cenpc-T29** (Figure 7 C,D), suggesting that CENP-C promotes cohesin levels at meiosis I.

The *sgo2*-1 mutant does not show any visible meiotic defect on its own, but enhances the cohesion defects of *sgo1-2*, provoking a complete loss of sister chromatid cohesion after anaphase I (Cromer et al., 2013). Strikingly, combining *sgo2-1* with *cenpc-T29** also provoked a complete loss of sister chromatid cohesion at telophase I (Figure 8 M-O), while both single mutants do not show this defect. This shows that CENP-C promotes centromeric sister chromatid cohesion during meiosis. *smc1-C624Y and sgo1-2sgo2-1* mutants also present a

complete loss of sister chromatid cohesion after meiosis I, which is equally observed when adding the *cenpc-T29* mutation (*Figure 8D-E and 8A-C*)*. Together, the findings implicate CENP-C in monopolar orientation and in the maintenance of centromeric cohesion at anaphase I.

The desumoylase ASP2 promotes monopolar orientation

Finally, we identified two independent mutations in the desumoylase *ASP2* (At4G33620), a frameshift mutation at position 385 (*mp150*) and a missense at codon 303 (D303>N) (*mp268*). The At4G33620 gene encodes for one of the two Arabidopsis homologs of the yeast desumoylase ULP2 (Novatchkova et al., 2004). It is known as ASP2 (Kong et al., 2017), but was also named in the literature ULP2-like1 (Novatchkova et al., 2004) and SPF2 (L. Liu et al., 2017). As the first functional characterization of the gene employed the name ASP2 (*Arabidopsis SUMO protease 2*) (Kong et al., 2017), we maintain this name in this manuscript. The *asp2-mp150* mutation provokes a frameshift upstream of the protease domain of ASP2, presumably abolishing its function. This allele significantly restored the fertility of *spo11-1 osd1* to levels similar to that of *smc1-C624Y* and *dcc1*, yet less than *rec8 (*Figure 3A). During male meiosis in *spo11-1* mutants, univalents randomly segregate during meiosis I. The *asp2-2* mutation invokes the equational 10:10 segregation of chromatids and mixed segregation in the *spo11-1* mutant at an intermediate penetrance similar *to cenpc-T29** or *dcc1*, but less than *rec8* (Figure 4J) and concordant with the intermediate penetrance observed in restored fertility. This result implicates the deSUMOylase ASP2 in monopolar orientation, suggesting that the sumoylation of an unknown target(s) favors bipolar orientation.

Single mutants of *asp2-mp150* show no visible growth or fertility defects (Figure 3B), as previously reported (Castro et al., 2018). However, immunostaining for CENH3 on *asp2-mp150* bivalents at metaphase I shows an average separation of sister kinetochores of 410nm, which is significantly greater than the 340nm measured in the wild type (Figure 6A-C). Immunostaining for REC8 revealed a significant reduction in meiotic cohesin broadly on chromosomes and at centromeres in *asp2-mp150* (Figure 7B-D). This suggests that the desumoylase ASP2 protects cohesin, which in turn favors close association of kinetochores and monopolar orientation.

The *asp2-3 cenpc-T29** double mutant strikingly shows loss of sister chromatid cohesion at telophase I (Figure 8H), reminiscent of *smc1-C624Y* and *sgo1sgo2* and implicating both *ASP2* and *CENP-C* in cohesion protection. *The asp2-3 smc1-C624Y* double mutants displays a stretched and fragmented bivalent structure at metaphase I (Figure 8J), suggesting a defect in chromosome compaction and/or DNA double strand-break repair, as observed in *rec8* (Chelysheva et al., 2005). Early loss of sister chromatid cohesion at telophase I is also observed (Figure 8K). Taken together, ASP2 promotes the monopolar orientation of kinetochores during meiosis in a cohesion-dependent manner.

Discussion

Monopolar orientation in plants is dependent on cohesin

Monopolar orientation in fission yeast and metazoans appears to be largely promoted by the specific regulation of meiotic cohesin in the vicinity of centromeres (Ogushi et al., 2021; Watanabe & Nurse, 1999). We show here that in addition to meiotic cohesin subunit REC8, mutations in cohesin subunits *SMC1*, *SMC3*, and *SCC3* promote monopolar orientation in Arabidopsis. Specific mutations in *SMC1* and *SMC3* reverse mono-orientation in achiasmatic chromosomes (Figure 4J) but are still viable (Figure 3B). The existence of cohesin mutants that affect monopolar orientation, but not the maintenance of cohesion, suggests that monopolar orientation requires a level of cohesion fidelity beyond that required for sister chromatid cohesion. They further support the role of chiasmata in promoting monopolar orientation (Hirose et al., 2011), as monopolar orientation remains intact in *smc1-C624Y* and *smc3-G129E* bivalents. We further find that CTF18 and DCC1, subunits of the Ctf18-RFC complex, support monopolar orientation. *ctf18* and *dcc1* similarly abolish monopolar orientation in univalent and may disrupt the cohesion threshold required for mono-orientation. Early separation of sister kinetochores (Figure 6) and reduced meiotic cohesin (Figure 7) in cohesin mutants suggest a disruption of meiosis I that favors bi-orientation over mono-orientation in the absence of chiasmata. Further support for this idea comes from work in mouse oocytes that demonstrated that specific extinction of REC8 in the immediate vicinity of centromeres, but not peri-centromeres, causes a significant increase in inter-sister kinetochore distance in metaphase I bivalents (Ogushi et al., 2021). Similar to our findings, this separation alone is not sufficient to reversion mono-orientation in bivalents, but effectively does so when univalents are present at

the metaphase I plate (Figure 4)(Ogushi et al., 2021). These findings broadly support a cohesion-centered model of monopolar orientation in plants.

Two plant shugoshins support mono-orientation

We show here that both plant shugoshins have a role in promoting monopolar orientation. Shugoshin (Sgo) proteins protect cohesion during meiosis by shuttling protein phosphatase 2A (PP2A) to pericentromeres to dephosphorylate REC8 and prevent its cleavage by separase (Kitajima et al., 2004, 2006; Riedel et al., 2006). Plants possess two shugoshin proteins that both function in cohesion protection during meiosis, but not mitosis (Cromer et al., 2013). Mutations in both *SGO1* and *SGO2* cause the 10:10 segregation of chromatids in *spo11* univalents (Figure 4J), implicating their role in monopolar orientation. The *sgo2-1* mutation shows higher penetrance in terms of bi-orientation (Figure 4J) and restored fertility of *spo11-1osd1* (Figure 3A), suggesting *SGO2* has a more important role in monopolar orientation in contrast to a less important role in cohesion protection (Cromer et al., 2013). In fission yeast, Sgo2 (no relation to plant SGO2 and orthologous to vertebrate SGOL1) but not Sgo1 was shown to promote mono-orientation through its regulation of Aurora kinase and thus microtubule attachment (Hauf et al., 2007; Rabitsch et al., 2004). Given that neither plant Shugoshin functions in mitosis in a manner orthologous to fission yeast Sgo2, plant *SGO1* and *SGO2* may promote mono-orientation entirely through cohesion protection. Double *sgo1-2 sgo2-1* (Cromer et al., 2013) and *cenpc-mp sgo2-1* (Figure 8 M-O) mutants both show early loss of sister chromatid cohesion in telophase I, firmly implicating both in cohesion protection. Taken together, plant Shugoshins are necessary for proper mono-orientation that may be ensured through multiple pathways including cohesion protection and error correction.

AtCENP-C functions in mono-orientation and cohesion protection

The inner kinetochore protein CENP-C is a crucial scaffold component that regulates kinetochore assembly during mitosis and meiosis (Gascoigne & Cheeseman, 2011). We show here that CENP-C also has a role in imposing mono-orientation during meiosis. The *cenpc-mp* allele is able to promote the equational 10:10 segregation of chromatids in *spo11-1* univalents (Figure 4J), indicating a loss of monopolar orientation. Reduced meiotic cohesin on *cenpc-mp* bivalents at metaphase I suggests that cohesion is affected by the mutation (Figure 7C, D), and combining *cenpc-mp* with mutations in the cohesion protector *SGO2* trigger the early loss of

sister chromatid cohesion after the first division (Figure 8). Taken together, CENP-C likely promotes monopolar orientation during meiosis I in a cohesion-dependent manner. One possible mechanism supporting this function could involve CENP-C interaction with cell cycle kinases. CENP-C was recently shown to possesses an Aurora B phosphorylation site in its N-terminus that regulates its interaction with the outer kinetochore component Mis12 in fission yeast (Zhou et al., 2017). During meiosis, *C. elegans* Aurora homolog AIR-2 was also found to be necessary for cohesin release and homolog segregation (Rogers et al., 2002), and Drosophila Aurora B and INCENP have also been shown to maintain cohesion through direction regulation of the shugoshin homolog MEI-S332 (Resnick et al., 2006). The *cenpc-mp* allele is predicted to have lost a significant portion of the N-terminus, including the Aurora B phosphorylation site and the Mis12 binding site. One explanation for bi-orienting univalents in *cenpc-T29* spo11* (Figure 4) could thus be a loss of error correction either through Aurora B-regulated CENPC-Mis12 interaction, or loss of the Mis12 binding site altogether. Disruption of Aurora B/CENP-C interaction may also affect cohesion given Aurora B's role in cohesion protection.

Do plants lack a meiosis I-specific cohesin regulator?

The findings presented here implicate a significant number of novel players in a crucial step of meiotic chromosome segregation in plants, namely the proper orientation of kinetochore complexes prior to the first meiotic division. While long recognized as an essential and unique feature of meiosis (Östergren, 1951), its precise mechanisms have remained elusive, notably in the plant kingdom. A shared mechanism among yeasts appears to involve meiosis-specific proteins, namely Moa1 in *S. pombe* (Yokobayashi & Watanabe, 2005). A likely functional homolog of Moa1, called MEIKIN, is conserved in mammals and similarly acts as a meiosis-specific shuttle for Polo kinase localization to the kinetochore (Kim et al., 2015). Mouse oocytes and spermatocytes lacking MEIKIN show signs of defective mono-orientation, with frequent splitting of kinetochores prior to anaphase I and bi-orienting kinetochores in cells lacking chiasmata (Kim et al., 2015), identical to the kinetochore and chromosome segregation phenotype of cohesin and *ASP2* mutants described here (Figures 4-6). Common properties that link all both kinetochore proteins is their meiosis specificity, their regulation of key cell cycle kinases, and their predicted intrinsic disorder (Galander & Marston, 2020). Despite finding numerous cohesion-related genes in our forward screen that support the REC8-dependent model described in *S. pombe* and metazoans, we did not find a meiosis-specific kinetochore protein. It cannot be concluded that Moa1/MEIKIN is absent from the plant kingdom on the basis of the screen; screening saturation may not have been reached, or gene duplication may

prevent the isolation of single mutant alleles. The absence however of Polo kinase/PLK in the plant kingdom may provide a clue (Okamura et al., 2017). One possibility could be that plants employ a different mechanism to ensure persistence of core centromeric cohesion, for example a functionally similar kinase. However, the early separation of kinetochores at metaphase I observed in cohesin mutants (Figure 6) mirrors that of MEIKIN mutants (Kim et al., 2015) and centromeric REC8 depleted oocytes (Ogushi et al., 2021), suggesting that a common mechanism may be conserved in plants.

SUMOylation promotes monopolar orientation

Our findings implicate the plant homolog of ULP2 in promoting monopolar orientation. Early separation of sister kinetochores and reduced meiotic cohesin at meiosis I suggest that ASP2 promotes monopolar orientation in a cohesion-dependent manner (Figures 6-7). *asp2-mp* paired with *cenpc-T29** show precocious loss of sister chromatid cohesion at telophase I, further implicating a role in cohesion (Figure 8). SUMOylation is known to have diverse roles in coordinating chromosome segregation in both mitosis and meiosis through means of kinetochore assembly, regulation of error correction, and cohesin establishment and maintenance (Almedawar et al., 2012; Ding et al., 2018; Huang et al., 2015; Mukhopadhyay et al., 2010; Su et al., 2021). The desumoylase ULP2 has been shown to prevent the build-up of SUMO chains on SMC complexes including cohesin in budding yeast (Psakhye & Branzei, 2021). Loss of ASP2 may lead to the premature removal of cohesin necessary to maintain monopolar orientation, leading to bi-orientation of univalents in *asp2 spo11* (Figure 4J). One cannot exclude however the possibility that a cohesin-independent role is also at play through SUMOylation of kinetochore or error correction-related proteins. Condensins, SMC complexes involved in chromosome condensation during meiosis and mitosis, are important for mono-orientation in budding yeast (Brito et al., 2010). ULP2/ASP2 also prevents SUMOylation-mediated removal of condensins (Psakhye & Branzei, 2021). Thus, *asp2* mutants may also perturb monopolar orientation through condensins, but would require further investigation of specific condensin complexes during Arabidopsis meiosis I.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana plants were grown in greenhouses or growth chambers under 16 hour day/8 hour night conditions at 20 degrees C. In addition to the EMS and CRISPR alleles produced in this study we used the following genetic material:

osd1-3 (Heyman et al., 2011)
rec8-3/SAIL_807_B08 (d'Erfurth et al., 2009)
spo11-1-3/SALK_146172 (Stacey et al., 2006)
spo11-2-3/GABI_749C12 (Hartung et al., 2007)
smc1-1/SALK_017437 (Schubert et al., 2009)
sgo1-2 and sgo2-1 (Cromer et al., 2013)
asp1-1/spf1-2/SALK_049255 (Kong et al., 2017; L. Liu et al., 2017)
asp2-2/*spf2-3*/SALK_140824 (Castro et al., 2018)
*asp2-3/*SAIL_182_F02 (Sessions et al., 2002)
*ctf18-3/*SALK_043339 (Alonso et al., 2003)
glabra1-hc1 (Elmayan et al., 1998)
GFP-tailswap (Ravi & Chan, 2010)
pans1-1/Salk_070337 (Cromer et al., 2013)

EMS mutagenesis

EMS mutagenesis of Arabidopsis plants was carried out as previously described (Capilla-Perez et al., 2018). Briefly, seeds were treated for 17 hours in 0.1% (v/v) EMS. EMS was neutralized by adding 1M sodium thiosulfate. Treatment was performed on seeds resulting from CENH3-*tailswap* (Marimuthu et al., 2011) crossed as female with plants heterozygous for *spo11-1* and *osd1* mutations and homozygous for the *glabra1* mutation.

CRISPR-Cas9 mutagenesis of *AtDCC1*

To assemble a complete CRISPR-Cas9 binary vector, four guide RNAs were first designed spanning the genomic sequence of AT2G44580 using CRISPOR (Haeussler et al., 2016) (http://crispor.tefor.net/). Cloning of the guide RNAs into the final Cas9-containing binary vector was carried out using the MoClo Golden Gate cloning toolkit (Weber et al., 2011) and integrating an intron-containing Cas9 (Grützner et al., 2021). Briefly, guide RNAs were

synthesized as an oligonucleotide comprising the target sequence and BsaI restriction enzyme recognition sequence. In the first cloning reaction, each guide was independently fused to an AtU6 promoter while simultaneous adding complementary BpiI recognition sites. In the final cloning reaction, all four guides were assembled with a selection marker (FastRED) (Ursache et al., 2021) and Cas9. This completed construct was then transformed into A. tumefaciens, and harvested cells were used to transform Col-0 A. thaliana plants using the floral dip method (Zhang et al., 2006). Positive transformants were selected by screening seeds for FastRED fluorescence.

Genotyping

To isolate genomic DNA, leaf tissue was harvested from young Arabidopsis plants and stored at -80 degrees C. Samples were pulverized using a tissue lyser, and then 200uL of extraction buffer (200mM Tris pH 7.5, 250mM NaCl, 25mM EDTA, 0.5% SDS) was added. Samples were briefly shaken and then incubated at 50 degrees C for 10 minutes. The mixture was then centrifuged for 15 minutes at 4000 x g. At least 100uL of the supernatant was removed and mixed with 100uL of isopropanol. Samples were again centrifuged for 15 minutes at 4000 x g. The supernatant was poured off and 100uL of 70% ethanol was added. Samples were centrifuged for 10 minutes at 4000 x g, then turned upside down and left to dry overnight. DNA was resuspended in 100uL of distilled water. Primers specific to T-DNA insertion alleles obtained from Salk or NASC collections or published alleles were used in 20ul PCR reactions to genotype plants. Primers used for novel alleles are listed in Appendix Table 1.

Chromosome preparations

Young inflorescences were harvested from Arabidopsis plants and immediately fixed in 3:1 ethanol:acetic acid. Flower buds roughly 0.5mm in size were isolated from inflorescences and washed in citrate buffer, then incubated in driselase enzyme mixture (Sigma) for 2 hours at 37 degrees C. Four to five digested buds were transferred to a clean slide and macerated with a bent dissection needle. Roughly 15uL of 60% acetic acid was added to the mixture and stirred gently at 45 degrees C on a hotplate for 1 minute. The slide was then flushed with ice-cold 3:1 fixative first around the droplet and then directly. Slides were left to dry tilted at room temperature, then 10uL of DAPI in mounting media was applied to the slide and a coverslip was added. Chromosomes were visualized using a Zeiss Axio Observer epifluorescence microscope.

Immunocytology

Slides that preserve the 3D structure of freshly fixed meiocytes were prepared and used for immunocytology using published methods (Capilla-Pérez et al., 2021). Briefly, fresh inflorescences were collected from Arabidopsis plants, dissected to expose young athers, and fixed in paraformaldehyde for 30 minutes under vacuum. Buds roughly 0.5mm in size were selected and digested in driselase cellulase/pectinase enzyme mixture for 30 minutes to remove cell walls. Anthers were then isolated and slightly ruptured to release meiocytes, then squashed with a polyacrylamide gel layer onto coverslips. Coverslips were stained with DAPI to stage meiocytes, then treated for 48 hours at 4 degrees C with primary antibodies for CENH3 (anti-chicken, 1:500) or REC8 (anti-rabbit, 1:250) prepared in blocking buffer. Secondary antibodies were then incubated for 24 hours at 4 degrees C before addition of mounting media with DAPI and the sealing of slides with nail polish. Slides were imaged using an Abberior Facility Line STED microscope using a 2D STED depletion laser. STED images were processed using Huygens Essential software (Scientific Volume Imaging, The Netherlands, http://svi.nl) to perform deconvolution and distances between STED CENH3 signals were measured using Fiji (ImageJ) on maximum intensity projections**.** To quantify relative fluorescence, non-deconvolved sum projection images were used in Fiji. Regions of interest (ROIs) were drawn over confocal CENH3 signals for centromeres or DAPI signals for chromatin. Mean background confocal REC8 signal was subtracted from mean REC8 signal on centromeres or chromatin, and then normalized by dividing this value by background-corrected CENH3. Thus, $(S_{REC8}-S_{REC8\ bg})/(S_{CENH3}-S_{CENH3\ bg})$, where S is the mean signal and bg is background. Statistical tests and plots were prepared using R and ggplot2. P values were established using Mann-Whitney-Wilcoxon tests.

Contribution statement

This manuscript is relevant to this work as it composed one of two major thesis projects undertaken during my studies. My contribution included the genetic mapping and identification of *AtDCC1* and *AtREC8-P60S* mutant alleles from the genetic screen, the generation of CRISPR-Cas9 mutant alleles in *AtDCC1*, chromosome segregation and fertility analysis of several of the described mutant alleles, all centromere separation analysis, all immunolocalization experiments, analysis of the data, and writing of the manuscript.

Figure legends

Figure 2. A forward genetic screen for monopolar orientation. (A) Schematic describing meiotic progression of the wildtype, haploids, haploid *spo11osd1* and haploid *spo11rec8osd1* (*MiMe*) (created with BioRender). (B) Schematic describing the creation an EMS mutant population to identifiy plants displaying restored fertility (created with BioRender). (C) Examples of M1 mutant individuals displaying chlorosis (left) and restored fertility (right) on single branches.

Figure 3. Fertility of monopolar orientation mutants in achiasmatic and chiasmatic contexts. Each dot represents the average number of seeds from 10 fruits of one plant. (A) Fertility of all monopolar orientation-defective mutant alleles in the *spo11osd1* mutant background. (B) Fertility of single monopolar orientation-defective mutant alleles.

Figure 4. Analysis of the effect of monopolar mutations on the segregation of achiasmate chromosomes. (A) Wild type metaphase I. (B) wild type metaphase II (C) wild type anaphase II. (D) spo11 metaphase I. (E) *spo11-1* metaphase II (e.g 7:3). (F) *spo11-1* metaphase II with split chromatids. (G) *rec8-3 spo11-1* metaphase I. (H) *rec8-3 spo11-1* anaphase I (I) *rec8-3 spo11-1* metaphase II. Scale bars, 10um. (J) Quantification of the mode of chromosome segregation.

Figure 5. Chromosome spreads of single mutant male meiocytes. (A) Wild type metaphase I. (B) wild type metaphase II (C) wild type telophase II. (D) *smc1-C624Y* metaphase I; stars indicate univalent (E) *smc1-C624Y* metaphase II. (F) *smc1-C624Y* telophase II. Scale bars, 10um. (G) Zoomed detail of wildtype metaphase I bivalents at centromeric region. (H) Zoomed detail of *smc1-C624Y* metaphase I bivalents at centromeric region. (I) Quantification of split centromeric regions in wild type and monopolar orientation mutants; each data point represents one centromeric region.

Figure 6. Inter-sister kinetochore distance in cohesin affected mutants at metaphase I. (A) Confocal/STED images of metaphase I bivalents in the wildtype and monopolar orientation mutants; blue = confocal DAPI, orange = confocal+STED CENH3. Scale bars, 2um. (B) Quantification of the distance between STED CENH3 signals in the wildtype and monopolar

orientation mutants, where one data point represents one pair of CENH3 signals. * indicates a p-value < 0.05, *** indicates a p-value < 0.01, and **** indicates a p-value < 0.0001. (C) Averaged and normalized 1um line scan profiles centered on CENH3 pairs in the wildtype and monopolar orientation mutants.

Figure 7. Meiotic cohesin at centromeres and chromatin in cohesin affected mutants. (A) Confocal/STED images of wildtype metaphase I bivalents; green = confocal REC8, orange = STED CENH3, blue = confocal DAPI. (B) Confocal/STED images of *asp2-mp* metaphase I bivalents. Scale bar represents 1um. (C) Quantification of normalized REC8 signals at centromeric regions; each data point represents one centromeric region. (D) Quantification of normalized REC8 signals on chromatin; each data point represents one metaphase I cell.

Figure 8. Chromosome spreads of double mutant male meiocytes. (A-C) *cenpc-T29* sgo1-2 sgo2-1*. (D-F) *cenpc-T29* smc1-C624Y*. (G-I) *asp2-3 cenpc-T29**. (J-L) *asp2-3 smc1-C624Y*. (M-O) *cenpc-T29* sgo2-1*. (A,D,G,J,M) metaphase I. (B,E,H,K,N) metaphase II. (C,F,I,L,O) telophase II.

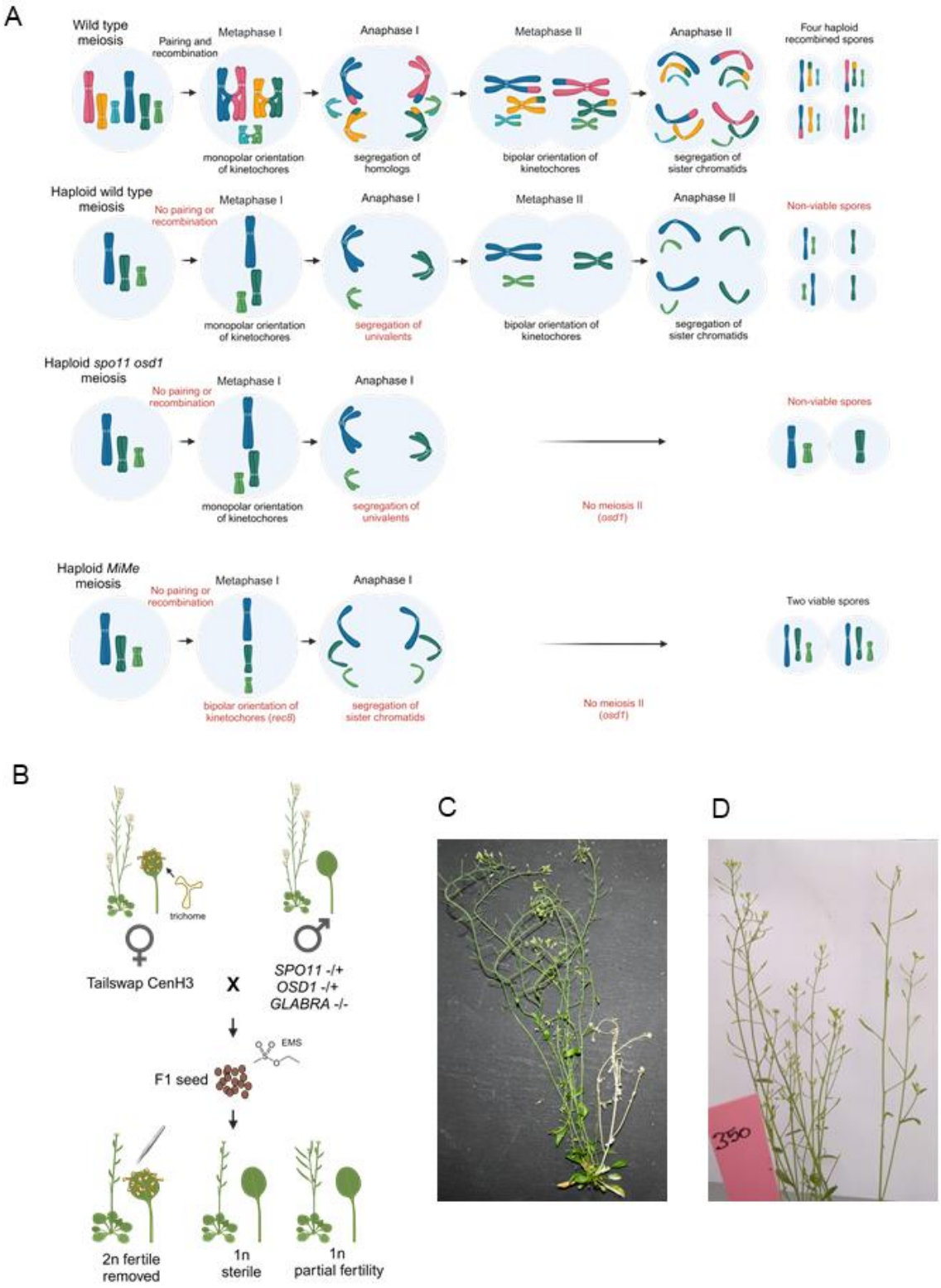

Figure 2. Equational meiosis and forward genetic screen

Protein	Function	Allele name	Gene	Position (TAIR10)		Effect on the protein	Method of confirmation	Comment
REC8	Cohesin subunit	mp257	AT5G05490	Chr5:1624739	C>T	R20>STOP	independant allele	meiosis specific
		mp305		Chr5:1625214	C>T	P60>S		
SMC1	Cohesin subunit	mp172	AT3G54670	Chr3:20239572	G>A	C644>Y	complementation test	null mutation is lethal
SMC3	Cohesin subunit	mp226	AT2G27170	Chr2:11615684	C>T	G129>E	mapping to a single mutation	null mutation is lethal
SCC3	Cohesin subunit	mp225	AT2G47980	Chr2:19633111	A>G	R326>G	independant allele	null mutation is lethal
CTF18	cohesin loading	mp193	AT1G04730	Chr1:1327905	C>T	splice site	independant allele	
		mp274		Chr1:1326791	C>T	splice site		
DCC1	cohesin loading	mp270	AT2G44580	Chr2:18403182	C>T	splice site	independant allele	
SGO2	cohesin protection	mp267	AT5G04320	Chr5:1210471	G>A	splice site	independant allele	
SGO1	cohesin protection	mp350	AT3G10440	Chr3:3248542	G>A	splice site	independent allele	
CENP-C	kinetochore	mp221	AT1G15660	Chr1:5381313	ins C	T29 frameshift, potential ATG at codon 56	mapping to a single mutation	null mutation is lethal
ASP2	desumoylase	mp150	AT4G33620	Chr4:16150537	del C	L385 frameshift	independant allele	
		mp268		Chr4:16150107	G>A	D303>N		

Table 1. Gene and mutations identified in the forward screen for monopolar orientation

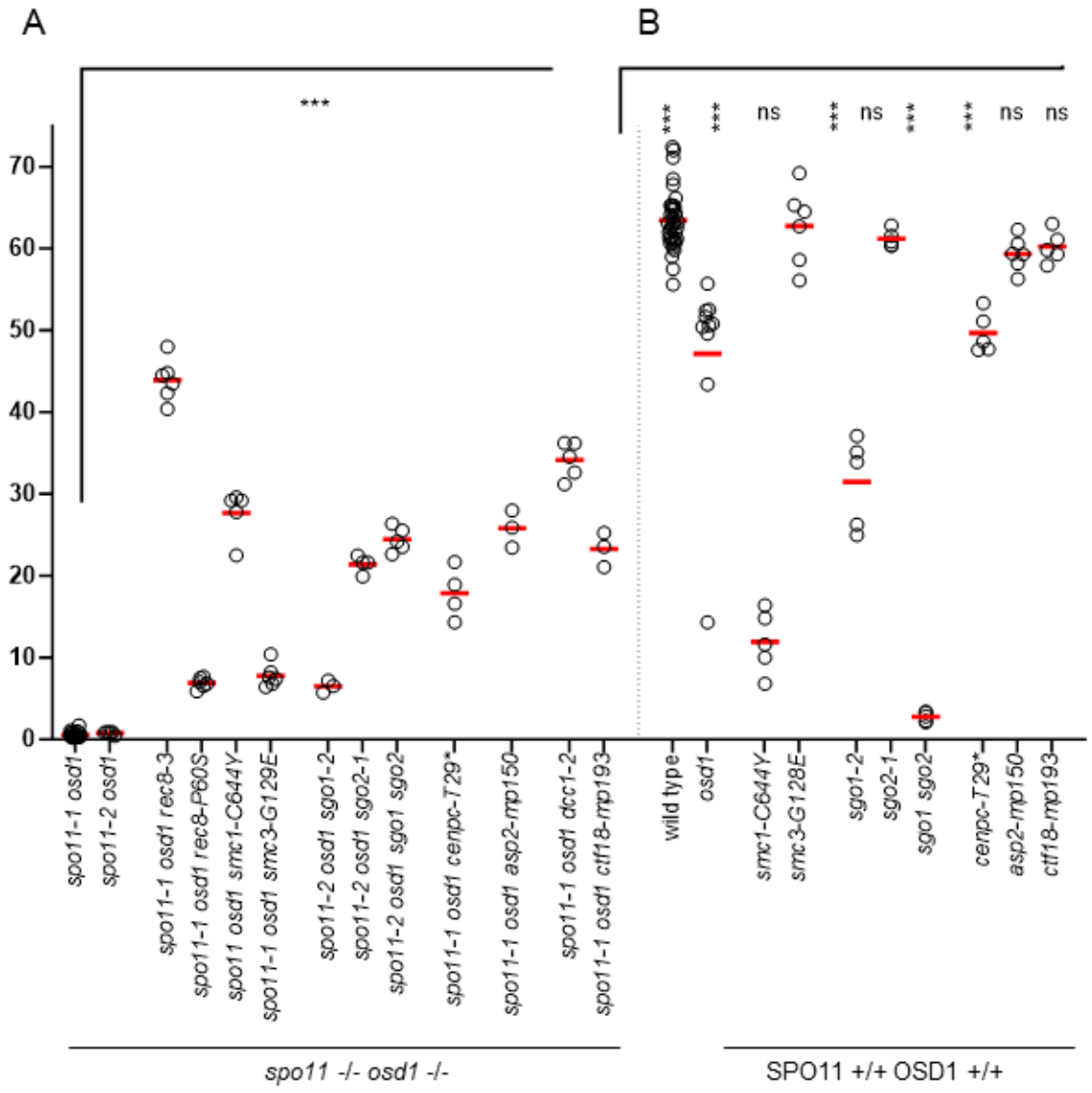

Figure 3. Fertility of monopolar orientation mutants in achiasmatic and chiasmatic contexts

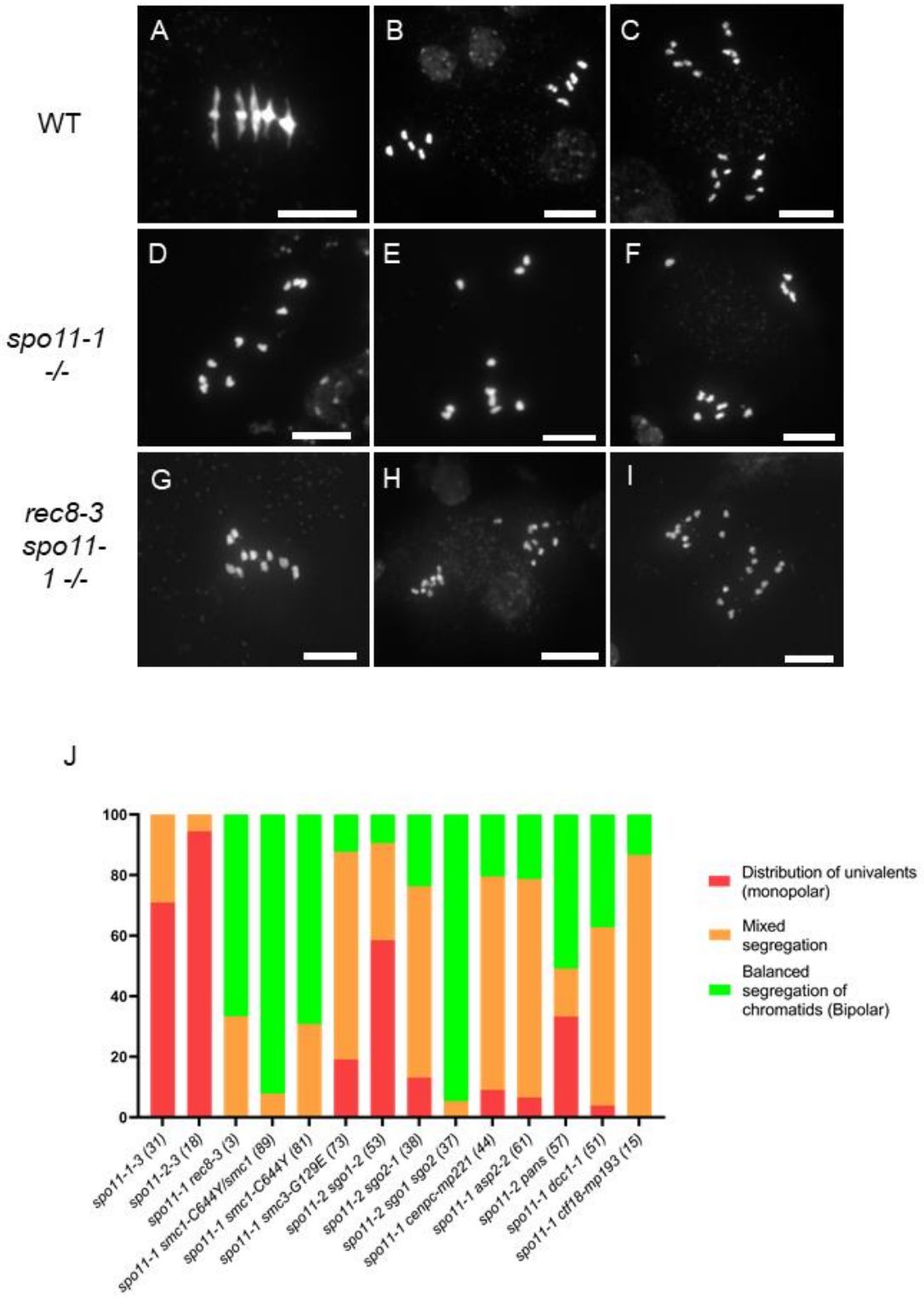

Figure 4. Analysis of the effect of monopolar mutations on the segregation of achiasmate chromosomes

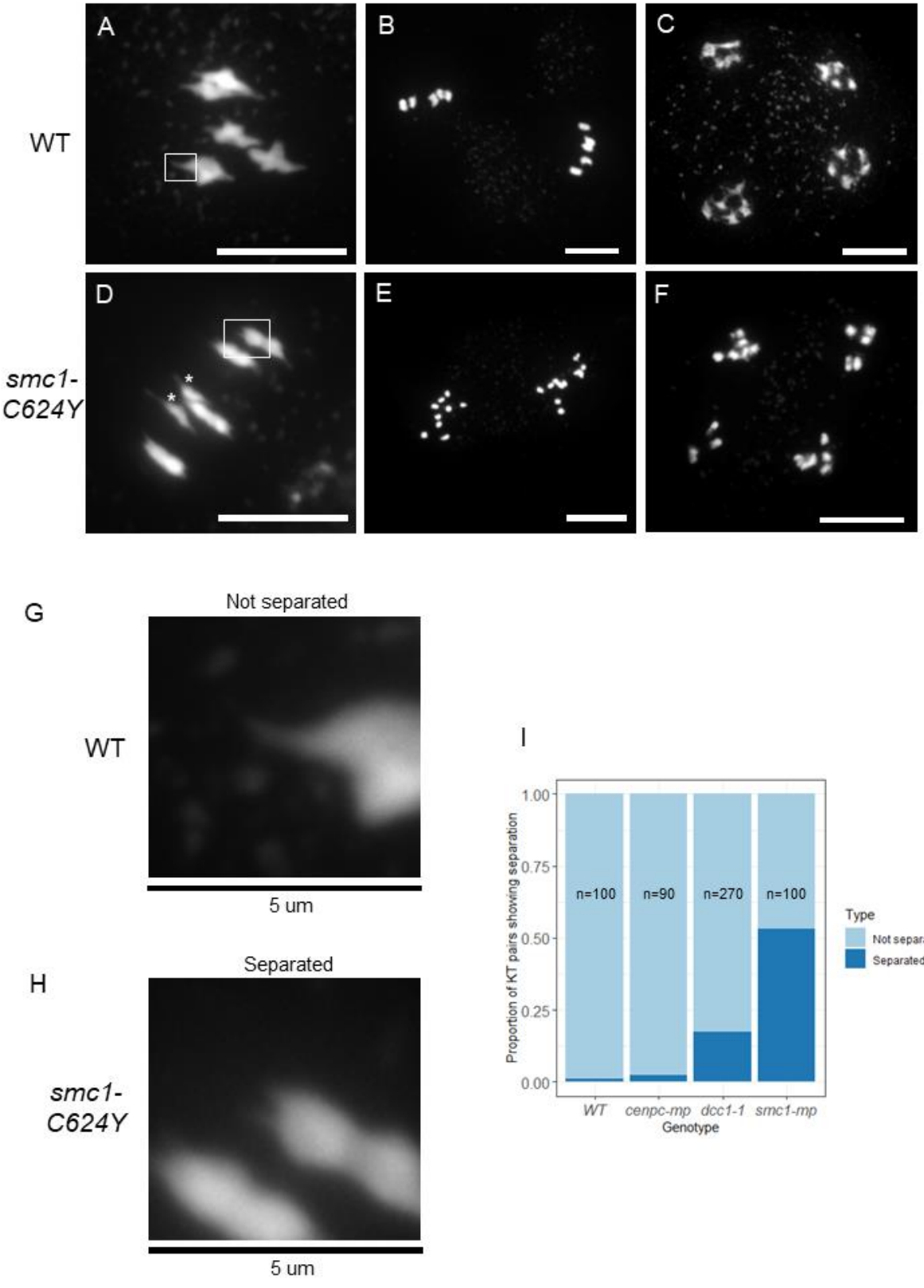

Figure 5. Chromosome spreads of single mutant male meiocytes.

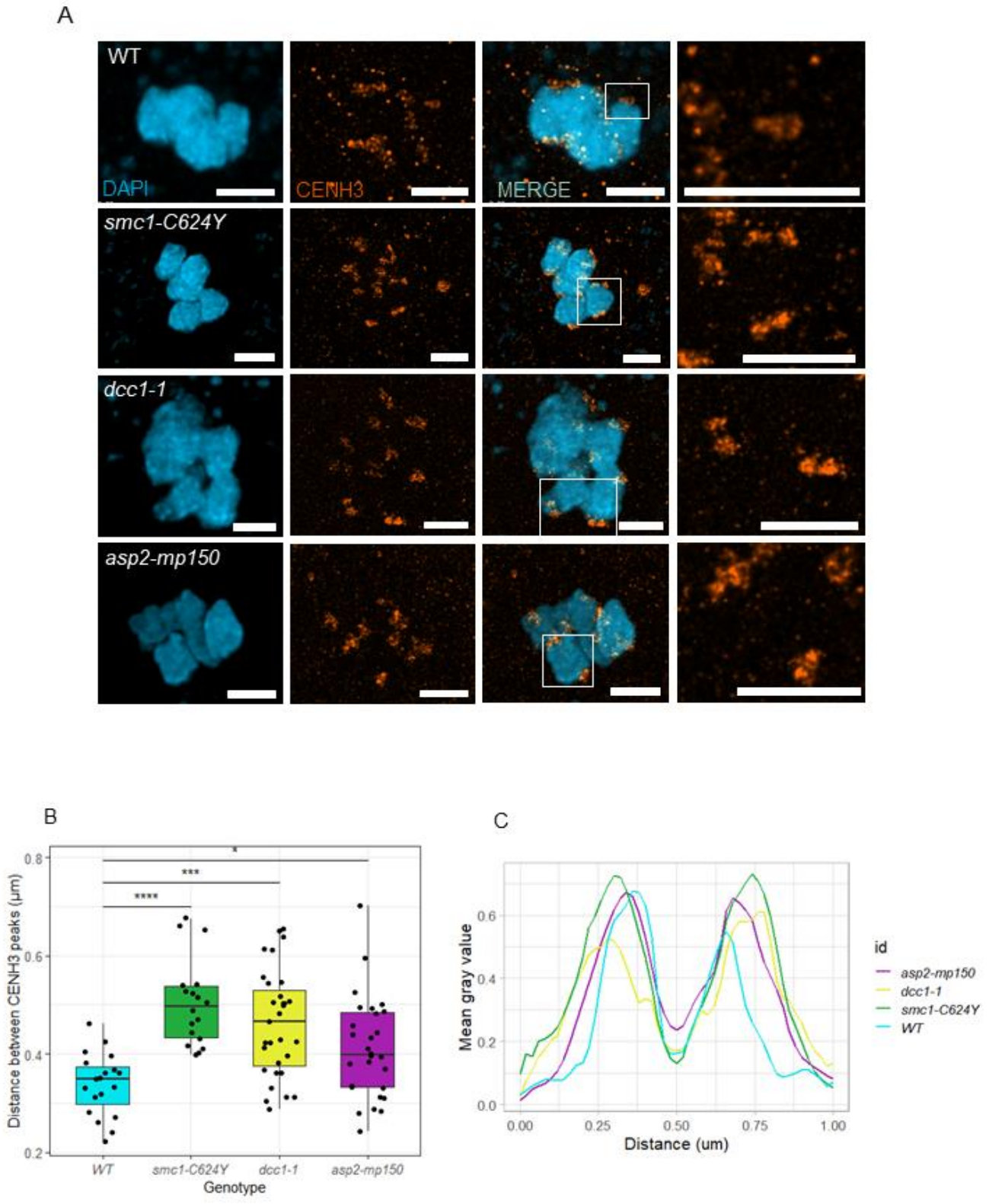

Figure 6. Inter-sister kinetochore distance in cohesin and *asp2* mutants at metaphase

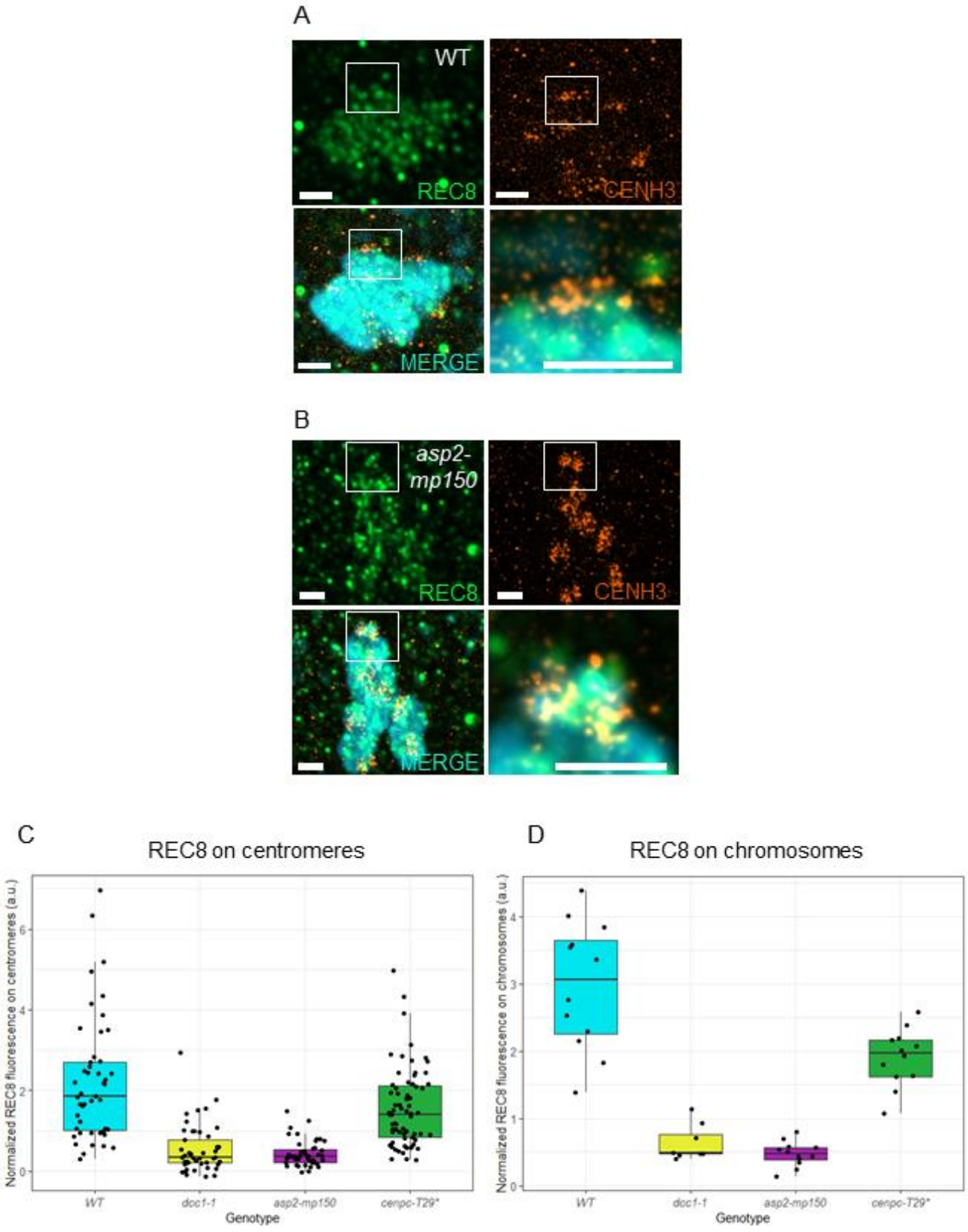

Figure 7. Meiotic cohesin at centromeres and on chromosomes

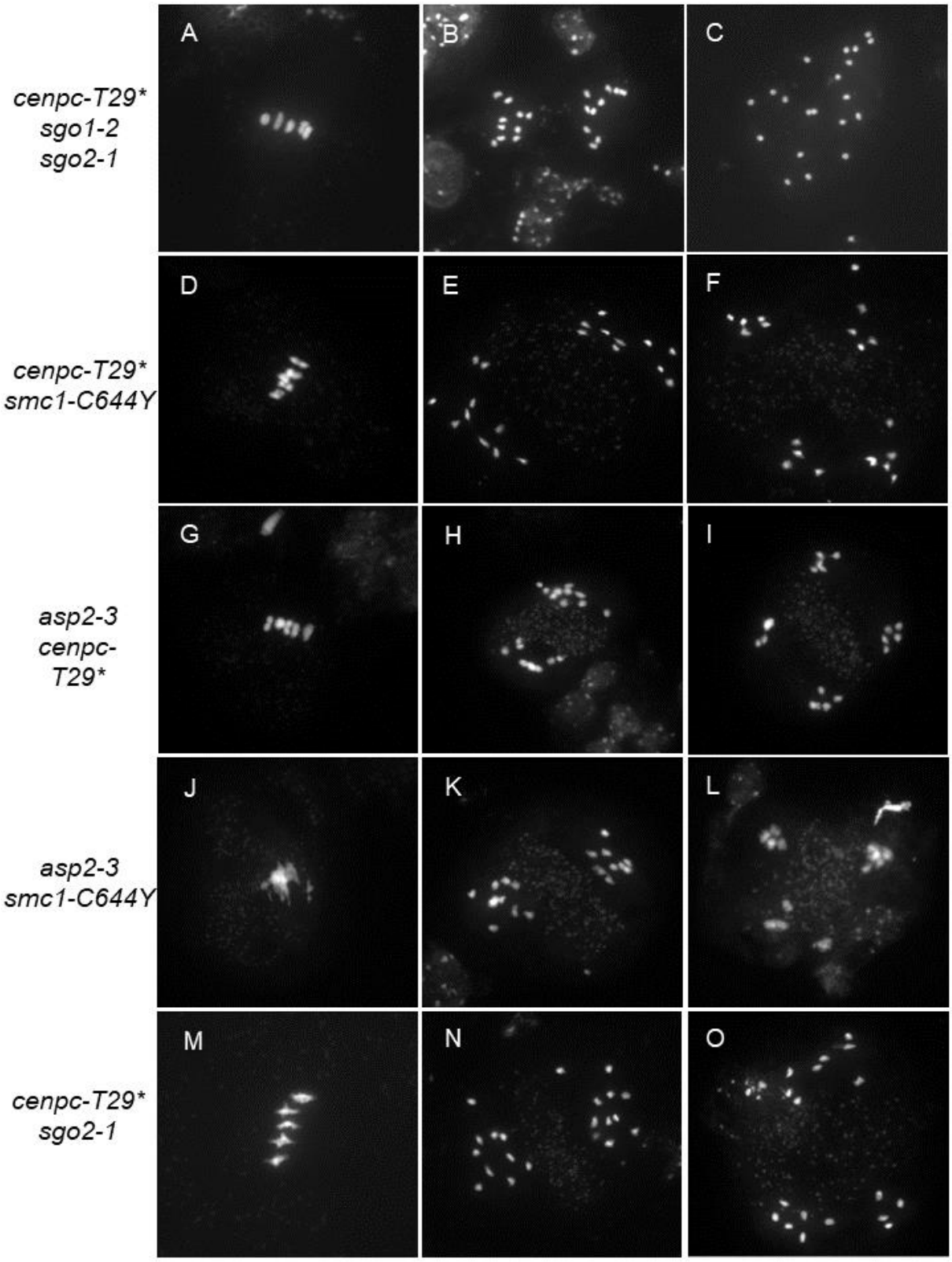

Figure 8. Chromosome spreads of double mutant male meiocytes

Chapter 3: Engineering clonal gametes and parthenogenesis for apomictic barley

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Abstract

Apomixis offers an attractive approach for fixing heterosis, or hybrid vigor, in modern agriculture and holds the potential to transform expensive and tedious hybrid seed production into stable asexual hybrid propagation through seeds. Altering meiosis to resemble a mitotic division (MiMe) while simultaneously rewiring genetic cues necessary for embryo development creates progeny identical to the maternal parent, a strategy proven to be feasible for engineering synthetic apomixis in rice. Notably missing is synthetic apomixis in Triticaceae, a family notably containing wheat and barley. Described here are progress towards synthetic apomixis to diploid barley, *Hordeum vulgare*. We show that meiosis can be altered to turn a reductional, recombined cell division into an equational one by showing that the double strand break initiator *SPO11-1* and the meiotic cohesin *REC8* are functionally conserved for recombination and sister chromatid cohesion in barley. We further show that *OSD1*, *TAM*, and *TDM1* are conserved in controlling barley meiotic progression, and display species-specific divergence and novel roles in post-meiotic development. We also find that the fertilization checkpoint can be avoided by expressing the embryogenesis regulator *BBM1* in the egg cell, inducing haploid barley plants. These findings suggest that apomixis can be engineered in the widely cultivated barley, with the potential to be extended to wheat and other cereal crops.

Introduction

A large number of economically important crops rely on the use of hybrid breeding, the crossing of selected lines to produce F1 progeny that outcompete their parents in desirable traits such as biomass and yield while also showing greater adaptability. Despite its precise mechanisms remaining unclear, hybrid vigor, or heterosis, has contributed to significant yield

gains in the past century (Hochholdinger & Baldauf, 2018). Hybrid production requires cost effective production of hybrid seeds, as the genome-wide heterozygosity present in F1 hybrids is lost in the next generation due to segregation during meiosis. While maize hybrid seed production benefits from the physical separation of male and female flowers, inbreeding crops such as wheat and barley present an added difficulty that can be overcome by utilizing male sterility, yet introduces additional limitations (Whitford et al., 2013). One proposal to fix heterozygosity in hybrids is the implementation of apomixis in modern crops (Hoisington et al., 1999; Spillane et al., 2004). Natural apomicts are able to skip meiosis and fertilization to yield diploid embryos identical to the maternal progenitor that in turn yield clonal seeds. Apomicts use various mechanisms to produce clonal seeds depending on the origin and formation of the embryo (Hand & Koltunow, 2014). Apomixis is found in roughly 250 plant species including those within the Poaceae, but absent in major crops (van Dijk et al., 2016). A synthetic strategy to circumvent meiosis to prevent recombination and reductional division was first described in the model plant Arabidopsis fifteen years ago by mutating three key meiotic genes (d'Erfurth et al., 2009), known as *MiMe*. *MiMe* plants carry mutations in SPO11-1, REC8, and OSD1, which respectively are required for recombination, monopolar orientation, and the onset of the second division. SPO11-1 initiates the formation of double strand breaks required for crossovers, while REC8 is a meiosis-specific cohesin that promotes monopolar orientation of sister kinetochores at meiosis I and sister chromatid cohesion until meiosis II (Bhatt et al., 1999; Chelysheva et al., 2005; Grelon et al., 2001). OSD1 regulates meiotic progression by repressing the anaphase promoting complex/cyclosome (APC/C), and possesses conserved KEN and D-boxes associated with this function (Cromer et al., 2012). Equational segregation of homologous chromosomes and a single division during meiosis in *MiMe* plants yields unrecombined diploid spores, phenocopying the "apomeiosis" observed in natural apomicts. To skip the second meiotic division, mutations in OSD1 can also effectively be replaced by null alleles of TAM (Tardy asynchronous meiosis), a Cyclin A1;2 gene that cooperates with OSD1 in meiotic progression (d'Erfurth et al., 2010). A third option to skip the second division involves disrupting a TAM phosphorylation site within TDM1, a proposed APC/C factor involved in meiotic exit (Cifuentes et al., 2016). *MiMe* using *osd1* has been shown to be feasible in rice due to conservation of key meiotic genes (Mieulet et al., 2016), and *MiMe* using tam has been recently described in tomato (Y. Wang et al., 2024).

While *MiMe* fulfills the requirement for a modified meiosis in the context of apomixis, the resulting male and female diploid gametes trigger a doubling of ploidy in the progeny following fertilization.

Numerous works in recent decades have identified means of preventing the contribution of one of the parent genomes, both of which can be used to mimic parthenogenesis observed in natural apomicts. The first route perturbs the proper segregation of one parent's chromosomes by altering a centromeric protein, excluding the paternal contribution in a process called genome elimination (GEM) (Ravi & Chan, 2010). Altering the tail domain of the centromeric protein CENH3 induces haploid plants upon crossing in Arabidopsis, and similarly produces diploids when crossing tetraploids. CENH3-tailswap thus acts as a haploid inducer while also mimicking parthenogenesis. Pairing of GEM with *MiMe* (synthetic parthenogenesis and apomeiosis) was successfully shown to produce clonal seeds in Arabidopsis shortly thereafter in the first demonstration of synthetic apomixis (Marimuthu et al., 2011). Important to note is that CENH3-tailswap haploid induction and synthetic apomixis requires a crossing step. An alternate strategy to induce genome elimination involves MATRILINEAL (ZmMTL/PLA1/NLD), a phospholipase gene involved in pollen development (Kelliher et al., 2017). Deletion of ZmMTL/PLA1/NLD causes elimination of the parental genome and has been shown to induce haploids in maize, rice, and wheat (Gilles et al., 2017; Kelliher et al., 2017; C. Liu et al., 2017; H. Liu et al., 2020; Yao et al., 2018). *Zmmtl/pla1/nld* can effectively induce synthetic apomixis when paired with *MiMe* (C. Wang et al., 2019). However, likely low penetrance of Zmmtl/pla1/nld and associated fertility loss keep clonal seed setting rates below 10% (C. Liu et al., 2022).

The second route to engineer parthenogenesis lies in the modification of genes driving embryogenesis, namely BABY BOOM (BBM) and PARTHENOGENESIS (PAR). BBM is an AP2/ERF transcription factor identified in a screen for genes upregulated during Brassica napus embryo development, and its overexpression was shown to induce the formation of somatic embryos on developing plants (Boutilier et al., 2002). A BBM homolog in the apomictic grass *Pennisetum squamulatum* was shown to be expressed preferentially in the egg cell, and its expression under its native promoter in sexual *Pennisetum glaucum* (pearl millet) can induce haploid embryos, confirming BBM as a viable parthenogenesis factor (Conner et al., 2015). In the dandelion *Taraxacum officinale*, a parthenogenesis-associated locus (Van Dijk et al., 2020) was recently shown to harbor PAR, a zinc finger containing protein that is

exclusively expressed in egg cells in apomictic plants due to a large transposon insertion in its promoter (Underwood et al., 2022). Dandelion PAR was further shown to induce parthenogenesis under an egg cell-specific promoter in lettuce (Underwood et al., 2022). Both BBM and PAR have been successfully paired with *MiMe* to introduce apomixis in rice with high efficiency (Khanday et al., 2019; Song et al., 2024; Vernet et al., 2022).

Means to engineer both apomeiosis and parthenogenesis by harnessing the genetic determinants of the meiotic program and embryogenesis have laid down a blueprint for synthetic apomixis in crops. Here, we ask whether these strategies can be extended beyond rice by attempting to induce clonal reproduction through seeds in the Triticeae member barley (*Hordeum vulgare*), a crop of significant economic and societal importance and a close relative of bread wheat. We find that key meiotic genes governing recombination and monopolar orientation are functionally conserved, but that the barley meiotic cell cycle displays signs of functional diversification. The identification and targeted mutation of barley *SPO11-1* and *REC8* is presented and shown to completely abolish recombination, monopolar orientation, and meiotic sister chromatid cohesion. Targeted mutation of barley *OSD1* homologs revealed that two OSD1-like genes differ in function at the second meiotic division, with the finding that *HvOSD1B* is necessary for meiotic progression, while *HvOSD1A* is not despite being the closest homolog of the rice OSD1. Mutation of barley *TAM* leads to skipping of the second division in both male and female meiosis, and barley *TDM1* mutants fail to exit meiosis after the second division, indicating broad conservation of both genes in flowering plants. We further investigate means of bypassing the fertilization checkpoint, and find that egg-cell expressed *BBM1* is sufficient to trigger parthenogenesis in barley. Combined, we demonstrate the feasibility of clonal seeds in other important cereal crops by providing the building blocks for barley apomixis.

Results

HvTAM is a conserved CYCA1 that regulates meiotic progression

We first aimed to develop *MiMe* in barley by asking whether the core meiotic machinery is conserved. We first set our sights on identifying a functional homolog of *TAM* (*TARDY ASYNCHRONOUS MEIOSIS*), a CYCA1;2 shown in Arabidopsis to be required for the onset of the second meiotic division (d'Erfurth et al., 2010). Using protein similarity searches with the BLAST algorithm against the barley reference genome (Monat et al., 2019) and curated

gene families from PLAZA (Van Bel et al., 2022), we were able to identify the CYCA1 clade in grass crops (Figure 9A). Full length protein sequences from barley, wheat, brachypodium, rice, tomato, and Arabidopsis were used to construct a phylogenetic tree representative of selected monocots and dicots. Clustering of monocot sequences with the described Arabidopsis CYCA1;1 and CYCA1;2 identified a conserved CYCA1 clade, including a potential barley TAM. Two CYCA1 copies are present in wheat and rice, but only one copy is present in the barley genome (Figure 9A). Reciprocal BLAST searches against the barley and Arabidopsis genome identified HORVU.MOREX.r3.3HG0249410 as having the highest similarity to Arabidopsis CYCA1;2. Phylogenetic analysis and synteny showed that HORVU.MOREX.r3.3HG0249410 is the likely sole barley member of the CYCA1 clade, as the syntenic locus of the CYCA1 duplications in other species is missing in barley (Figure 9A, Supplemental figure 1). Sequence alignment with other plant CYCA1 protein sequences and human Cyclin-A1 shows high levels of conservation with the barley CYCA1, including a conserved MRAIL motif (Schulman et al., 1998) (Figure 9B), further support for HORVU.MOREX.r3.3HG0249410 as the likely barley TAM homolog. To test if the candidate gene is involved in meiosis, we developed a CRISPR-Cas9 genome editing construct targeting HORVU.MOREX.r3.3HG0249410 with the aim of obtaining full deletions of the coding sequence (Figure 9C). We transformed immature barley embryos using the genome editing construct, obtaining 5 independent calli yielding in total 60 T0 plants. Sequencing of the targeted locus in 5 independent T0 plants showed the presence of biallelic insertions or deletions in three T0 plants. To assess meiotic progression, we performed male meiocyte squashes. In the wildtype ‘Golden promise fast’, tetrads are observed corresponding to the four haploid spores produced at the conclusion of meiosis (Figure 9D, top left). In contrast, a T0 plant displaying a 1bp insertion and 2bp deletion expected to cause a frameshift within exon 10 (Figure 9C) showed a high proportion of dyads (two spores with a distinct cleavage furrow, Figure 9D, top right), suggesting that a single division occurs. These dyads further develop into distinct microspores (Figure 9D, bottom right), suggesting intact post-meiotic development. Small numbers of polyads (2/29 cells) or tetrads (1/29 cells) were observed, suggesting incomplete penetrance (Figure 9D, bottom left). T0 plants with biallelic mutations displayed significant reductions in fertility, yielding between 0 to 10 seeds per spike compared to the roughly 20 observed in the wildtype (Figure 9E). We next sowed the progeny of T0 biallelic mutants. Strikingly, all progeny of the T0 plants (n=64) displayed developmental defects including severely reduced plant height, thicker and greener leaves,

and a failure to extend beyond the 5-6 leaf stage (Figure 9F). Leaves from sixteen plants displaying this phenotype were subjected to flow cytometry and all were found to contain predominantly 4C nuclei, suggesting that all T1 progeny are tetraploid (Figure 9G). This suggests that functional unreduced diploid spores are produced at the conclusion of male and female meiosis in the mutants that develop into gametes and fuse during fertilization to yield tetraploid progeny. Given these findings, we conclude that HORVU.MOREX.r3.3HG0249410 is the barley ortholog of Arabidopsis *TAM*, playing a similar role in meiosis, and hereby refer to it as *HvTAM*. These results further demonstrate the first *TAM* ortholog in monocots and support broad conservation among flowering plants.

HvTDM is conserved in controlling meiotic termination

We next asked whether other regulators of the meiotic cell cycle are conserved in barley to extend the toolbox for synthetic apomeiosis. TDM1 (THREE DIVISION MUTANT) is a proposed APC/C subunit that controls exit from meiosis after meiosis II (Cromer et al., 2012), but has also been suggested to function by inhibiting translation of meiotic transcripts to trigger a cell fate transition (A. Cairo et al., 2022). Thus, *tdm1* loss of function mutants in Arabidopsis fail to exit meiosis and undergo additional meiotic divisions following meiosis II (Cromer et al., 2012). TDM1 is negatively regulated by TAM-coordinated phosphorylation, and mutation of this phosphorylation site strikingly triggers a premature exit from meiosis after the first division, yielding unreduced diploid gametes in the same manner as *tam* mutants (Cifuentes et al., 2016). We thus explored whether TDM1 is conserved among grasses, and again probed the barley reference genome and gene family curations. Arabidopsis TDM1 contains a tetratricopeptide repeat region (TPR) (Cifuentes et al., 2016) and thereby shows similarity to other TPR-containing proteins including AtSDI1 (Rakpenthai et al., 2022). We constructed a phylogenetic tree using barley, wheat, brachypodium, rice, tomato, and Arabidopsis full length protein sequences from AtTDM1- and AtSDI1-like genes (Figure 10A). Arabidopsis SDI1 and the likely tomato SDI1 act as an outgroup to delineate the TDM1 clade. Interestingly, the monocots wheat and barley show two *TDM1*-like gene copies, whereas rice possesses only one copy (Figure 10A). All proteins within the TDM1 clade show conservation of a ‘TP’ motif that is a phosphorylation site required for TAM activity (Cifuentes et al., 2016). An additional ‘TPP’ motif is present among monocots (Figure 10B), suggesting the presence of an additional phosphorylation site. The *Triticeae*-specific *TDM1*-like subclade however is missing this second motif in both the wheat and barley gene copies.

On the basis of this observation and higher protein sequence similarity, we developed a CRISPR-Cas9 genome editing construct *HORVU5Hr1G088430* with the aim of obtaining deletion alleles (Figure 10C). Immature barley embryos were transformed with the genome editing construct, yielding two independent calli and in total six T0 plants. The presence of insertions and deletions were observed by PCR amplification and sanger sequencing, with one line showing a 2bp insertion and a 1bp insertion expected to cause a frameshift within the fifth coding exon. Meiocyte squashes using anthers from this plant revealed the occurrence of more than two meiotic divisions, and resulting meiotic products with greater than four spores (Figure 10D). This plant further showed complete sterility, suggesting high penetrance of polyad production and subsequent failure to produce viable gametes. With these findings, *HORVU5Hr1G088430* is the barley *TDM1* ortholog, hereafter referred to as *HvTDM1*. These results demonstrate that *TDM1* and its role in governing meiotic exit is broadly conserved in flowering plants.

HvOSD1B is an OSD1 paralog that regulates meiotic progression

We next set our sights on a third means of altering the barley meiotic cell cycle to complete the apomeiosis toolkit. Mutations in *OSD1* produces unreduced diploid gametes at high penetrance and clonal diploid gametes when paired with mutations in *SPO11-1* and *REC8* (d'Erfurth et al., 2009), and its meiotic functions are conserved in rice and sufficient for *MiMe* (Mieulet et al., 2016). We thus asked whether *OSD1* is conserved in barley. We created a phylogenetic tree using full length protein sequences of selected monocots and dicots from a curated homologous gene family built from AtOSD1 (PLAZA) (Figure 11A). The phylogenetic tree confirmed the previous observation of a duplication of *OSD1*-like genes in monocots (Mieulet et al., 2016) (Figure 11A). One barley OSD1-like gene copy groups with the described rice OSD1, which we hereafter refer to as *HvOSD1A*. The alternative subclade contains the barley paralog *HvOSD1B* and a not-yet described rice gene. Notably, both clades show high levels of conservation within the described KEN and D-boxes (Figure 11B). We targeted *HvOSD1B* by developing a CRISPR-Cas9 genome editing construct with the aim of obtaining deletion alleles (Figure 11C). Transformation of immature barley embryos yielded three independent calli and in total 28 T0 plantlets. PCR amplification and illumina short read amplicon sequencing of three independent T0 plants revealed biallelic insertion/deletions in all three plants at both guide RNA target positions (Figure 11C). Meiocyte squashes of late-stage anthers using acetocarmine revealed the formation of dyads (two spores with a clear

cleavage furrow) and resulting microspores in *Hvosd1b* mutants (Figure 11D). Dyads rather than tetrads were almost exclusively observed, suggesting the absence of a second division (Figure 11E). In contrast to fertile Arabidopsis and rice *osd1* mutants, *Hvosd1b* biallelic mutants with insertions and deletions at both guide RNA positions show complete sterility, failing to yield seeds (Figure 11F). Pollination of *Hvosd1b* flowers with wildtype (haploid) pollen failed to produce seeds. Further, the rescue of immature embryo-like structures and transfer to in vitro culture from *Hvosd1b* plants did not result in the development of plantlets, suggesting that triploid embryos, or embryos of any ploidy, fail to develop. One possibility thus may be that *HvOSD1B* impacts post-meiotic development or female meiotic progression. Transformation of immature embryos with a genome editing construct containing known functional gRNAs failed to yield *Hvosd1aHvosd1b* double mutants (Figure 11G), suggesting synthetic lethality and a role of both barley *OSD1* orthologs in somatic development as previously shown in Arabidopsis (Cromer et al., 2012).

Turning the barley first division of meiosis into mitosis

The findings above illuminate the fact that the meiotic cell cycle shows a degree of functional divergence among plants, and notably among closely related plant species. We next sought to investigate whether other key components of synthetic apomeiosis, namely those that control recombination and monopolar orientation, are conserved in barley. Protein similarity searches of the barley reference genome identified clear orthologs of AtSPO11-1 and AtREC8; the barley genome notably contains two highly similar copies of REC8 that likely represent a recent duplication, hereby referred to as HvREC8A and HvREC8B. Two gRNAs targeting *HvSPO11-1*, *HvREC8A/B* (showing perfect complementarity to both genes), and *HvOSD1A* respectively were assembled into a single genome editing construct and used to transform immature barley embryos (Figure 12A). Fifteen independent calli were obtained, from which 24 total T0 plants developed. PCR screening, sanger sequencing, and multiplexed illumina sequencing identified 11 T0 transformants showing CRISPR activity conferring biallelic or homozygous insertions and deletions in all four genes targeted (Figure 12B). During barley meiosis, seven bivalents form at the metaphase I plate following the association of homologous chromosomes during prophase I (Figure 12D). At anaphase I, homologous chromosomes segregate to opposing spindle poles while maintaining the association of sister chromatids. At anaphase II, sister chromatids segregate equationally to yield four haploid meiotic products. Chromosome squashes of *Hvspo11 Hvrec8ab Hvosd1a* quadruple mutant

anthers revealed that univalents rather than bivalents form at metaphase I, suggesting complete failure of recombination (Figure 12C, left). Strikingly, at late anaphase I/telophase I, sister chromatids appear in a 14:14 balanced segregation ratio, suggesting complete loss of monopolar orientation and sister chromatid cohesion (Figure 12C, middle). At anaphase II, alignment and stretching of sister chromatids were observed, suggesting an intact meiosis I-meiosis II transition (Figure 12C, right). Correspondingly, tetrads (four spores) were observed in late *Hvspo11Hvrec8a/bHvosd1a* meiocytes (Supplemental figure 2). Thus, *SPO11* and *REC8* are functionally conserved in barley and their mutation is sufficient to turn the first meiotic division into a mitotic one. The *OSD1* ortholog (*HvOSD1A*) more closely related to rice *OSD1* does not however function in the meiosis I-meiosis II transition, and thus combining *Hvspo11 Hvrec8ab* with mutations in *Hvosd1b* or *Hvtam* would be of interest.

OsBBM1 expression in the egg cell can induce haploids in barley

One component of synthetic apomixis is the engineering of apomeiosis through *MiMe*, while the other is the engineering parthenogenesis. To next ask whether complete synthetic apomixis is feasible and sufficient to produce clonal diploid barley seeds, we turned our sights to parthenogenesis induction. Rice BBM1 was shown to be effective in inducing haploids when expressed in the egg cell, where it is normally turned off, driven by either an Arabidopsis-derived *DD45* promoter or a rice-derived *OsECA1.1* promoter (Khanday et al., 2019), effectively mimicking parthenogenesis. Given high sequence conservation of *BBM1* between rice and barley (Supplemental figure 3), we transformed immature barley embryos with two constructs expressing *OsBBM1* under either the *DD45* (pMP226) or *OsECA1.1* (pMP227) promoter, utilizing identical constructs effective for parthenogenesis induction in rice (Khanday et al., 2019)(Figure 13A). We obtained six independent calli from pMP226 and thirteen independent calli from pBM227, yielding in total twelve and twenty-four T0 plants from each construct respectively. T0 transformants from either construct were indistinguishable from the wildtype in terms of development or fertility. Seeds from three independent T0 plants were sown to yield six total T1 families. In all families originating from *pDD45::BBM1* T0 parents, normal germination was observed and all progeny yielded seeds. In one family containing *pOsECA1.1::BBM1*, a single plant was found to show severely reduced plant height and sterility. Plants from this family also showed unusual inflorescence morphology and reduced fertility (Figure 13B). Flow cytometry was performed using leaf tissue from the sterile plant and showed a peak significantly shifted from a known

2C (diploid) peak, demonstrating that this plant was haploid (Figure 13C). Seeds from two plants displaying reduced fertility (Figure 13B, '2n*') were sown to yield two T2 families. Both families yielded multiple developmentally-stunted and sterile plants, which were later confirmed to be haploid by flow cytometry (Figure 13D). Among the T2 progeny containing *pOsECA1.1::BBM1*, no sterile plants were observed. Chromosome squashes prepared from haploid inflorescences confirmed their ploidy and showed signs of haploid meiosis signatures such as foldback pairing (Figure 13E). Combined, the results demonstrate that the *OsECA1.1* egg-cell specific promoter driving rice *BBM1* is sufficient to induce haploid embryos in barley at rates of over 10% (Figure 13G).

Discussion

Conservation of core meiotic machinery brings clonal gametes to *Triticeae*

One of the fundamental characteristics of natural apomictic species is the avoidance of meiosis. While the developmental pathways and a few genetic loci have been narrowed down in some species, the underlying genes and molecular mechanisms remain elusive (Cornaro et al., 2023; Vijverberg et al., 2010). As of now, altering key steps of the meiotic program have proven to be most effective in mimicking natural apomeiosis. Mutations in fundamental genes necessary for recombination, chromosome segregation, and meiotic progression functionally disassemble the hallmarks of meiosis to yield unreduced clonal gametes (d'Erfurth et al., 2009). To date, *MiMe* has been successfully implemented in Arabidopsis (d'Erfurth et al., 2009), rice (Mieulet et al., 2016), and tomato (Y. Wang et al., 2024), and shown to be sufficient for producing clonal seeds in the former two species (Khanday et al., 2019; Marimuthu et al., 2011). Greater penetrancy is notably obtained when simultaneously combining MiMe and BBM1-egg cell expression in a single construct (Vernet et al., 2022). *MiMe* has also shown potential in progressive heterosis through the creation of hybrids-of-hybrids (Y. Wang et al., 2024; Ward et al., 2024). Here, we find that the key meiotic genes *SPO11-1* and *REC8* are functionally conserved in barley and sufficient to provoke an equational meiotic division by preventing recombination, monopolar orientation of sister kinetochores, and meiotic sister chromatid cohesion. These findings add to the synthetic apomeiosis toolkit while reaffirming a widely conserved meiotic program across kingdoms. Identification of a barley *SPO11* homolog opens avenues for studying double-strand break formation and other aspects of meiotic prophase in a new diploid model. Further, conserved

functions of *HvREC8* support the model that monopolar orientation is dependent on cohesin in plants (Chelysheva et al., 2005; Mieulet et al., 2016; Y. Wang et al., 2024).

HvTAM, HvTDM1, and *HvOSD1B* control meiotic progression in barley

Foundational studies in Arabidopsis identified key players in meiotic cell cycle regulation in plants, with proven applications in synthetic apomixis beyond model systems. It was therefore striking to uncover both sequence and functional divergence among what are thought of as closely related plant families. *OSD1*-like genes underwent a duplication event common to the grasses, yet our findings suggest that only one copy is important for meiotic cell cycle control in barley (HvOSD1b, LOC_Os04g39670), whereas the other copy is functional in rice (OsOSD1, HvOSD1a) (Figure 11), suggesting species-specific functional divergence. One possibility is that expression differences, perhaps at the tissue level, may underly the functional divergence. Similarly, *TDM1* also appears to have undergone a duplication event, although only among the Triticaeae (Figure 10A). The described TAM phosphorylation site (TP motif) is however present in only one Triticeae TDM1 copy, HvTDM1, and thus may define meiotic function as *HvTDM1* mutants fail to exit meiosis after the second division. *Hvosd1b* mutants notably fail to produce viable embryos in contrast to *Atosd1* and *Ososd1*, an outcome that could result from post-meiotic development or differences between male and female meiosis. Further, independent duplications of OSD1 exist in both grasses and dicots, as *AtOSD1* and *AtUVI4* are distinct from the monocot OSD1 clade. Despite being independent, both duplications are essential in Arabidopsis (Cromer et al., 2012) and barley (Figure 11G). Strikingly, species-specific divergence is observed among the grass duplication given that HvOSD1b rather than HvOSD1a/OsOSD1 functions in meiosis, suggesting rapid evolution. Sterility in *Hvosd1b* suggests that it may not be a viable candidate for unreduced gamete production in the context of *Triticeae* synthetic apomixis, despite being the best candidate in Arabidopsis and rice due to high penetrance and tetraploid seed production. OSD1 however exists in only single copy in many species beyond Arabidopsis and rice, and expected lethality may hinder its application for synthetic apomixis in other crops.

Cyclins are notorious for diversification (Bulankova et al., 2013), and it is not entirely surprising that closely related species differ in gene copy number within highly conserved clades. What is surprising is that post-meiotic outcomes appear to differ in *TAM* mutants between Arabidopsis and barley, as *Hvtam* mutants show loss of fertility (Figure 9E). Loss of

fertility in *Hvtam* could be attributed to reproductive barriers such as the triploid block (Köhler et al., 2010). Triploid embryos may be less tolerated in barley, and may thus manifest as reduced fertility and the exclusive production of tetraploid seeds (Figure 9D-F). Embryo rescue experiments could help clarify whether this is indeed the case, and a closer look at female meiosis would also be helpful to explore penetrance of the mutation between the sexes. High penetrance during male meiosis and the exclusive production of tetraploid seed renders *Hvtam* the best candidate for unreduced gamete production in barley synthetic apomixis.

Expression of *OsBBM1* can drive synthetic parthenogenesis in barley

Inducing parthenogenesis in non-apomictic species has been shown to be feasible by two methods: by exclusion of the paternal genome using various mutations, or by the mis-expression of dominant genes that trigger embryogenesis by skipping the fertilization requirement. The latter seems to follow the path that natural apomicts employ (Underwood et al., 2022) and shows greater efficacy in synthetic apomixis induction (Vernet et al., 2022). Two different genes have been identified, *BBM* and *PAR*, in which specific alleles present in natural apomicts drive their expression in the egg cell, where they are normally turned off (Conner et al., 2015; Underwood et al., 2022). Cell type-specific expression of these genes within the egg cell in non-apomictic sexual plants is sufficient to trigger embryogenesis independent of the cue normally provided by the sperm. The result in diploid species is the formation of haploid embryos developing from reduced gametes, and when paired with apomeiosis, diploid clonal embryos arising from unreduced gametes. We found that the expression of rice *BBM1* under the control of an egg-cell specific promoter was capable of yielding haploid barley embryos. Interestingly, *BBM1* misexpression appears to have an effect on fertility (Figure 13B); failed embryogenesis is one possible cause. Haploid induction rates reached levels comparable to those described in rice (Figure 13G) (Khanday et al., 2019), providing a new method to generate haploids in barley that may find value in doubled haploid production. The efficiency of clonal seed production will thus be interesting to clarify in barley, as varied rates have been observed in rice (Vernet et al., 2022). The recent findings that *PAR* can also effectively yield clonal progeny in rice (Song et al., 2024) provide a hint that alternative or complementary strategies to *BBM* may also be useful in *Triticeae*.

The building blocks of barley apomixis

The goal of obtaining clonal seeds in modern crops is a long-standing one, and evidence that two distinct processes underly naturally occurring apomixis has been clear for decades (Spillane et al., 2001). The quest to identify genetic components driving these processes remains a challenge, but a significant body of work now suggests that both can be mimicked in one's species of choice. Major leaps in our understanding of the plant meiotic cell cycle, gamete transmission, and embryogenesis have been gleaned from both model systems and lesser-tamed plant genera. Here we aimed to extend these findings and ask whether they can be readily applied to a crop species of interest. We find that indeed they largely can, while also identifying unexpected cases of specialization. By presenting effective tools for engineering apomixis in barley, we suggest that clonal gametes are on the horizon across important cereals. Clarification of the major players in diploid barley will hopefully pave the way for later implementation in other *Triticeae* such as wheat, though genome complexity may present additional challenges.

Materials and methods

Chromosome preparations

For chromosome squashes, immature barley inflorescences (spikes) roughly 1-2cm in size were harvested from the sheaths of primary or secondary tillers and immediately fixed in 3:1 ethanol:acetic acid. Following the development gradient, spikelets were selected starting from the bottom of the spike. A single anther was isolated and placed on a clean slide with a large drop of 1% acetocarmine. Slides were passed 1-2 times over a flame, then a coverslip was added and force was applied directly to the slide. Anthers were immediately visualized with a widefield light microscope at 20x magnification for staging. Additional stain was added to the sides of the coverslips before imaging using a Zeiss Axioplan microscope. To visualize chromatin using DAPI, the squashes were first flash frozen in liquid nitrogen and the coverslip was flipped. Slides were then dehydrated using an ethanol series by immersion in first 70%, 90%, and lastly 100% ethanol for two minutes each. Vectashield mounting media containing DAPI was then applied, and cells were imaged using a Zeiss Axio Observer epifluorescence microscope. Plots were prepared using ggplot2 within R.

Construct design, cloning, and transformation

After selection of gene homologs, guide RNAs were designed using CRISPOR (Haeussler et al., 2016). To assemble genome editing constructs in barley, guide RNAs were fused to HvU6 promoters and assembled into a final binary vector using golden gate cloning, following previously described methods (Kumar et al., 2018). Transformation of barley plants was carried out using the agrobacterium strain AGL-1 (GoldBio) using previously described methods (Hensel et al., 2009). Briefly, immature embryos from barley cultivar Golden Promise Fast were rescued and co-cultivated with agrobacterium containing the genome editing constructs under selective and sterile tissue culture conditions until the development of young plantlets.

Protein sequence and phylogenetic analysis

Protein BLAST (P-blast) within the IPK Galaxy web tool (*IPK Galaxy Blast Suite*, 2024) (https://galaxy-web.ipk-gatersleben.de/) was used to search for high confidence proteins from the barley reference genome (Mascher et al., 2021). Multple sequence alignment was performed using MUSCLE (Edgar, 2004) within the EMBL-EBI (Madeira et al., 2022). For phylogenetic analysis, full amino acid sequences were input to PhylogenyFR, using MUSCLE for alignment and PhyML for phylogenetic tree construction (Dereeper et al., 2008).

Short-read sequencing and data analysis

To assess CRISPR-induced mutations in genome edited barley plants, 150 base-pair PCR amplicons were first amplified from T0 transformant genomic DNA that spanned each gRNA containing specific sequence overhangs placed within the oligos. Secondary oligos were then designed that are complementary to the initial oligo and contain additional random sequences, acting as barcodes. Barcoded secondary amplicons were amplified for each gRNA and for each individual plant. PCR product from all samples were then mixed in a single tube and PCR purified. Samples were then provided to the Max Planck Genome Center, Cologne. TPase-based sequencing libraries were prepared, and six million 150bp paired-end reads were sequenced using an Illumina HiSeq3000. Raw data was processed using Galaxy (Goecks et al., 2010) by demultiplexing according to barcodes and mapping reads to the genes of interest using Bowtie2 (Langmead & Salzberg, 2012). Variant calling was performed on the output BAM files using FreeBayes.

Flow Cytometry

Fresh barley leaves were chopped in Galbraith's buffer to extract nuclei and filtered using a 30um sieve (Loureiro et al., 2006). Nuclei were then stained for 15 minutes with 100ug/mL DAPI and processed with a Beckman Coulter CytoFlex flow cytometer to determine ploidy using CytExpert software.

Contribution statement

This manuscript is relevant to this work as it composed the second of two major thesis projects undertaken during my studies. My contribution included the identification of putative barley homologous genes, the design and cloning of CRISPR-Cas9 mutagenesis constructs, the characterization of mutations, all cytological experiments and imaging, fertility analysis, flow cytometry experiments, all data analysis, and writing of the manuscript.

Figure legends

Figure 9. HvTAM is a conserved CYCA1 that regulates meiotic progression. (A) Maximum-likelihood phylogenetic tree of cyclin family proteins in barley (HORVU), wheat (TraesCS), rice (LOC_Os), brachypodium (Bradi), tomato (Solyc), and Arabidopsis (AT). Trees were generated using PhylogenyFR by performing MUSCLE sequence alignment of full-length sequences, curation using G-blocks, and maximum likelihood tree generation. (B) MUSCLE alignment of human Cyclin-A1 and selected plant CYCA1 protein sequences. Alignments were visualized using SnapGene software; colors represent conservation of amino acid properties according to ClustalX. (C) Schematic of *HORVU.MOREX.r3.3HG0249410/HvTAM*. Green boxes represent coding regions, and arrows indicate the position of guide RNAs for Cas9 targeting. Sanger sequence trace files show a biallelic mutation in plant 23011 at the second guide RNA. (D) Male meiotic products stained with acetocarmine. Top left, tetrads in the wildtype (WT) Golden promise fast. Top right, dyads in *Hvtam*. Bottom left, quantification of tetrads, polyads, and dyads in the wildtype and *Hvtam*. Bottom right, microspore development after dyad production in *Hvtam*. (E) Fertility on wildtype Golden promise fast barley plants and T0 *Hvtam* transformants.

23014 and 23011 are biallelic *Hvtam* T0 plants. Each data point represents one spike. (F) Plant development in the wildtype (left) and *Hvtam* T0 progeny (right). (G) Flow cytometry of developing leaves in the wildtype (left) and *Hvtam* T0 progeny (right). Peaks show total DNA content present in isolated nuclei, corresponding to ploidy.

Figure 10. HvTDM1 is conserved in controlling meiotic termination. (A) Maximum-likelihood phylogenetic tree of TDM1-like proteins in *A. thaliana*, tomato, and selected grass species. Trees were generated using PhylogenyFR by performing MUSCLE sequence alignment of full-length sequences, curation using G-blocks, and maximum likelihood tree generation. (B) MUSCLE alignment of TDM1-like proteins in *A. thaliana*, tomato, and selected grass species. Alignments were visualized using SnapGene software; colors represent conservation of amino acid properties according to ClustalX. (C) Schematic of *HORVU5Hr1G088430/HvTDM1*. Green boxes represent coding regions, and arrows indicate the position of guide RNAs for Cas9 targeting. Sanger sequence trace files show a biallelic mutation in plant 23001 at the second guide RNA. (D) Male meiotic products stained with acetocarmine. Left, tetrads in the wildtype (WT) Golden promise fast. Right, polyads/anaphase III in *Hvtdm1*; red arrows indicate cleavage furrow after anaphase III.

Figure 11. HvOSD1b is an OSD1 paralog that regulates meiotic progression. (A) Maximum-likelihood phylogenetic tree of OSD1-like proteins in selected dicots and monocots. (B) MUSCLE alignment of *A. thaliana* OSD1 and UVI4, rice OSD1A and OSD1B, and barley OSD1A and OSD1B. (C) Schematic of *HORVU2Hr1G082000/HvOSD1B*. IGV view of Illumina amplicon sequences at the first guide RNA position indicate an insertion and deletion in plant 22056. (D) Male meiotic products stained with acetocarmine. Left, tetrads in the wildtype. Top right, dyads in *hvosd1b*. Bottom right, developing microspores in *hvosd1b*. (E) Quantification of tetrads, polyads, and dyads in the wildtype (WT) and *Hvosd1b*. (F) Fertile spikes in the wildtype (left), and sterile spikes in *Hvosd1b*. (G) Top, schematic of *HvOSD1A* and *HvOSD1B*. Bottom, insertion/deletion alleles recovered in *Hvosd1b* and *Hvosd1aHvosd1b* transformants; -/- denotes biallelic mutations, -/+ denotes monoallelic mutations, and +/+ denotes no mutations.

Figure 12. Turning barley meiosis I into mitosis. (A) Schematic of multiplexed genome editing construct targeting *MiMe* homologs with two guide RNAs. (B) Insertion/deletion

alleles recovered in T0 transformants and fertility phenotypes; -/- denotes biallelic mutations, -/+ denotes monoallelic mutations, and +/+ denotes no mutations. (C) Top, wildtype Golden promise fast meiosis. Bottom, *Hvosd1a Hvrec8a/b Hvspo11-1* meiosis. (D) Schematic of wildtype and *Hvosd1a Hvrec8a/b Hvspo11-1* meiosis and expected meiotic products. Created using BioRender

Figure 13. BBM1 expression in the egg cell can induce haploids in barley. (A) Schematic of constructs containing either *A. thaliana* (DD45) or rice-derived (OsEC1.1) egg-cell promoters driving expression of OsBBM1. (B) Spike phenotype of T1 plants expressing *pOsEC1.1::OsBBM1*. (C) Flow cytometry of sterile *pOsEC1.1::OsBBM1* T1 plant (left) and mixture with wildtype nuclei (right). (D) Plant development of T1 progeny expressing *pOsEC1.1::OsBBM1*. (E) Mitotic and meiotic cells in developing haploid T2 barley plants. (G) Haploid induction rates among both constructs.

Supplemental figure 1. CYCA1 syntenic alignments between wheat and barley. Chromosome-level syntenic LASTZ alignments obtained from EnsemblPlants (Harrison et al., 2024) between barley and wheat at CYCA1 genic loci.

Supplemental figure 2. *Hvspo11Hvrec8a/bHvosd1a* mutants produce tetrads. Acetocarmine staining of meiotic products in *Hvspo11Hvrec8a/bHvosd1a*, and insertion/deletion alleles identified after multiplexed illumina amplicon sequencing.

Supplemental figure 3. BBM1 is conserved between rice and barley. MUSCLE alignment between the top two barley BLAST hits for OsBBM1 and OsBBM1.

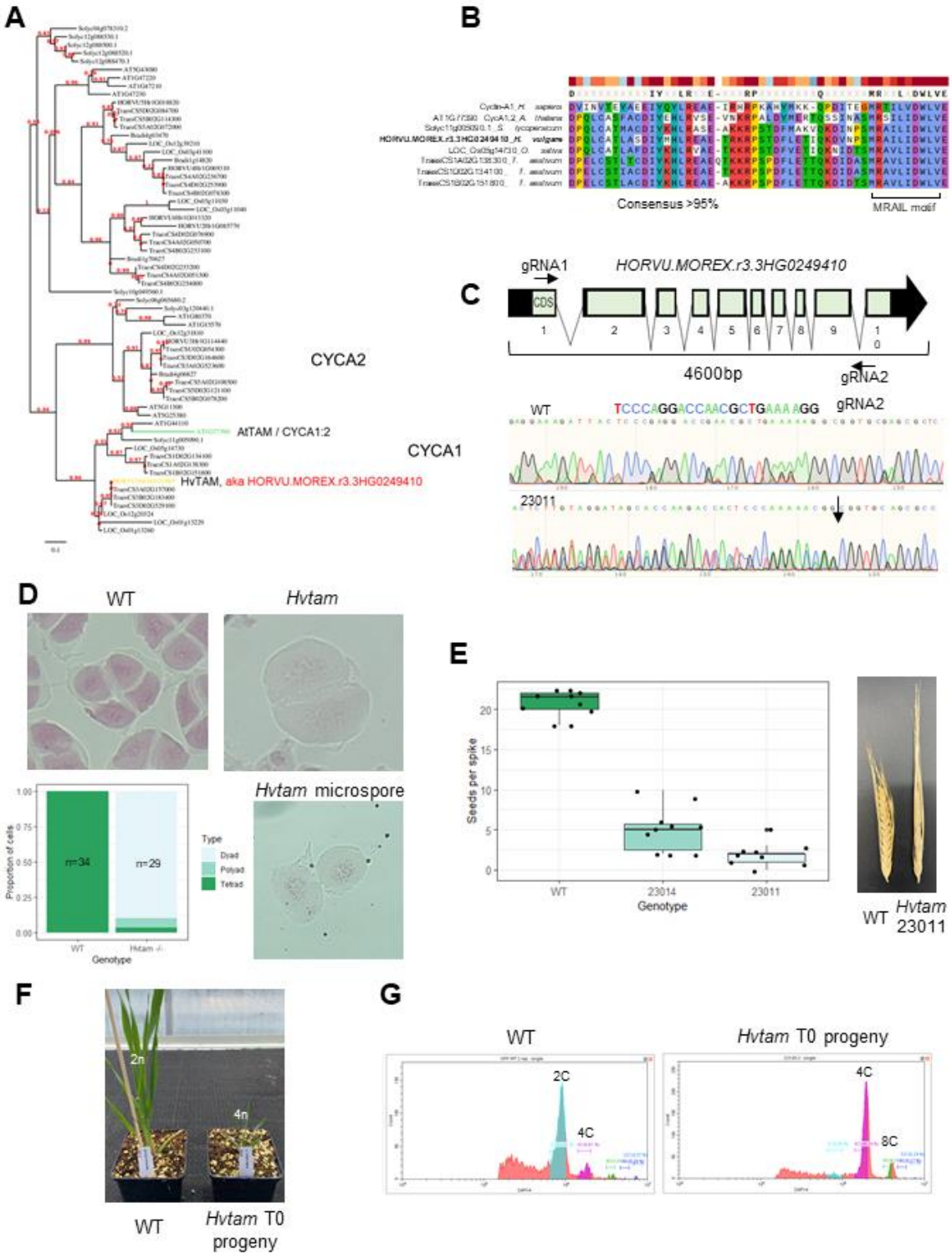


Figure 9. HvTAM is a conserved CYCA1 that regulates meiotic progression

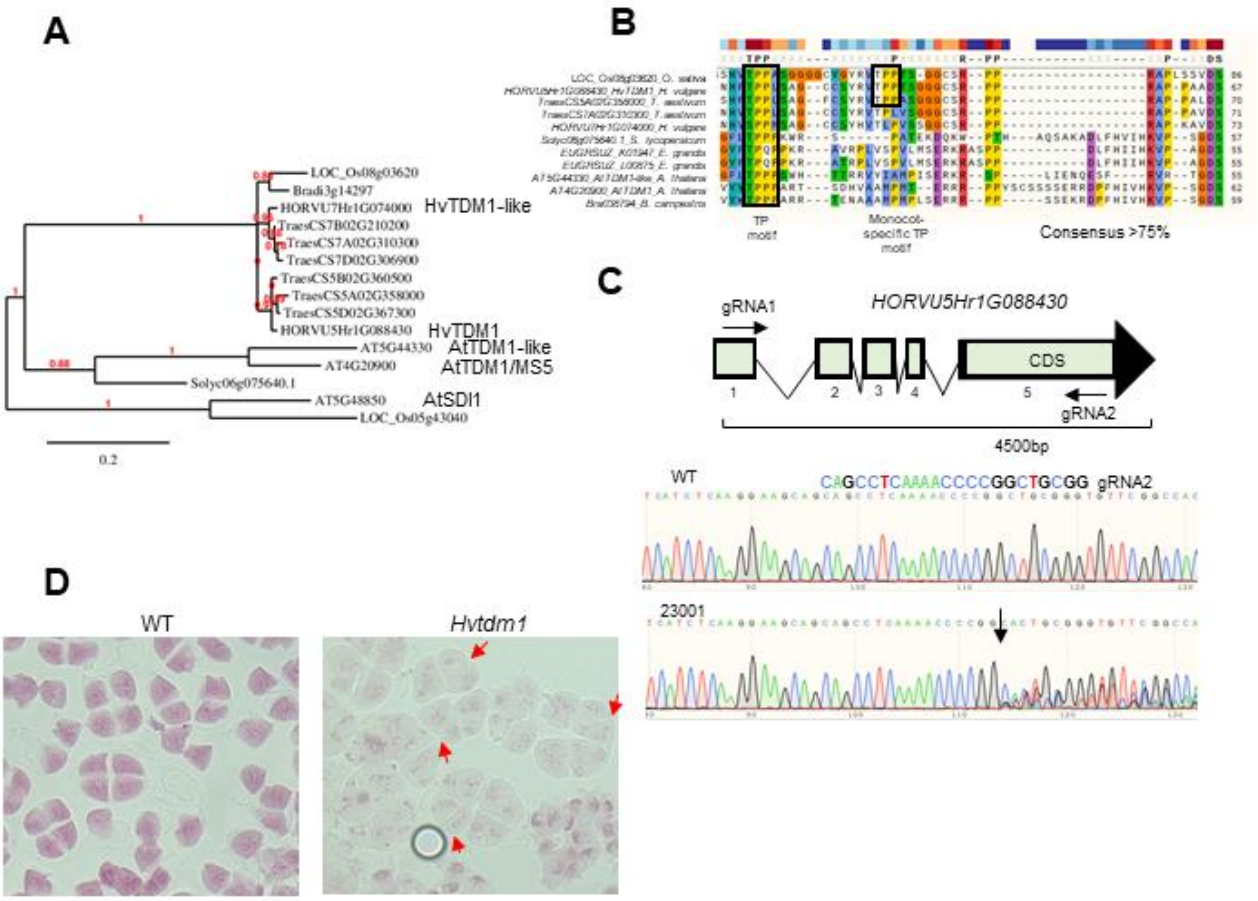
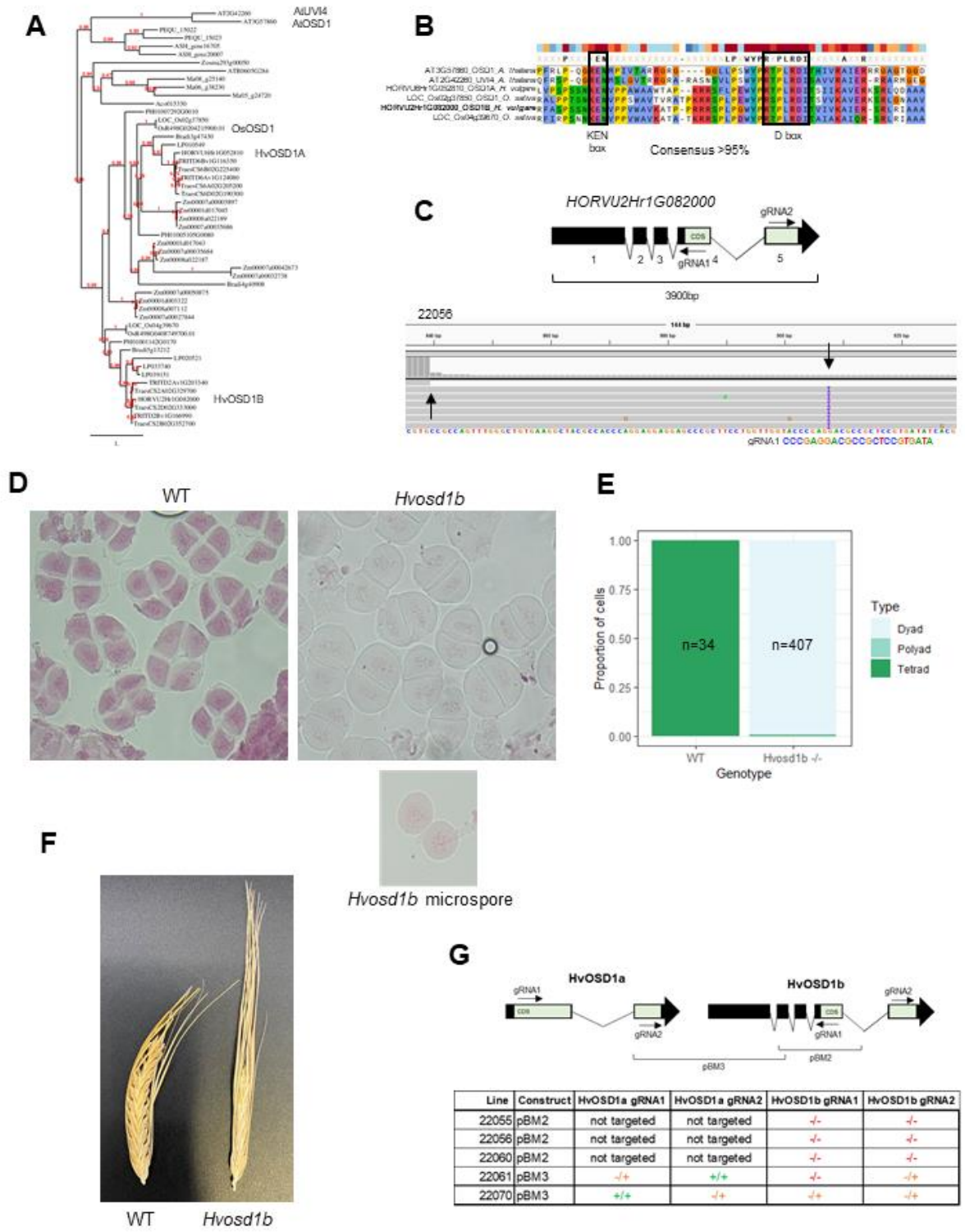


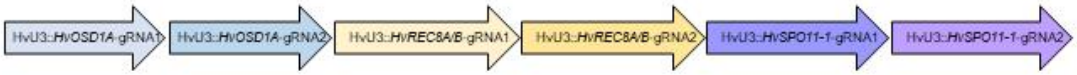
Figure 10. HvTDM1 is conserved in controlling meiotic termination



Line	Construct	HvOSD1a gRNA1	HvOSD1a gRNA2	HvOSD1b gRNA1	HvOSD1b gRNA2
22055	pBM2	not targeted	not targeted	-/-	-/-
22056	pBM2	not targeted	not targeted	-/-	-/-
22060	pBM2	not targeted	not targeted	-/-	-/-
22061	pBM3	-/+	+/+	-/-	-/+
22070	pBM3	+/+	-/+	-/+	-/+

Figure 11. HvOSD1b is an OSD1 paralog that regulates meiotic progression

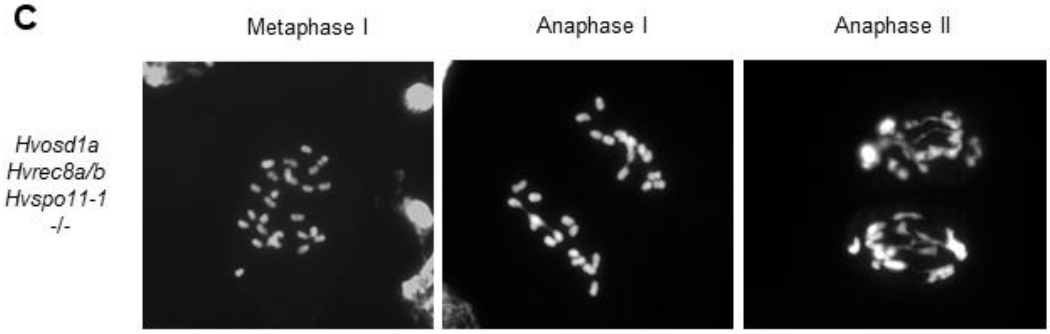
A



B

Genotype	T0 transformants	Expected phenotype	Observed phenotype
Hvosd1 -/- Hvrec8a/b -/- Hvspo11 -/-	11	fertile	sterile
Hvosd1 -/- Hvrec8a/b -/- HvSPO11 -/+	5	sterile	sterile
Hvosd1 -/- Hvrec8a/b -/- HvSPO11 +/+	2	sterile	sterile
HvOSD1 -/+ HvREC8a/b +/+ *Hvspo11 -/-*	1	sterile	sterile

C



D

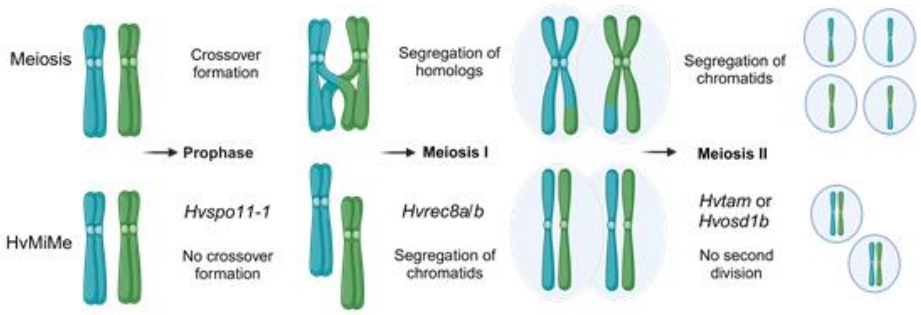
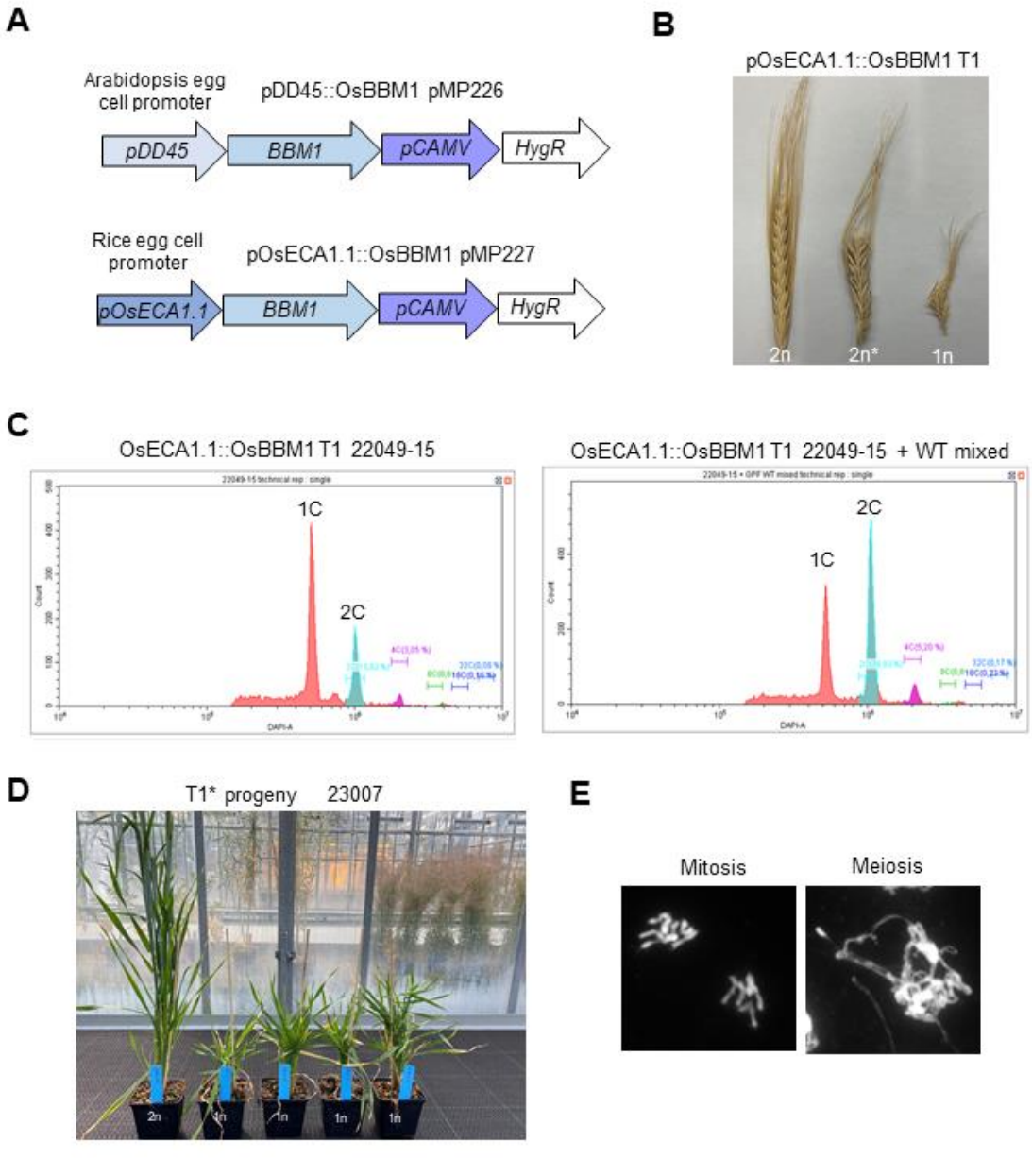


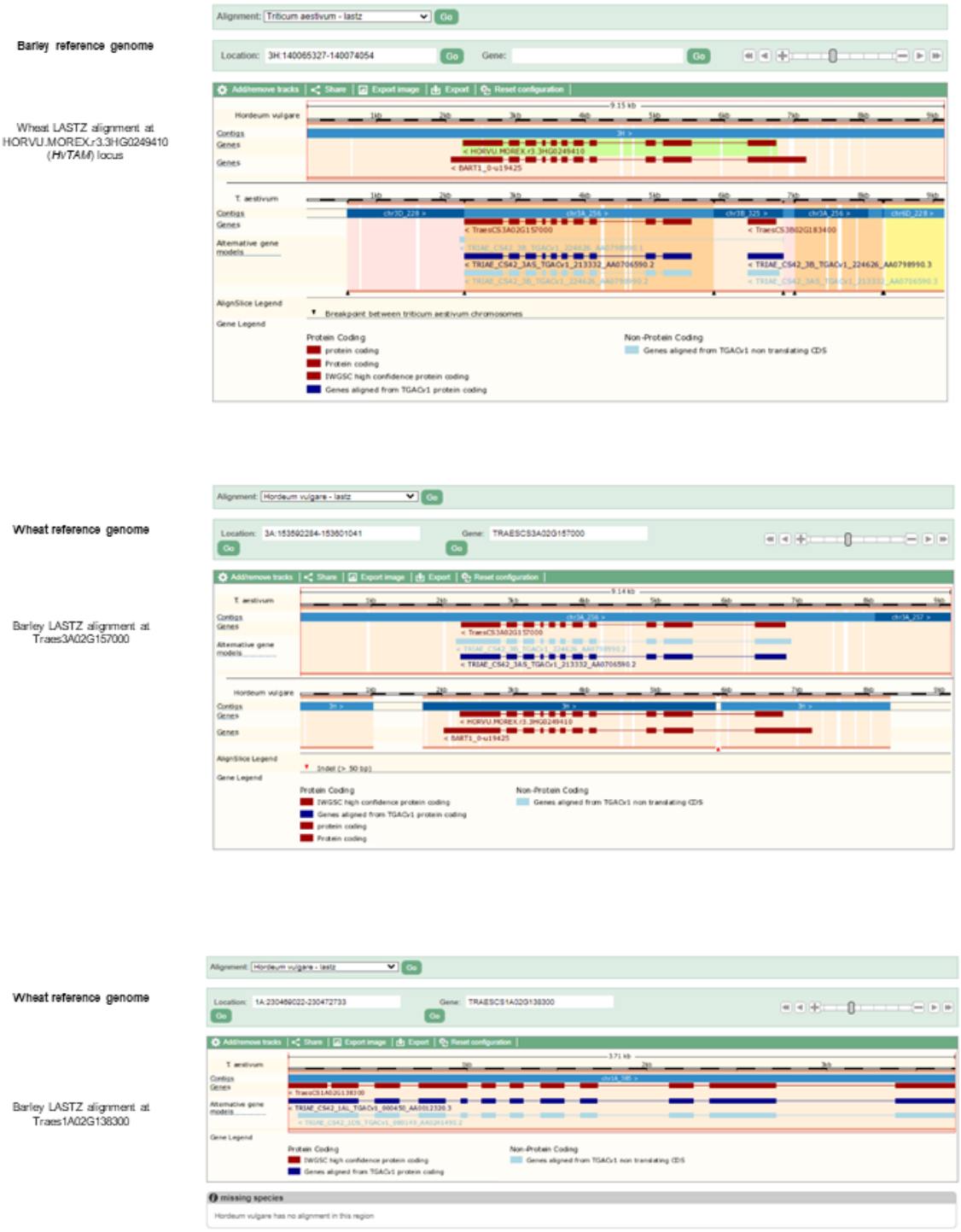
Figure 12. Turning barley meiosis I into mitosis



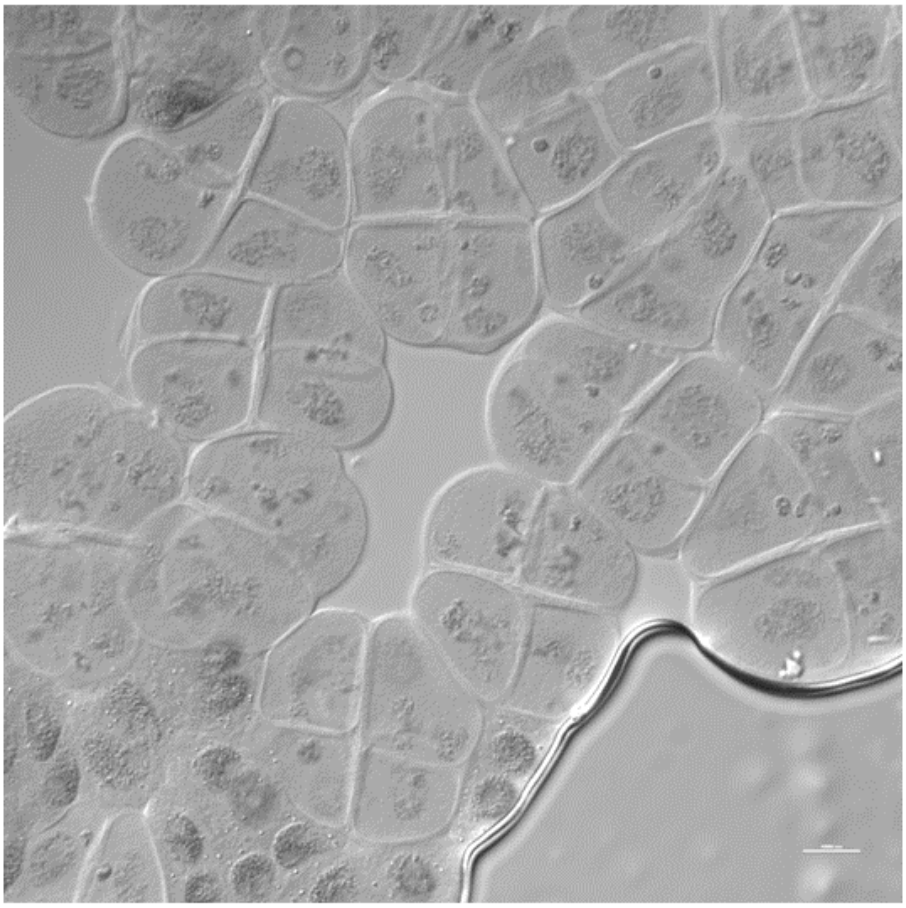
G

Family	Promoter	Number of plants	Number of haploids	Haploid induction (%)
23007	proOsECA1	33	4	12.1
23008	proOsECA1	18	3	16.7
23009	proAtDD45	43	0	0
23010	proAtDD45	38	0	0

Figure 13. OsBBM1 expression in the egg cell can induce haploids in barley



Supplemental figure 1. CYCA1 syntenic alignments between wheat and barley.

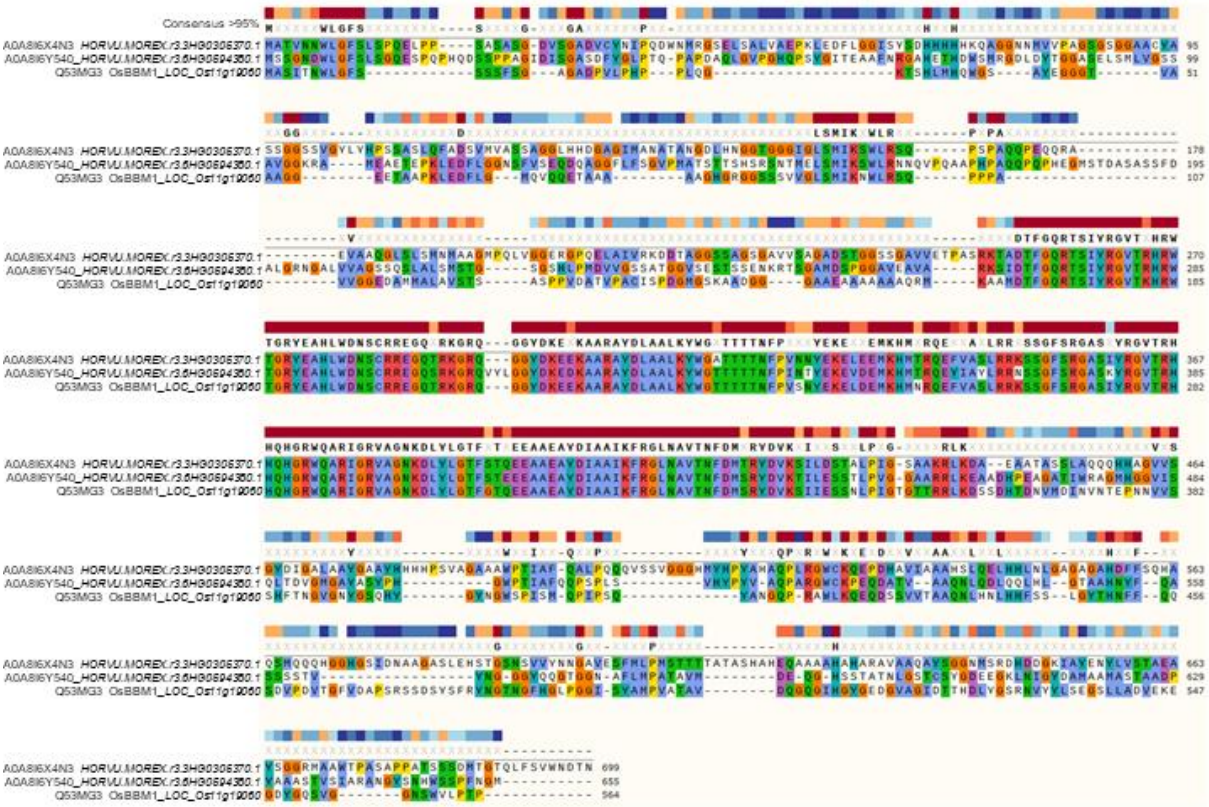


pMP213 21009 *Hvspo11Hvrec8a/bHvosd1a*

Line	BAM file	Guide target	BAM file allele 1	allele 2	allele 3
21009	L349	HvOSD1 guide 1	1bp ins T	1bp del	
21009	L349	HvREC8a/b guide	27bp del	1bp ins G	1bp ins A
21009	L349	HvREC8a/b guide	1bp ins A	1bp ins T	1bp ins A
21009	L349	HvSpo11 guide 1	3bp del (TCA)	1bp ins A	

Mutations within mapped illumina short reads

Supplemental figure 2. Hvspo11Hvrec8a/bHvosd1a mutants produce tetrads



Supplemental figure 3. BBM1 is conserved between rice and barley

Chapter 4: Conclusions and perspectives

Sister kinetochore orientation at meiosis I

Chromosomes are divided up in an intensely regulated manner during cell division to ensure new daughter cells have exactly the correct number and complement. How this is precisely carried out remains a major topic of study, but it is clear that properties specific to the chromosomes and the proteins that coordinate their movement drive this process. Position and orientation of kinetochores on chromosomes is key and differs between meiosis and mitosis. Kinetochores and their respective chromosomes differ in architecture before mitotic or meiotic divisions, and this in turn dictates how tension is established when kinetochores are pulled on by spindle microtubules (Nicklas, 1997). During mitosis, sister kinetochores face opposing spindle poles (bi-orient) to favor microtubule attachment from opposite poles. During meiosis, sister kinetochores face the same spindle poles (mono-orient) to favor microtubule attachment from the same pole. Both attachment states generate tension on the spindle needed to trigger chromosome segregation. An absence of this tension is sensed by a regulatory network (Chromosomal passenger complex, or CPC) that removes incorrect attachments. The presence of tension satisfies a checkpoint (spindle assembly checkpoint, or SAC) that in turn gives the green light to segregate chromosomes by activating a large E3 ubiquitin ligase complex (the anaphase promoting complex/cyclosome, or APC/C). The APC/C finally triggers chromosome segregation by releasing a protease (Separase) that removes the physical ties between chromosomes (cohesin). Thus, the orientation of kinetochores dictates a regulatory program that determines when and how chromosomes divide. Over seventy years after a description of sister kinetochore co-orientation in lilies and other plant chromosomes (Östergren, 1951), we now know that kinetochore orientation during meiosis is itself tightly regulated. Sister kinetochores are tethered together during meiosis I, either through physical connections (Monopolin in budding yeast) or close association (REC8 cohesin in fission yeast, mouse, and plants). This promotes their orientation to a single pole and favors mono-polar attachment by microtubules, an outcome further favored by the architecture of joined homologous chromosomes (Hirose et al., 2011). How REC8 precisely promotes monopolar orientation remains unclear. Meiosis-specific proteins (Moa1 in fission yeast, Meikin in mouse) that localize to the kinetochore shuttle the cell cycle kinase Polo kinase (Plk1 in mouse) to the kinetochore have a major role. Both Moa1/Meikin and Polo kinase at the kinetochore are needed for mono-orientation likely by regulating REC8 cohesin,

and this may underly a common mechanism among fission yeast and mammals. The work described here provides support for a REC8-dependent model of monopolar orientation in plants, and further implicates kinetochore components and SUMOylation in promoting plant monopolar orientation through cohesion. Defects in cohesion trigger a weakened association of sister kinetochores that mirrors that seen in mouse Meikin mutants, suggesting a common mechanism across kingdoms. Proper kinetochore orientation has implications in cancer, human development, and plant breeding. As demonstrated in chapter 2, knowledge of the precise mechanisms behind kinetochore orientation during plant meiosis perhaps most readily holds promise for implementing apomixis in modern agriculture.

Introducing synthetic apomixis to barley

The use of hybrid breeding is widespread in modern agriculture, most notably in maize (Andorf et al., 2019). Commercial hybrids exhibit heterosis, or hybrid vigor, that contributes higher agronomic performance compared to the parents. Crops such as wheat have had comparatively less success with hybrid breeding, in large part owing to cost constraints of hybrid seed production (Longin et al., 2012). Utilizing apomixis in crops has long been suggested to be of benefit for fixing hybrids to shorten breeding cycles and significantly reduce costs (Spillane et al., 2004). The elucidation of the molecular mechanisms coordinating meiosis and embryogenesis have since offered a template to synthesize apomixis in plants from scratch. Many wild apomicts produce clonal seeds first by skipping meiosis and second by avoiding a fertilization checkpoint. Work in model plants and the major crop rice has shown that both of these characteristics can be induced by genetic manipulation of just four genes. Deletion of three genes that control major characteristics of meiosis induce a mitotic-like division to prevent genetic mixing and ploidy reduction in gametes. Expression of a single embryogenesis factor in the female egg cell, where it is normally turned off, causes the development of an unfertilized, unreduced embryo. Simultaneous manipulation of both processes is sufficient to trigger the production of clonal seeds. While thus far successful in rice, it has been unclear whether synthetic apomixis can be broadly extended to other economically important crops, such as wheat or barley. This work provides evidence that synthetic apomixis in barley, a relative of wheat, is within reach. A conserved meiotic program and fertilization checkpoint in barley, shown in Chapter 3, suggest that clonal seeds can be engineered beyond rice. Synthetic apomixis may thus be broadly applicable to given species of interest, and holds the potential for a place in the modern breeding scheme.

Appendices

Appendix Table 1. List of oligonucleotides used in this work

Oligo	Sequence (5'-3')	Chapter	Purpose	Note
1	TGCTTCAAATCGTCACTGCG	2	REC8-P60S genotyping F	NlaIV digestion for WT
2	CGTGAATATGATTTTGACGGGGA	2	REC8-P60S genotyping R	NlaIV digestion for WT
3	TCAGTCAGTACGTGTCAAGCA	2	SMC1-C624Y genotyping F	Acc1 digestion for mutant
4	CCAGTCATTGTTCCCGCCTT	2	SMC1-C624Y genotyping R	Acc1 digestion for mutant
5	CATCGTGAAGCTCTTTGTCGT	2	SMC3-G129E genotyping F	Apo1 digestion for mutant
6	aatcttgcagAAAAGGTGAAGTT	2	SMC3-G129E genotyping R	Apo1 digestion for mutant
7	ATTCGCTCTTAGTGCTCTTGC	2	ctf18-mp193 genotyping	HinFI digestion for WT
8	GAGTGTTCAAGCAAGAGCGAATGTCAGATT	2	ctf18-mp193 genotyping	HinFI digestion for WT
9	GGACCCTACCCAGACGATTC	2	dcc1-1, dcc1-2 genotyping F	31+32 for wt
10	CTCATCACTCGCTTGAACCG	2	dcc1-1, dcc1-2	31+32 for wt

			genotyping F	
11	TCTTCAATTTTGGGCCCAGC	2	dcc1-1, dcc1-2 genotyping R	31+34 for mut
12	TTCAAGCTTACTCAGGGCTTTCGTTGTTTCCTGGC	2	cenpc-T29* genotyping	BsuRI digestion for mutant
13	TGCAGTTTACCACAGACTTTTCGTC	2	cenpc-T29* genotyping	BsuRI digestion for mutant
14	CGGAAGCTGGCTAACTTAG	2	asp2-mp150 genotyping	MboI digestion for WT
15	caatcaaatgcaagaataag	2	asp2-mp150 genotyping	MboI digestion for WT
16	agcaAAGATCGCCGTCGTAACGGC	3	hvtam gRNA1 forward oligo	For sgRNA assembly
17	aaacGCCGTTACGACGGCGATCTT	3	hvtam gRNA1 reverse oligo	For sgRNA assembly
18	agcaTCCCAGGACCAACGCTGAAA	3	hvtam gRNA2 forward oligo	For sgRNA assembly
19	aaacTTTCAGCGTTGGTCCTGGGA	3	hvtam gRNA2 reverse oligo	For sgRNA assembly
20	GCTTCTCCTCGGCGATGT	3	HvTAM Guide 1F genotyping/sequencing	
21	ACGAACAAAAGAGGCAATCCC	3	HvTAM Guide 1R genotyping/sequencing	

22	GAAGCAGAACTCCACCCTTG	3	HvTAM Guide 2F genotyping/sequencing	
23	GGACATTGCTTCTTCCCGAC	3	HvTAM Guide 2R genotyping/sequencing	
24	GATGTCGAGCAACCCCGC	3	HvTAM Guide 1F genotyping/sequencing	
25	TCCCACAACAACGAACAAAAGA	3	HvTAM Guide 1R genotyping/sequencing	
26	TGCTTCTTCCCGACAAATTTG	3	HvTAM Guide 2F genotyping/sequencing	
27	TGCAGCTTTCACAAATCTTCTGA	3	HvTAM Guide 2R genotyping/sequencing	
28	agcaTGCACCCCCGATGGCTTCCC	3	hvtdm sgRNA1 F	For sgRNA assembly
29	aaacGGGAAGCCATCGGGGGTGCA	3	hvtdm sgRNA1 R	For sgRNA assembly
30	agcaCAGCCTCAAAACCCCGGCTG	3	hvtdm sgRNA2 F	For sgRNA assembly
31	aaacCAGCCGGGGTTTTGAGGCTG	3	hvtdm sgRNA2 R	For sgRNA assembly
32	TACTAGTCAGCCACCGAACG	3	HvTDM guide 1 seq F	
33	GTGGTGGTCGATGGTGTTAC	3	HvTDM guide 1 seq R	
34	TGGAGGAGGATGAACAGCAG	3	HvTDM guide 2 seq F	

35	GCCTTCCTGACAAAACTCCG	3	HvTDM guide 2 seq R	
36	agcaTATCACGGAGCGGCGTCCTC	3	HvOSD1B sgRNA1 F	For sgRNA assembly
37	aaacGAGGACGCCGCTCCGTGATA	3	HvOSD1B sgRNA1 R	For sgRNA assembly
38	agcaATCTACGCAACCTGCGAACT	3	HvOSD1B sgRNA2 F	For sgRNA assembly
39	aaacAGTTCGCAGGTTGCGTAGAT	3	HvOSD1B sgRNA2 R	For sgRNA assembly
40	TCCAGCAACAAGGAGAACGT	3	HVOSD1B Guide 1 seq F	
41	TGGCAAAGAAGAAAAGGCAC	3	HvOSD1B Guide 1 seq R	
42	GGCTGCAGGTAGTTCTAGCT	3	HvOSDB Guide 2 seq F	
43	GGCCTGCAACCATTTCAGTT	3	HvOSDB Guide 2 seq R	
44	agcaCTGAAGTGAGGATTGCCAGC	3	HvOSD1A sgRNA1 F	For sgRNA assembly
45	aaacGCTGGCAATCCTCACTTCAG	3	HvOSD1A sgRNA1 R	For sgRNA assembly
46	agcaCCTGAAGCGAACTGATCCCA	3	HvOSD1A sgRNA2 F	For sgRNA assembly

47	aaacTGGGATCAGTTCGCTTCAGG	3	HvOSD1A sgRNA2 R	For sgRNA assembly
48	AGGACCACGTATTCATCTTGGAT	3	HvOSD1A -seq-F	
49	ACTGTCATGATCCATGGAGGAG	3	HvOSD1A -seq-R	
50	CCTTGCAGTCTCCATCCAGA	3	HvOSD1A Guide 2 seq F	
51	AGAACCGAGCACCATGGAAG	3	HvOSD1A Guide 2 seq R	
52	agcaACGCGAATCCGTACTGAGGC	3	SPO11-1 sgRNA1 F	For sgRNA assembly
53	aaacGCCTCAGTACGGATTCGCGT	3	SPO11-1 sgRNA1 R	For sgRNA assembly
54	agcaTTGATTGCTTCGATGCTAAC	3	SPO11-1 sgRNA2 F	For sgRNA assembly
55	aaacGTTAGCATCGAAGCAATCAA	3	SPO11-1 sgRNA2 R	For sgRNA assembly
56	CGTCAGATTCTTACCGGTGCG	3	HvSPO11-1-sgRNA1-seq-F	
57	ATCTGCACATCAACGACGCAT	3	HvSPO11-1-sgRNA1-seq-R	
58	TTCCATCTAATCACACAGTACG	3	HvSPO11-1-sgRNA2-seq-F	
59	ACTGAATTTCCTAAGGAAACAG	3	HvSPO11-1-	

			sgRNA2-seq-R	
60	agcaACTGATACTGCCAGGTTCCG	3	REC8a/b sgRNA1 F	For sgRNA assembly
61	aaacCGGAACCTGGCAGTATCAGT	3	REC8a/b sgRNA1 R	For sgRNA assembly
62	agcaCCTCTAAAAGGCGTCAAGTC	3	REC8a/b sgRNA2 F	For sgRNA assembly
63	aaacGACTTGACGCCTTTTAGAGG	3	REC8a/b sgRNA2 R	For sgRNA assembly
64	TGCTTCTTGCAGGTATGAAGCA	3	HvREC8a-sgRNA1-seq-F	
65	CAGACAGAAGCTAGGAGACAGG	3	HvREC8a-sgRNA1-seq-R	
66	CCCCCAAGTGATCATGGATAAC	3	HvREC8a-sgRNA2-seq-F	
67	AATTCGGTAAGTGCAATGCATG	3	HvREC8a-sgRNA2-seq-R	

Bibliography

Almedawar, S., Colomina, N., Bermudez-Lopez, M., Pocino-Merino, I., & Torres-Rosell, J. (2012). A SUMO-Dependent Step during Establishment of Sister Chromatid Cohesion. *CURRENT BIOLOGY*, *22*(17), 1576–1581. https://doi.org/10.1016/j.cub.2012.06.046

Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C. C., Parker, H., Prednis, L., Ansari, Y., Choy, N., … Ecker, J. R. (2003). Genome-wide insertional mutagenesis of Arabidopsis thaliana. *Science*, *301*(5633), 653–657. https://doi.org/10.1126/science.1086391

Andorf, C., Beavis, W. D., Hufford, M., Smith, S., Suza, W. P., Wang, K., Woodhouse, M., Yu, J., & Lubberstedt, T. (2019). Technological advances in maize breeding: Past, present and future. *Theoretical and Applied Genetics*, *132*(3), 817–849. https://doi.org/10.1007/s00122-019-03306-3

Ariyoshi, M., & Fukagawa, T. (2023). An updated view of the kinetochore architecture. *Trends in Genetics*, *39*(12), 941–953. https://doi.org/10.1016/j.tig.2023.09.003

Bai, X., Peirson, B. N., Dong, F., Xue, C., & Makaroff, C. A. (1999). Isolation and Characterization of SYN1, a RAD21-like Gene Essential for Meiosis in Arabidopsis. *The Plant Cell*, *11*(3), 417–430. https://doi.org/10.1105/tpc.11.3.417

Barton, R. E., Massari, L. F., Robertson, D., & Marston, A. L. (2022). Eco1-dependent cohesin acetylation anchors chromatin loops and cohesion to define functional meiotic chromosome domains. *eLife*, *11*, e74447. https://doi.org/10.7554/eLife.74447

Ben-Shahar, T. R., Heeger, S., Lehane, C., East, P., Flynn, H., Skehel, M., & Uhlmann, F. (2008). Eco1-Dependent Cohesin Acetylation During Establishment of Sister

Chromatid Cohesion. *Science*, *321*(5888), 563–566. https://doi.org/10.1126/science.1157774

Benyahya, F., Nadaud, I., Da Ines, O., Rimbert, H., White, C., & Sourdille, P. (2020). SPO11.2 is essential for programmed double-strand break formation during meiosis in bread wheat (Triticum aestivum L.). *The Plant Journal*, *104*(1), 30–43. https://doi.org/10.1111/tpj.14903

Berkowitz, K. M., Sowash, A. R., Koenig, L. R., Urcuyo, D., Khan, F., Yang, F., Wang, P. J., Jongens, T. A., & Kaestner, K. H. (2012). Disruption of Chtf18 Causes Defective Meiotic Recombination in Male Mice. *Plos Genetics*, *8*(11), e1002996. https://doi.org/10.1371/journal.pgen.1002996

Bermudez, V. P., Maniwa, Y., Tappin, I., Ozato, K., Yokomori, K., & Hurwitz, J. (2003). The alternative Ctf18-Dcc1-Ctf8-replication factor C complex required for sister chromatid cohesion loads proliferating cell nuclear antigen onto DNA. *Proceedings of the National Academy of Sciences*, *100*(18), 10237–10242. https://doi.org/10.1073/pnas.1434308100

Bhatt, A. M., Lister, C., Page, T., Fransz, P., Findlay, K., Jones, G. H., Dickinson, H. G., & Dean, C. (1999). The DIF1 gene of Arabidopsis is required for meiotic chromosome segregation and belongs to the REC8/RAD21 cohesin gene family. *The Plant Journal*, *19*(4), 463–472. https://doi.org/10.1046/j.1365-313X.1999.00548.x

Bicknell, R. A., & Koltunow, A. M. (2004). Understanding apomixis: Recent advances and remaining conundrums. *The Plant Cell*, *16*(Suppl), S228–S245. https://doi.org/10.1105/tpc.017921

Blattner, A. C., Chaurasia, S., Mckee, B. D., & Lehner, C. F. (2016). Separase Is Required for Homolog and Sister Disjunction during Drosophila melanogaster Male Meiosis, but

Not for Biorientation of Sister Centromeres. *Plos Genetics*, *12*(4), Article 4. https://doi.org/10.1371/journal.pgen.1005996

Bolaños-Villegas, P., Yang, X., Wang, H.-J., Juan, C.-T., Chuang, M.-H., Makaroff, C. A., & Jauh, G.-Y. (2013). Arabidopsis CHROMOSOME TRANSMISSION FIDELITY 7 (AtCTF7/ECO1) is required for DNA repair, mitosis and meiosis. *The Plant Journal: For Cell and Molecular Biology*, *75*(6), 927–940. https://doi.org/10.1111/tpj.12261

Bonner, M. K., Haase, J., Swinderman, J., Halas, H., Jenkins, L. M. M., & Kelly, A. E. (2019). Enrichment of Aurora B kinase at the inner kinetochore controls outer kinetochore assembly. *JOURNAL OF CELL BIOLOGY*, *218*(10), 3237–3257. https://doi.org/10.1083/jcb.201901004

Boutilier, K., Offringa, R., Sharma, V. K., Kieft, H., Ouellet, T., Zhang, L. M., Hattori, J., Liu, C. M., van Lammeren, A. a. M., Miki, B. L. A., Custers, J. B. M., & Campagne, M. M. V. (2002). Ectopic expression of BABY BOOM triggers a conversion from vegetative to embryonic growth. *Plant Cell*, *14*(8), 1737–1749. https://doi.org/10.1105/tpc.001941

Brito, I. L., Yu, H.-G., & Amon, A. (2010). Condensins Promote Coorientation of Sister Chromatids During Meiosis I in Budding Yeast. *Genetics*, *185*(1), Article 1. https://doi.org/10.1534/genetics.110.115139

Buheitel, J., & Stemmann, O. (2013). Prophase pathway-dependent removal of cohesin from human chromosomes requires opening of the Smc3–Scc1 gate. *The EMBO Journal*, *32*(5), 666–676. https://doi.org/10.1038/emboj.2013.7

Bulankova, P., Akimcheva, S., Fellner, N., & Riha, K. (2013). Identification of Arabidopsis Meiotic Cyclins Reveals Functional Diversification among Plant Cyclin Genes. *PLOS Genetics*, *9*(5), e1003508. https://doi.org/10.1371/journal.pgen.1003508

Cairo, A., Vargova, A., Shukla, N., Capitao, C., Mikulkova, P., Valuchova, S., Pecinkova, J., Bulankova, P., & Riha, K. (2022). Meiotic exit in Arabidopsis is driven by P-body–mediated inhibition of translation. *Science*, *377*(6606), 629–634. https://doi.org/10.1126/science.abo0904

Cairo, G., & Lacefield, S. (2020). Establishing correct kinetochore-microtubule attachments in mitosis and meiosis. *Essays in Biochemistry*, *64*(2), 277–287. https://doi.org/10.1042/EBC20190072

Capilla-Pérez, L., Durand, S., Hurel, A., Lian, Q., Chambon, A., Taochy, C., Solier, V., Grelon, M., & Mercier, R. (2021). The synaptonemal complex imposes crossover interference and heterochiasmy in Arabidopsis. *Proceedings of the National Academy of Sciences*, *118*(12), e2023613118. https://doi.org/10.1073/pnas.2023613118

Capilla-Perez, L., Solier, V., Portemer, V., Chambon, A., Hurel, A., Guillebaux, A., Vezon, D., Cromer, L., Grelon, M., & Mercier, R. (2018). The HEM Lines: A New Library of Homozygous Arabidopsis thaliana EMS Mutants and its Potential to Detect Meiotic Phenotypes. *Frontiers in Plant Science*, *9*. https://doi.org/10.3389/fpls.2018.01339

Castro, P. H., Santos, M. Â., Freitas, S., Cana-Quijada, P., Lourenço, T., Rodrigues, M. A. A., Fonseca, F., Ruiz-Albert, J., Azevedo, J. E., Tavares, R. M., Castillo, A. G., Bejarano, E. R., & Azevedo, H. (2018). Arabidopsis thaliana SPF1 and SPF2 are nuclear-located ULP2-like SUMO proteases that act downstream of SIZ1 in plant development. *Journal of Experimental Botany*, *69*(19), 4633–4649. https://doi.org/10.1093/jxb/ery265

Challa, K., Fajish, G., Shinohara, M., Klein, F., Gasser, S. M., & Shinohara, A. (2019). Meiosis-specific prophase-like pathway controls cleavage-independent release of cohesin by Wapl phosphorylation. *PLoS GENETICS*, *15*(1), e1007851. https://doi.org/10.1371/journal.pgen.1007851

Challa, K., Lee, M.-S., Shinohara, M., Kim, K. P., & Shinohara, A. (2016). Rad61/Wpl1 (Wapl), a cohesin regulator, controls chromosome compaction during meiosis. *Nucleic Acids Research*, *44*(7), 3190–3203. https://doi.org/10.1093/nar/gkw034

Cheeseman, I. M. (2014). The Kinetochore. *Cold Spring Harbor Perspectives in Biology*, *6*(7), a015826. https://doi.org/10.1101/cshperspect.a015826

Chelysheva, L., Diallo, S., Vezon, D., Gendrot, G., Vrielynck, N., Belcram, K., Rocques, N., Márquez-Lema, A., Bhatt, A. M., Horlow, C., Mercier, R., Mézard, C., & Grelon, M. (2005). AtREC8 and AtSCC3 are essential to the monopolar orientation of the kinetochores during meiosis. *Journal of Cell Science*, *118*(20), 4621–4632. https://doi.org/10.1242/jcs.02583

Chen, B., Maas, L., Figueiredo, D., Zhong, Y., Reis, R., Li, M., Horstman, A., Riksen, T., Weemen, M., Liu, H., Siemons, C., Chen, S., Angenent, G. C., & Boutilier, K. (2022). BABY BOOM regulates early embryo and endosperm development. *Proceedings of the National Academy of Sciences of the United States of America*, *119*(25), e2201761119. https://doi.org/10.1073/pnas.2201761119

Cifuentes, M., Jolivet, S., Cromer, L., Harashima, H., Bulankova, P., Renne, C., Crismani, W., Nomura, Y., Nakagami, H., Sugimoto, K., Schnittger, A., Riha, K., & Mercier, R. (2016). TDM1 Regulation Determines the Number of Meiotic Divisions. *PLOS Genetics*, *12*(2), e1005856. https://doi.org/10.1371/journal.pgen.1005856

Cifuentes, M., Rivard, M., Pereira, L., Chelysheva, L., & Mercier, R. (2013). Haploid Meiosis in Arabidopsis: Double-Strand Breaks Are Formed and Repaired but Without Synapsis and Crossovers. *PLOS ONE*, *8*(8), e72431. https://doi.org/10.1371/journal.pone.0072431

Conner, J. A., Mookkan, M., Huo, H., Chae, K., & Ozias-Akins, P. (2015). A parthenogenesis gene of apomict origin elicits embryo formation from unfertilized eggs in a sexual

plant. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(36), 11205–11210. https://doi.org/10.1073/pnas.1505856112

Corbett, K. D., Yip, C. K., Ee, L.-S., Walz, T., Amon, A., & Harrison, S. C. (2010). The Monopolin Complex Crosslinks Kinetochore Components to Regulate Chromosome-Microtubule Attachments. *Cell*, *142*(4), Article 4. https://doi.org/10.1016/j.cell.2010.07.017

Cornaro, L., Banfi, C., Cucinotta, M., Colombo, L., & van Dijk, P. J. (2023). Asexual reproduction through seeds: The complex case of diplosporous apomixis. *Journal of Experimental Botany*, *74*(8), 2462–2478. https://doi.org/10.1093/jxb/erad054

Cromer, L., Heyman, J., Touati, S., Harashima, H., Araou, E., Girard, C., Horlow, C., Wassmann, K., Schnittger, A., Veylder, L. D., & Mercier, R. (2012). OSD1 Promotes Meiotic Progression via APC/C Inhibition and Forms a Regulatory Network with TDM and CYCA1;2/TAM. *PLOS Genetics*, *8*(7), e1002865. https://doi.org/10.1371/journal.pgen.1002865

Cromer, L., Jolivet, S., Horlow, C., Chelysheva, L., Heyman, J., De Jaeger, G., Koncz, C., De Veylder, L., & Mercier, R. (2013). Centromeric Cohesion Is Protected Twice at Meiosis, by SHUGOSHINs at Anaphase I and by PATRONUS at Interkinesis. *Current Biology*, *23*(21), 2090–2099. https://doi.org/10.1016/j.cub.2013.08.036

Cromer, L., Jolivet, S., Singh, D. K., Berthier, F., De Winne, N., De Jaeger, G., Komaki, S., Prusicki, M. A., Schnittger, A., Guerois, R., & Mercier, R. (2019). Patronus is the elusive plant securin, preventing chromosome separation by antagonizing separase. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(32), 16018–16027. https://doi.org/10.1073/pnas.1906237116

d'Erfurth, I., Cromer, L., Jolivet, S., Girard, C., Horlow, C., Sun, Y., To, J. P. C., Berchowitz, L. E., Copenhaver, G. P., & Mercier, R. (2010). The CYCLIN-A CYCA1;2/TAM Is

Required for the Meiosis I to Meiosis II Transition and Cooperates with OSD1 for the Prophase to First Meiotic Division Transition. *PLOS Genetics*, *6*(6), e1000989. https://doi.org/10.1371/journal.pgen.1000989

d'Erfurth, I., Jolivet, S., Froger, N., Catrice, O., Novatchkova, M., & Mercier, R. (2009). Turning Meiosis into Mitosis. *PLOS Biology*, *7*(6), e1000124. https://doi.org/10.1371/journal.pbio.1000124

Davidson, I. F., Bauer, B., Goetz, D., Tang, W., Wutz, G., & Peters, J.-M. (2019). DNA loop extrusion by human cohesin. *Science*, *366*(6471), 1338–1345. https://doi.org/10.1126/science.aaz3418

Davidson, I. F., & Peters, J.-M. (2021). Genome folding through loop extrusion by SMC complexes. *Nature Reviews Molecular Cell Biology*, *22*(7), 445–464. https://doi.org/10.1038/s41580-021-00349-7

De, K., Sterle, L., Krueger, L., Yang, X., & Makaroff, C. A. (2014). Arabidopsis thaliana WAPL Is Essential for the Prophase Removal of Cohesin during Meiosis. *PLoS Genetics*, *10*(7). https://doi.org/10.1371/journal.pgen.1004497

De Muyt, A., Pereira, L., Vezon, D., Chelysheva, L., Gendrot, G., Chambon, A., Lainé-Choinard, S., Pelletier, G., Mercier, R., Nogué, F., & Grelon, M. (2009). A High Throughput Genetic Screen Identifies New Early Meiotic Recombination Functions in Arabidopsis thaliana. *PLOS Genetics*, *5*(9), e1000654. https://doi.org/10.1371/journal.pgen.1000654

De Muyt, A., Vezon, D., Gendrot, G., Gallois, J.-L., Stevens, R., & Grelon, M. (2007). AtPRD1 is required for meiotic double strand break formation in Arabidopsis thaliana. *The EMBO Journal*, *26*(18), 4126–4137. https://doi.org/10.1038/sj.emboj.7601815

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.-F., Guindon, S., Lefort, V., Lescot, M., Claverie, J.-M., & Gascuel, O. (2008).

Phylogeny.fr: Robust phylogenetic analysis for the non-specialist. *Nucleic Acids Research*, *36*(suppl_2), W465–W469. https://doi.org/10.1093/nar/gkn180

Ding, Y., Kaido, M., Llano, E., Pendas, A. M., & Kitajima, T. S. (2018). The Post-anaphase SUMO Pathway Ensures the Maintenance of Centromeric Cohesion through Meiosis I-II Transition in Mammalian Oocytes. *Current Biology*, *28*(10), 1661-1669.e4. https://doi.org/10.1016/j.cub.2018.04.019

Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, *32*(5), 1792–1797. https://doi.org/10.1093/nar/gkh340

Eichinger, C. S., Kurze, A., Oliveira, R. A., & Nasmyth, K. (2013). Disengaging the Smc3/kleisin interface releases cohesin from Drosophila chromosomes during interphase and mitosis. *The EMBO Journal*, *32*(5), 656–665. https://doi.org/10.1038/emboj.2012.346

Elmayan, T., Balzergue, S., Béon, F., Bourdon, V., Daubremet, J., Guénet, Y., Mourrain, P., Palauqui, J. C., Vernhettes, S., Vialle, T., Wostrikoff, K., & Vaucheret, H. (1998). Arabidopsis mutants impaired in cosuppression. *The Plant Cell*, *10*(10), 1747–1758. https://doi.org/10.1105/tpc.10.10.1747

Galander, S., & Marston, A. L. (2020). Meiosis I Kinase Regulators: Conserved Orchestrators of Reductional Chromosome Segregation. *BioEssays*, *42*(10), 2000018. https://doi.org/10.1002/bies.202000018

Gandhi, R., Gillespie, P. J., & Hirano, T. (2006). Human Wapl is a cohesin-binding protein that promotes sister-chromatid resolution in mitotic prophase. *Current Biology: CB*, *16*(24), 2406–2417. https://doi.org/10.1016/j.cub.2006.10.061

Ganji, M., Shaltiel, I. A., Bisht, S., Kim, E., Kalichava, A., Haering, C. H., & Dekker, C. (2018). Real-time imaging of DNA loop extrusion by condensin. *Science*, *360*(6384), 102–105. https://doi.org/10.1126/science.aar7831

Gascoigne, K. E., & Cheeseman, I. M. (2011). Kinetochore assembly: If you build it, they will come. *Current Opinion in Cell Biology*, *23*(1), 102–108. https://doi.org/10.1016/j.ceb.2010.07.007

Gilles, L. M., Khaled, A., Laffaire, J.-B., Chaignon, S., Gendrot, G., Laplaige, J., Berges, H., Beydon, G., Bayle, V., Barret, P., Comadran, J., Martinant, J.-P., Rogowsky, P. M., & Widiez, T. (2017). Loss of pollen-specific phospholipase NOT LIKE DAD triggers gynogenesis in maize. *Embo Journal*, *36*(6), 707–717. https://doi.org/10.15252/embj.201796603

Goecks, J., Nekrutenko, A., Taylor, J., & The Galaxy Team. (2010). Galaxy: A comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome Biology*, *11*(8), R86. https://doi.org/10.1186/gb-2010-11-8-r86

Grelon, M., Vezon, D., Gendrot, G., & Pelletier, G. (2001). AtSPO11-1 is necessary for efficient meiotic recombination in plants. *The EMBO Journal*, *20*(3), 589–600. https://doi.org/10.1093/emboj/20.3.589

Gruber, S., Haering, C. H., & Nasmyth, K. (2003). Chromosomal Cohesin Forms a Ring. *Cell*, *112*(6), 765–777. https://doi.org/10.1016/S0092-8674(03)00162-4

Grützner, R., Martin, P., Horn, C., Mortensen, S., Cram, E. J., Lee-Parsons, C. W. T., Stuttmann, J., & Marillonnet, S. (2021). High-efficiency genome editing in plants mediated by a Cas9 gene containing multiple introns. *Plant Communications*, *2*(2), 100135. https://doi.org/10.1016/j.xplc.2020.100135

Haarhuis, J. H. I., Elbatsh, A. M. O., van den Broek, B., Camps, D., Erkan, H., Jalink, K., Medema, R. H., & Rowland, B. D. (2013). WAPL-mediated removal of cohesin protects against segregation errors and aneuploidy. *Current Biology: CB*, *23*(20), 2071–2077. https://doi.org/10.1016/j.cub.2013.09.003

Haeussler, M., Schönig, K., Eckert, H., Eschstruth, A., Mianné, J., Renaud, J.-B., Schneider-Maunoury, S., Shkumatava, A., Teboul, L., Kent, J., Joly, J.-S., & Concordet, J.-P. (2016). Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. *Genome Biology*, *17*(1), 148. https://doi.org/10.1186/s13059-016-1012-2

Hand, M. L., & Koltunow, A. M. G. (2014). The Genetic Control of Apomixis: Asexual Seed Formation. *Genetics*, *197*(2), 441–450. https://doi.org/10.1534/genetics.114.163105

Harrison, P. W., Amode, M. R., Austine-Orimoloye, O., Azov, A. G., Barba, M., Barnes, I., Becker, A., Bennett, R., Berry, A., Bhai, J., Bhurji, S. K., Boddu, S., Branco Lins, P. R., Brooks, L., Ramaraju, S. B., Campbell, L. I., Martinez, M. C., Charkhchi, M., Chougule, K., … Yates, A. D. (2024). Ensembl 2024. *Nucleic Acids Research*, *52*(D1), D891–D899. https://doi.org/10.1093/nar/gkad1049

Hartung, F., Wurz-Wildersinn, R., Fuchs, J., Schubert, I., Suer, S., & Puchta, H. (2007). The Catalytically Active Tyrosine Residues of Both SPO11-1 and SPO11-2 Are Required for Meiotic Double-Strand Break Induction in Arabidopsis. *The Plant Cell*, *19*(10), 3090–3099. https://doi.org/10.1105/tpc.107.054817

Hauf, S., Biswas, A., Langegger, M., Kawashima, S. A., Tsukahara, T., & Watanabe, Y. (2007). Aurora controls sister kinetochore mono-orientation and homolog bi-orientation in meiosis-I. *The EMBO Journal*, *26*(21), 4475–4486. https://doi.org/10.1038/sj.emboj.7601880

Hauf, S., Roitinger, E., Koch, B., Dittrich, C. M., Mechtler, K., & Peters, J.-M. (2005). Dissociation of Cohesin from Chromosome Arms and Loss of Arm Cohesion during Early Mitosis Depends on Phosphorylation of SA2. *PLOS Biology*, *3*(3), e69. https://doi.org/10.1371/journal.pbio.0030069

Hensel, G., Kastner, C., Oleszczuk, S., Riechen, J., & Kumlehn, J. (2009). Agrobacterium-Mediated Gene Transfer to Cereal Crop Plants: Current Protocols for Barley, Wheat, Triticale, and Maize. *International Journal of Plant Genomics*, *2009*(1), 835608. https://doi.org/10.1155/2009/835608

Heyman, J., Van den Daele, H., De Wit, K., Boudolf, V., Berckmans, B., Verkest, A., Kamei, C. L. A., De Jaeger, G., Koncz, C., & De Veylder, L. (2011). Arabidopsis ULTRAVIOLET-B-INSENSITIVE4 Maintains Cell Division Activity by Temporal Inhibition of the Anaphase-Promoting Complex/Cyclosome. *The Plant Cell*, *23*(12), 4394–4410. https://doi.org/10.1105/tpc.111.091793

Higashi, T. L., & Uhlmann, F. (2022). SMC complexes: Lifting the lid on loop extrusion. *CURRENT OPINION IN CELL BIOLOGY*, *74*, 13–22. https://doi.org/10.1016/j.ceb.2021.12.003

Hirose, Y., Suzuki, R., Ohba, T., Hinohara, Y., Matsuhara, H., Yoshida, M., Itabashi, Y., Murakami, H., & Yamamoto, A. (2011). Chiasmata Promote Monopolar Attachment of Sister Chromatids and Their Co-Segregation toward the Proper Pole during Meiosis I. *Plos Genetics*, *7*(3), Article 3. https://doi.org/10.1371/journal.pgen.1001329

Hochholdinger, F., & Baldauf, J. A. (2018). Heterosis in plants. *Current Biology*, *28*(18), R1089–R1092. https://doi.org/10.1016/j.cub.2018.06.041

Hoencamp, C., & Rowland, B. D. (2023). Genome control by SMC complexes. *Nature Reviews Molecular Cell Biology*, *24*(9), 633–650. https://doi.org/10.1038/s41580-023-00609-8

Hoisington, D., Khairallah, M., Reeves, T., Ribaut, J.-M., Skovmand, B., Taba, S., & Warburton, M. (1999). Plant genetic resources: What can they contribute toward increased crop productivity? *Proceedings of the National Academy of Sciences*, *96*(11), 5937–5943. https://doi.org/10.1073/pnas.96.11.5937

Hornig, N. C. D., Knowles, P. P., McDonald, N. Q., & Uhlmann, F. (2002). The dual mechanism of separase regulation by securin. *Current Biology: CB*, *12*(12), 973–982. https://doi.org/10.1016/s0960-9822(02)00847-3

Huang, C.-J., Wu, D., Khan, F. A., & Huo, L.-J. (2015). The SUMO Protease SENP3 Orchestrates G2-M Transition and Spindle Assembly in Mouse Oocytes. *SCIENTIFIC REPORTS*, *5*, 15600. https://doi.org/10.1038/srep15600

IPK Galaxy Blast Suite. (2024). https://galaxy-web.ipk-gatersleben.de/

Ivanov, D., Schleiffer, A., Eisenhaber, F., Mechtler, K., Haering, C. H., & Nasmyth, K. (2002). Eco1 Is a Novel Acetyltransferase that Can Acetylate Proteins Involved in Cohesion. *Current Biology*, *12*(4), 323–328. https://doi.org/10.1016/S0960-9822(02)00681-4

Jiang, C., Sun, J., Li, R., Yan, S., Chen, W., Guo, L., Qin, G., Wang, P., Luo, C., Huang, W., Zhang, Q., Fernie, A. R., Jackson, D., Li, X., & Yan, J. (2022). A reactive oxygen species burst causes haploid induction in maize. *Molecular Plant*, *15*(6), 943–955. https://doi.org/10.1016/j.molp.2022.04.001

Kawashima, S. A., Tsukahara, T., Langegger, M., Hauf, S., Kitajima, T. S., & Watanabe, Y. (2007). Shugoshin enables tension-generating attachment of kinetochores by loading Aurora to centromeres. *GENES & DEVELOPMENT*, *21*(4), 420–435. https://doi.org/10.1101/gad.1497307

Kawasumi, R., Abe, T., Psakhye, I., Miyata, K., Hirota, K., & Branzei, D. (2021). Vertebrate CTF18 and DDX11 essential function in cohesion is bypassed by preventing WAPL-

mediated cohesin release. *Genes & Development*. https://doi.org/10.1101/gad.348581.121

Kelliher, T., Starr, D., Richbourg, L., Chintamanani, S., Delzer, B., Nuccio, M. L., Green, J., Chen, Z., McCuiston, J., Wang, W., Liebler, T., Bullock, P., & Martin, B. (2017). MATRILINEAL, a sperm-specific phospholipase, triggers maize haploid induction. *Nature*, *542*(7639), 105-+. https://doi.org/10.1038/nature20827

Kelliher, T., Starr, D., Wang, W., McCuiston, J., Zhong, H., Nuccio, M. L., & Martin, B. (2016). Maternal Haploids Are Preferentially Induced by CENH3-tailswap Transgenic Complementation in Maize. *Frontiers in Plant Science*, *7*, 414. https://doi.org/10.3389/fpls.2016.00414

Khanday, I., Skinner, D., Yang, B., Mercier, R., & Sundaresan, V. (2019). A male-expressed rice embryogenic trigger redirected for asexual propagation through seeds. *Nature*, *565*(7737), 91–95. https://doi.org/10.1038/s41586-018-0785-8

Kim, J., Ishiguro, K., Nambu, A., Akiyoshi, B., Yokobayashi, S., Kagami, A., Ishiguro, T., Pendas, A. M., Takeda, N., Sakakibara, Y., Kitajima, T. S., Tanno, Y., Sakuno, T., & Watanabe, Y. (2015). Meikin is a conserved regulator of meiosis-I-specific kinetochore function. *Nature*, *517*(7535), Article 7535. https://doi.org/10.1038/nature14097

Kitajima, T. S., Kawashima, S. A., & Watanabe, Y. (2004). The conserved kinetochore protein shugoshin protects centromeric cohesion during meiosis. *Nature*, *427*(6974), 510–517. https://doi.org/10.1038/nature02312

Kitajima, T. S., Miyazaki, Y., Yamamoto, M., & Watanabe, Y. (2003). Rec8 cleavage by separase is required for meiotic nuclear divisions in fission yeast. *The EMBO Journal*, *22*(20), 5643–5653. https://doi.org/10.1093/emboj/cdg527

Kitajima, T. S., Sakuno, T., Ishiguro, K., Iemura, S., Natsume, T., Kawashima, S. A., & Watanabe, Y. (2006). Shugoshin collaborates with protein phosphatase 2A to protect cohesin. *Nature*, *441*(7089), 46–52. https://doi.org/10.1038/nature04663

Köhler, C., Mittelsten Scheid, O., & Erilova, A. (2010). The impact of the triploid block on the origin and evolution of polyploid plants. *Trends in Genetics*, *26*(3), 142–148. https://doi.org/10.1016/j.tig.2009.12.006

Kong, X., Luo, X., Qu, G.-P., Liu, P., & Jin, J. B. (2017). Arabidopsis SUMO protease ASP1 positively regulates flowering time partially through regulating FLC stability. *Journal of Integrative Plant Biology*, *59*(1), 15–29. https://doi.org/10.1111/jipb.12509

Kueng, S., Hegemann, B., Peters, B. H., Lipp, J. J., Schleiffer, A., Mechtler, K., & Peters, J.-M. (2006). Wapl controls the dynamic association of cohesin with chromatin. *Cell*, *127*(5), 955–967. https://doi.org/10.1016/j.cell.2006.09.040

Kumar, N., Galli, M., Ordon, J., Stuttmann, J., Kogel, K.-H., & Imani, J. (2018). Further analysis of barley MORC1 using a highly efficient RNA-guided Cas9 gene-editing system. *Plant Biotechnology Journal*, *16*(11), 1892–1903. https://doi.org/10.1111/pbi.12924

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods*, *9*(4), 357–359. https://doi.org/10.1038/nmeth.1923

Lee, J., Kitajima, T. S., Tanno, Y., Yoshida, K., Morita, T., Miyano, T., Miyake, M., & Watanabe, Y. (2008). Unified mode of centromeric protection by shugoshin in mammalian oocytes and somatic cells. *Nature Cell Biology*, *10*(1), 42–52. https://doi.org/10.1038/ncb1667

Li, X., & Dawe, R. K. (2009). Fused sister kinetochores initiate the reductional division in meiosis I. *Nature Cell Biology*, *11*(9), Article 9. https://doi.org/10.1038/ncb1923

Liu, C., He, Z., Zhang, Y., Hu, F., Li, M., Liu, Q., Huang, Y., Wang, J., Zhang, W., Wang, C., & Wang, K. (2022). Synthetic apomixis enables stable transgenerational transmission of heterotic phenotypes in hybrid rice. *Plant Communications*, 100470. https://doi.org/10.1016/j.xplc.2022.100470

Liu, C., Li, X., Meng, D., Zhong, Y., Chen, C., Dong, X., Xu, X., Chen, B., Li, W., Li, L., Tian, X., Zhao, H., Song, W., Luo, H., Zhang, Q., Lai, J., Jin, W., Yan, J., & Chen, S. (2017). A 4-bp Insertion at ZmPLA1 Encoding a Putative Phospholipase A Generates Haploid Induction in Maize. *Molecular Plant*, *10*(3), 520–522. https://doi.org/10.1016/j.molp.2017.01.011

Liu, C., McElver, J., Tzafrir, I., Joosen, R., Wittich, P., Patton, D., Van Lammeren, A. A. M., & Meinke, D. (2002). Condensin and cohesin knockouts in Arabidopsis exhibit a titan seed phenotype. *The Plant Journal: For Cell and Molecular Biology*, *29*(4), 405–415. https://doi.org/10.1046/j.1365-313x.2002.01224.x

Liu, H., Rankin, S., & Yu, H. (2013). Phosphorylation-enabled binding of SGO1–PP2A to cohesin protects sororin and centromeric cohesion during mitosis. *Nature Cell Biology*, *15*(1), 40–49. https://doi.org/10.1038/ncb2637

Liu, H. W., Bouchoux, C., Panarotto, M., Kakui, Y., Patel, H., & Uhlmann, F. (2020). Division of Labor between PCNA Loaders in DNA Replication and Sister Chromatid Cohesion Establishment. *Molecular Cell*, *78*(4), 725-+. https://doi.org/10.1016/j.molcel.2020.03.017

Liu, H., Wang, K., Jia, Z., Gong, Q., Lin, Z., Du, L., Pei, X., & Ye, X. (2020). Efficient induction of haploid plants in wheat by editing of TaMTL using an optimized Agrobacterium-mediated CRISPR system. *Journal of Experimental Botany*, *71*(4), 1337–1349. https://doi.org/10.1093/jxb/erz529

Liu, L., Jiang, Y., Zhang, X., Wang, X., Wang, Y., Han, Y., Coupland, G., Jin, J. B., Searle, I., Fu, Y.-F., & Chen, F. (2017). Two SUMO Proteases SUMO PROTEASE RELATED TO FERTILITY1 and 2 Are Required for Fertility in Arabidopsis. *Plant Physiology*, *175*(4), 1703–1719. https://doi.org/10.1104/pp.17.00021

Longin, C. F. H., Mühleisen, J., Maurer, H. P., Zhang, H., Gowda, M., & Reif, J. C. (2012). Hybrid breeding in autogamous cereals. *Theoretical and Applied Genetics*, *125*(6), 1087–1096. https://doi.org/10.1007/s00122-012-1967-7

Loureiro, J., Rodriguez, E., Dolezel, J., & Santos, C. (2006). Comparison of four nuclear isolation buffers for plant DNA flow cytometry. *Annals of Botany*, *98*(3), 679–689. https://doi.org/10.1093/aob/mcl141

Lv, J., Yu, K., Wei, J., Gui, H., Liu, C., Liang, D., Wang, Y., Zhou, H., Carlin, R., Rich, R., Lu, T., Que, Q., Wang, W. C., Zhang, X., & Kelliher, T. (2020). Generation of paternal haploids in wheat by genome editing of the centromeric histone CENH3. *Nature Biotechnology*, 1–5. https://doi.org/10.1038/s41587-020-0728-4

Madeira, F., Pearce, M., Tivey, A. R. N., Basutkar, P., Lee, J., Edbali, O., Madhusoodanan, N., Kolesnikov, A., & Lopez, R. (2022). Search and sequence analysis tools services from EMBL-EBI in 2022. *Nucleic Acids Research*, *50*(W1), W276–W279. https://doi.org/10.1093/nar/gkac240

Maier, N. K., Ma, J., Lampson, M. A., & Cheeseman, I. M. (2021). Separase cleaves the kinetochore protein Meikin at the meiosis I/II transition. *Developmental Cell*, *56*(15), 2192-2206.e8. https://doi.org/10.1016/j.devcel.2021.06.019

Marimuthu, M. P. A., Jolivet, S., Ravi, M., Pereira, L., Davda, J. N., Cromer, L., Wang, L., Nogué, F., Chan, S. W. L., Siddiqi, I., & Mercier, R. (2011). Synthetic Clonal Reproduction Through Seeds. *Science*, *331*(6019), 876–876. https://doi.org/10.1126/science.1199682

Marques, A., & Pedrosa-Harand, A. (2016). Holocentromere identity: From the typical mitotic linear structure to the great plasticity of meiotic holocentromeres. *Chromosoma*, *125*(4), 669–681. https://doi.org/10.1007/s00412-016-0612-7

Mascher, M., Wicker, T., Jenkins, J., Plott, C., Lux, T., Koh, C. S., Ens, J., Gundlach, H., Boston, L. B., Tulpová, Z., Holden, S., Hernández-Pinzón, I., Scholz, U., Mayer, K. F. X., Spannagl, M., Pozniak, C. J., Sharpe, A. G., Šimková, H., Moscou, M. J., … Stein, N. (2021). Long-read sequence assembly: A technical evaluation in barley. *The Plant Cell*, *33*(6), 1888–1906. https://doi.org/10.1093/plcell/koab077

Mayer, M. L., Gygi, S. P., Aebersold, R., & Hieter, P. (2001). Identification of RFC(Ctf18p, Ctf8p, Dcc1p): An Alternative RFC Complex Required for Sister Chromatid Cohesion in S. cerevisiae. *Molecular Cell*, *7*(5), 959–970. https://doi.org/10.1016/S1097-2765(01)00254-4

Mayer, M. L., Pot, I., Chang, M., Xu, H., Aneliunas, V., Kwok, T., Newitt, R., Aebersold, R., Boone, C., Brown, G. W., & Hieter, P. (2004). Identification of Protein Complexes Required for Efficient Sister Chromatid Cohesion. *Molecular Biology of the Cell*, *15*(4), 1736–1745. https://doi.org/10.1091/mbc.e03-08-0619

McAinsh, A. D., & Marston, A. L. (2022). The Four Causes: The Functional Architecture of Centromeres and Kinetochores. *Annual Review of Genetics*, *56*(1), 279–314. https://doi.org/10.1146/annurev-genet-072820-034559

McKinley, K. L., & Cheeseman, I. M. (2016). The molecular basis for centromere identity and function. *Nature Reviews Molecular Cell Biology*, *17*(1), 16–29. https://doi.org/10.1038/nrm.2015.5

Mieulet, D., Jolivet, S., Rivard, M., Cromer, L., Vernet, A., Mayonove, P., Pereira, L., Droc, G., Courtois, B., Guiderdoni, E., & Mercier, R. (2016). Turning rice meiosis into mitosis. *Cell Research*, *26*(11), 1242–1254. https://doi.org/10.1038/cr.2016.117

Mishra, A., Hu, B., Kurze, A., Beckouët, F., Farcas, A.-M., Dixon, S. E., Katou, Y., Khalid, S., Shirahige, K., & Nasmyth, K. (2010). Both interaction surfaces within cohesin's hinge domain are essential for its stable chromosomal association. *Current Biology: CB*, *20*(4), 279–289. https://doi.org/10.1016/j.cub.2009.12.059

Monat, C., Padmarasu, S., Lux, T., Wicker, T., Gundlach, H., Himmelbach, A., Ens, J., Li, C., Muehlbauer, G. J., Schulman, A. H., Waugh, R., Braumann, I., Pozniak, C., Scholz, U., Mayer, K. F. X., Spannagl, M., Stein, N., & Mascher, M. (2019). TRITEX: Chromosome-scale sequence assembly of Triticeae genomes with open-source tools. *Genome Biology*, *20*(1), 284. https://doi.org/10.1186/s13059-019-1899-5

Mukhopadhyay, D., Arnaoutov, A., & Dasso, M. (2010). The SUMO protease SENP6 is essential for inner kinetochore assembly. *Journal of Cell Biology*, *188*(5), 681–692. https://doi.org/10.1083/jcb.200909008

Musacchio, A. (2015). The Molecular Biology of Spindle Assembly Checkpoint Signaling Dynamics. *Current Biology: CB*, *25*(20), R1002-1018. https://doi.org/10.1016/j.cub.2015.08.051

Musacchio, A., & Desai, A. (2017). A Molecular View of Kinetochore Assembly and Function. *Biology*, *6*(1), Article 1. https://doi.org/10.3390/biology6010005

Musacchio, A., & Salmon, E. D. (2007). The spindle-assembly checkpoint in space and time. *Nature Reviews Molecular Cell Biology*, *8*(5), 379–393. https://doi.org/10.1038/nrm2163

Nakajima, M., Kumada, K., Hatakeyama, K., Noda, T., Peters, J.-M., & Hirota, T. (2007). The complete removal of cohesin from chromosome arms depends on separase. *Journal of Cell Science*, *120*(Pt 23), 4188–4196. https://doi.org/10.1242/jcs.011528

Nasmyth, K. (2015). A meiotic mystery: How sister kinetochores avoid being pulled in opposite directions during the first division. *BioEssays*, *37*(6), 657–665. https://doi.org/10.1002/bies.201500006

Nasmyth, K., & Haering, C. H. (2009). Cohesin: Its Roles and Mechanisms. *Annual Review of Genetics*, *43*(1), 525–558. https://doi.org/10.1146/annurev-genet-102108-134233

Nerusheva, O. O., Galander, S., Fernius, J., Kelly, D., & Marston, A. L. (2014). Tension-dependent removal of pericentromeric shugoshin is an indicator of sister chromosome biorientation. *GENES & DEVELOPMENT*, *28*(12), 1291–1309. https://doi.org/10.1101/gad.240291.114

Nicklas, R. B. (1997). How Cells Get the Right Chromosomes. *Science*, *275*(5300), 632–637. https://doi.org/10.1126/science.275.5300.632

Nishiyama, T., Ladurner, R., Schmitz, J., Kreidl, E., Schleiffer, A., Bhaskara, V., Bando, M., Shirahige, K., Hyman, A. A., Mechtler, K., & Peters, J.-M. (2010). Sororin mediates sister chromatid cohesion by antagonizing Wapl. *Cell*, *143*(5), 737–749. https://doi.org/10.1016/j.cell.2010.10.031

Nishiyama, T., Sykora, M. M., Huis in ’t Veld, P. J., Mechtler, K., & Peters, J.-M. (2013). Aurora B and Cdk1 mediate Wapl activation and release of acetylated cohesin from chromosomes by phosphorylating Sororin. *Proceedings of the National Academy of Sciences*, *110*(33), 13404–13409. https://doi.org/10.1073/pnas.1305020110

Novatchkova, M., Budhiraja, R., Coupland, G., Eisenhaber, F., & Bachmair, A. (2004). SUMO conjugation in plants. *Planta*, *220*(1), 1–8. https://doi.org/10.1007/s00425-004-1370-y

Ogushi, S., Rattani, A., Godwin, J., Metson, J., Schermelleh, L., & Nasmyth, K. (2021). Loss of sister kinetochore co-orientation and peri-centromeric cohesin protection after

meiosis I depends on cleavage of centromeric REC8. *Developmental Cell*, *56*(22), 3100-3114.e4. https://doi.org/10.1016/j.devcel.2021.10.017

Okamura, E., Sakamoto, T., Sasaki, T., & Matsunaga, S. (2017). A Plant Ancestral Polo-Like Kinase Sheds Light on the Mystery of the Evolutionary Disappearance of Polo-Like Kinases in the Plant Kingdom. *CYTOLOGIA*, *82*(3), 261–266. https://doi.org/10.1508/cytologia.82.261

Östergren, G. (1951). THE MECHANISM OF CO-ORIENTATION IN BIVALENTS AND MULTIVALENTS. *Hereditas*, *37*(1–2), Article 1–2. https://cyberleninka.org/article/n/359347

Petronczki, M., Chwalla, B., Siomos, M. F., Yokobayashi, S., Helmhart, W., Deutschbauer, A. M., Davis, R. W., Watanabe, Y., & Nasmyth, K. (2004). Sister-chromatid cohesion mediated by the alternative RF-CCtf18/Dcc1/Ctf8, the helicase Chl1 and the polymerase-alpha-associated protein Ctf4 is essential for chromatid disjunction during meiosis II. *Journal of Cell Science*, *117*(Pt 16), 3547–3559. https://doi.org/10.1242/jcs.01231

Petronczki, M., Matos, J., Mori, S., Gregan, J., Bogdanova, A., Schwickart, M., Mechtler, K., Shirahige, K., Zachariae, W., & Nasmyth, K. (2006). Monopolar Attachment of Sister Kinetochores at Meiosis I Requires Casein Kinase 1. *Cell*, *126*(6), 1049–1064. https://doi.org/10.1016/j.cell.2006.07.029

Plohl, M., Meštrović, N., & Mravinac, B. (2014). Centromere identity from the DNA point of view. *Chromosoma*, *123*(4), 313–325. https://doi.org/10.1007/s00412-014-0462-0

Prusén Mota, I., Galova, M., Schleiffer, A., Nguyen, T.-T., Kovacikova, I., Farias Saad, C., Litos, G., Nishiyama, T., Gregan, J., Peters, J.-M., & Schlögelhofer, P. (2024). Sororin is an evolutionary conserved antagonist of WAPL. *Nature Communications*, *15*(1), 4729. https://doi.org/10.1038/s41467-024-49178-0

Psakhye, I., & Branzei, D. (2021). SMC complexes are guarded by the SUMO protease Ulp2 against SUMO-chain-mediated turnover. *Cell Reports*, *36*(5), 109485. https://doi.org/10.1016/j.celrep.2021.109485

Psakhye, I., Kawasumi, R., Abe, T., Hirota, K., & Branzei, D. (2023). PCNA recruits cohesin loader Scc2 to ensure sister chromatid cohesion. *Nature Structural & Molecular Biology*, *30*(9), 1286–1294. https://doi.org/10.1038/s41594-023-01064-x

Rabitsch, K. P., Gregan, J., Schleiffer, A., Javerzat, J.-P., Eisenhaber, F., & Nasmyth, K. (2004). Two fission yeast homologs of Drosophila Mei-S332 are required for chromosome segregation during meiosis I and II. *Current Biology: CB*, *14*(4), 287–301. https://doi.org/10.1016/j.cub.2004.01.051

Rabitsch, K. P., Petronczki, M., Javerzat, J. P., Genier, S., Chwalla, B., Schleiffer, A., Tanaka, T. U., & Nasmyth, K. (2003). Kinetochore recruitment of two nucleolar proteins is required for homolog segregation in meiosis I. *Developmental Cell*, *4*(4), Article 4. https://doi.org/10.1016/S1534-5807(03)00086-8

Rakpenthai, A., Apodiakou, A., Whitcomb, S. J., & Hoefgen, R. (2022). In silico analysis of cis-elements and identification of transcription factors putatively involved in the regulation of the OAS cluster genes SDI1 and SDI2. *The Plant Journal*, *110*(5), 1286–1304. https://doi.org/10.1111/tpj.15735

Rankin, S., Ayad, N. G., & Kirschner, M. W. (2005). Sororin, a substrate of the anaphase-promoting complex, is required for sister chromatid cohesion in vertebrates. *Molecular Cell*, *18*(2), 185–200. https://doi.org/10.1016/j.molcel.2005.03.017

Ravi, M., & Chan, S. W. L. (2010). Haploid plants produced by centromere-mediated genome elimination. *Nature*, *464*(7288), Article 7288. https://doi.org/10.1038/nature08842

Resnick, T. D., Satinover, D. L., MacIsaac, F., Stukenberg, P. T., Earnshaw, W. C., Orr-Weaver, T. L., & Carmena, M. (2006). INCENP and Aurora B Promote Meiotic Sister

Chromatid Cohesion through Localization of the Shugoshin MEI-S332 in Drosophila. *Developmental Cell*, *11*(1), 57–68. https://doi.org/10.1016/j.devcel.2006.04.021

Riedel, C. G., Katis, V. L., Katou, Y., Mori, S., Itoh, T., Helmhart, W., Gálova, M., Petronczki, M., Gregan, J., Cetin, B., Mudrak, I., Ogris, E., Mechtler, K., Pelletier, L., Buchholz, F., Shirahige, K., & Nasmyth, K. (2006). Protein phosphatase 2A protects centromeric sister chromatid cohesion during meiosis I. *NATURE*, *441*(7089), 53–61. https://doi.org/10.1038/nature04664

Rogers, E., Bishop, J. D., Waddle, J. A., Schumacher, J. M., & Lin, R. (2002). The aurora kinase AIR-2 functions in the release of chromosome cohesion in Caenorhabditis elegans meiosis. *The Journal of Cell Biology*, *157*(2), 219–229. https://doi.org/10.1083/jcb.200110045

Rojas, J., Oz, T., Jonak, K., Lyzak, O., Massaad, V., Biriuk, O., & Zachariae, W. (2023). Spo13/MEIKIN ensures a Two-Division meiosis by preventing the activation of APC/CAma1 at meiosis I. *The EMBO Journal*, *42*(20), e114288. https://doi.org/10.15252/embj.2023114288

Rowland, B. D., Roig, M. B., Nishino, T., Kurze, A., Uluocak, P., Mishra, A., Beckouët, F., Underwood, P., Metson, J., Imre, R., Mechtler, K., Katis, V. L., & Nasmyth, K. (2009). Building sister chromatid cohesion: Smc3 acetylation counteracts an antiestablishment activity. *Molecular Cell*, *33*(6), 763–774. https://doi.org/10.1016/j.molcel.2009.02.028

Sakuno, T., Tada, K., & Watanabe, Y. (2009). Kinetochore geometry defined by cohesion within the centromere. *Nature*, *458*(7240), Article 7240. https://doi.org/10.1038/nature07876

Sakuno, T., Tanaka, K., Hauf, S., & Watanabe, Y. (2011). Repositioning of Aurora B Promoted by Chiasmata Ensures Sister Chromatid Mono-Orientation in Meiosis I. *Developmental Cell*, *21*(3), 534–545. https://doi.org/10.1016/j.devcel.2011.08.012

Sarangapani, K. K., Duro, E., Deng, Y., Alves, F. de L., Ye, Q., Opoku, K. N., Ceto, S., Rappsilber, J., Corbett, K. D., Biggins, S., Marston, A. L., & Asbury, C. L. (2014). Sister kinetochores are mechanically fused during meiosis I in yeast. *Science*, *346*(6206), Article 6206. https://doi.org/10.1126/science.1256729

Sarkar, S., Shenoy, R. T., Dalgaard, J. Z., Newnham, L., Hoffmann, E., Millar, J. B. A., & Arumugam, P. (2013). Monopolin Subunit Csm1 Associates with MIND Complex to Establish Monopolar Attachment of Sister Kinetochores at Meiosis I. *Plos Genetics*, *9*(7), Article 7. https://doi.org/10.1371/journal.pgen.1003610

Schubert, V., Weissleder, A., Ali, H., Fuchs, J., Lermontova, I., Meister, A., & Schubert, I. (2009). Cohesin gene defects may impair sister chromatid alignment and genome stability in Arabidopsis thaliana. *Chromosoma*, *118*(5), 591–605. https://doi.org/10.1007/s00412-009-0220-x

Schulman, B. A., Lindstrom, D. L., & Harlow, E. (1998). Substrate recruitment to cyclin-dependent kinase 2 by a multipurpose docking site on cyclin A. *Proceedings of the National Academy of Sciences*, *95*(18), 10453–10458. https://doi.org/10.1073/pnas.95.18.10453

Sessions, A., Burke, E., Presting, G., Aux, G., McElver, J., Patton, D., Dietrich, B., Ho, P., Bacwaden, J., Ko, C., Clarke, J. D., Cotton, D., Bullis, D., Snell, J., Miguel, T., Hutchison, D., Kimmerly, B., Mitzel, T., Katagiri, F., … Goff, S. A. (2002). A high-throughput Arabidopsis reverse genetics system. *The Plant Cell*, *14*(12), 2985–2994. https://doi.org/10.1105/tpc.004630

Shao, T., Tang, D., Wang, K., Wang, M., Che, L., Qin, B., Yu, H., Li, M., Gu, M., & Cheng, Z. (2011). OsREC8 Is Essential for Chromatid Cohesion and Metaphase I Monopolar Orientation in Rice Meiosis. *Plant Physiology*, *156*(3), Article 3. https://doi.org/10.1104/pp.111.177428

Singh, D. K., Andreuzza, S., Panoli, A. P., & Siddiqi, I. (2013). AtCTF7 is required for establishment of sister chromatid cohesion and association of cohesin with chromatin during meiosis in Arabidopsis. *BMC Plant Biology*, *13*(1), 117. https://doi.org/10.1186/1471-2229-13-117

Singh, D. K., Spillane, C., & Siddiqi, I. (2015). PATRONUS1 is expressed in meiotic prophase I to regulate centromeric cohesion in Arabidopsis and shows synthetic lethality with OSD1. *BMC Plant Biology*, *15*, 201. https://doi.org/10.1186/s12870-015-0558-6

Siomos, M. F., Badrinath, A., Pasierbek, P., Livingstone, D., White, J., Glotzer, M., & Nasmyth, K. (2001). Separase is required for chromosome segregation during meiosis I in Caenorhabditis elegans. *Current Biology: CB*, *11*(23), 1825–1835. https://doi.org/10.1016/s0960-9822(01)00588-7

Song, M., Wang, W., Ji, C., Li, S., Liu, W., Hu, X., Feng, A., Ruan, S., Du, S., Wang, H., Dai, K., Guo, L., Qian, Q., Si, H., & Hu, X. (2024). Simultaneous production of high-frequency synthetic apomixis with high fertility and improved agronomic traits in hybrid rice. *Molecular Plant*, *17*(1), 4–7. https://doi.org/10.1016/j.molp.2023.11.007

Spillane, C., Curtis, M. D., & Grossniklaus, U. (2004). Apomixis technology development—Virgin births in farmers' fields? *Nature Biotechnology*, *22*(6), 687–691. https://doi.org/10.1038/nbt976

Spillane, C., Steimer, A., & Grossniklaus, U. (2001). Apomixis in agriculture: The quest for clonal seeds. *Sexual Plant Reproduction*, *14*(4), 179–187. https://doi.org/10.1007/s00497-001-0117-1

Srinivasan, M., Fumasoni, M., Petela, N. J., Murray, A., & Nasmyth, K. A. (2020). Cohesion is established during DNA replication utilising chromosome associated cohesin rings as well as those loaded de novo onto nascent DNAs. *eLife*, *9*, e56611. https://doi.org/10.7554/eLife.56611

Stacey, N. J., Kuromori, T., Azumi, Y., Roberts, G., Breuer, C., Wada, T., Maxwell, A., Roberts, K., & Sugimoto-Shirasu, K. (2006). Arabidopsis SPO11-2 functions with SPO11-1 in meiotic recombination. *The Plant Journal*, *48*(2), 206–216. https://doi.org/10.1111/j.1365-313X.2006.02867.x

Su, X. B., Wang, M., Schaffner, C., Nerusheva, O. O., Clift, D., Spanos, C., Kelly, D. A., Tatham, M., Wallek, A., Wu, Y., Rappsilber, J., Jeyaprakash, A. A., Storchova, Z., Hay, R. T., & Marston, A. L. (2021). SUMOylation stabilizes sister kinetochore biorientation to allow timely anaphase. *JOURNAL OF CELL BIOLOGY*, *220*(7), e202005130. https://doi.org/10.1083/jcb.202005130

Sun, Q., Liu, F., Mo, X., Yao, B., Liu, G., Chen, S., & Ren, Y. (2023). Shugoshin Regulates Cohesin, Kinetochore-Microtubule Attachments, and Chromosomal Instability. *CYTOGENETIC AND GENOME RESEARCH*, *162*(6), 283–296. https://doi.org/10.1159/000528141

Sutani, T., Kawaguchi, T., Kanno, R., Itoh, T., & Shirahige, K. (2009). Budding yeast Wpl1(Rad61)-Pds5 complex counteracts sister chromatid cohesion-establishing reaction. *Current Biology: CB*, *19*(6), 492–497. https://doi.org/10.1016/j.cub.2009.01.062

Takahashi, N., Quimbaya, M., Schubert, V., Lammens, T., Vandepoele, K., Schubert, I., Matsui, M., Inze, D., Berx, G., & De Veylder, L. (2010). The MCM-Binding Protein ETG1 Aids Sister Chromatid Cohesion Required for Postreplicative Homologous Recombination Repair. *Plos Genetics*, *6*(1), e1000817. https://doi.org/10.1371/journal.pgen.1000817

Talbert, P. B., Bryson, T. D., & Henikoff, S. (2004). Adaptive evolution of centromere proteins in plants and animals. *Journal of Biology*, *3*(4), 18. https://doi.org/10.1186/jbiol11

Tan, E. H., Henry, I. M., Ravi, M., Bradnam, K. R., Mandakova, T., Marimuthu, M. P., Korf, I., Lysak, M. A., Comai, L., & Chan, S. W. (2015). Catastrophic chromosomal restructuring during genome elimination in plants. *eLife*, *4*, e06516. https://doi.org/10.7554/eLife.06516

Tanaka, K., Chang, H. L., Kagami, A., & Watanabe, Y. (2009). CENP-C functions as a scaffold for effectors with essential kinetochore functions in mitosis and meiosis. *Developmental Cell*, *17*(3), 334–343. https://doi.org/10.1016/j.devcel.2009.08.004

Taylor, S. J., Bel Borja, L., Soubigou, F., Houston, J., Cheerambathur, D. K., & Pelisch, F. (2023). BUB-1 and CENP-C recruit PLK-1 to control chromosome alignment and segregation during meiosis I in C. elegans oocytes. *eLife*, *12*, e84057. https://doi.org/10.7554/eLife.84057

Toth, A., Rabitsch, K. P., Galova, M., Schleiffer, A., Buonomo, S. B. C., & Nasmyth, K. (2000). Functional genomics identifies Monopolin: A kinetochore protein required for segregation of homologs during meiosis I. *Cell*, *103*(7), Article 7. https://doi.org/10.1016/S0092-8674(00)00217-8

Touati, S. A., & Wassmann, K. (2016). How oocytes try to get it right: Spindle checkpoint control in meiosis. *CHROMOSOMA*, *125*(2), 321–335. https://doi.org/10.1007/s00412-015-0536-7

Uhlmann, F. (2016). SMC complexes: From DNA to chromosomes. *Nature Reviews Molecular Cell Biology*, *17*(7), 399–412. https://doi.org/10.1038/nrm.2016.30

Uhlmann, F., Wernic, D., Poupart, M. A., Koonin, E. V., & Nasmyth, K. (2000). Cleavage of cohesin by the CD clan protease separin triggers anaphase in yeast. *Cell*, *103*(3), 375–386. https://doi.org/10.1016/s0092-8674(00)00130-6

Underwood, C. J., Vijverberg, K., Rigola, D., Okamoto, S., Oplaat, C., Camp, R. H. M. O. den, Radoeva, T., Schauer, S. E., Fierens, J., Jansen, K., Mansveld, S., Busscher, M., Xiong, W., Datema, E., Nijbroek, K., Blom, E.-J., Bicknell, R., Catanach, A., Erasmuson, S., … van Dijk, P. J. (2022). A PARTHENOGENESIS allele from apomictic dandelion can induce egg cell division without fertilization in lettuce. *Nature Genetics*, *54*(1), Article 1. https://doi.org/10.1038/s41588-021-00984-y

Ursache, R., Fujita, S., Dénervaud Tendon, V., & Geldner, N. (2021). Combined fluorescent seed selection and multiplex CRISPR/Cas9 assembly for fast generation of multiple Arabidopsis mutants. *Plant Methods*, *17*(1), 111. https://doi.org/10.1186/s13007-021-00811-9

Van Bel, M., Silvestri, F., Weitz, E. M., Kreft, L., Botzki, A., Coppens, F., & Vandepoele, K. (2022). PLAZA 5.0: Extending the scope and power of comparative and functional genomics in plants. *NUCLEIC ACIDS RESEARCH*, *50*(D1), D1468–D1474. https://doi.org/10.1093/nar/gkab1024

Van Dijk, P. J., Op den Camp, R., & Schauer, S. E. (2020). Genetic Dissection of Apomixis in Dandelions Identifies a Dominant Parthenogenesis Locus and Highlights the

Complexity of Autonomous Endosperm Formation. *Genes*, *11*(9), 961. https://doi.org/10.3390/genes11090961

van Dijk, P. J., Rigola, D., & Schauer, S. E. (2016). Plant Breeding: Surprisingly, Less Sex Is Better. *Current Biology*, *26*(3), R122–R124. https://doi.org/10.1016/j.cub.2015.12.010

Vernet, A., Meynard, D., Lian, Q., Mieulet, D., Gibert, O., Bissah, M., Rivallan, R., Autran, D., Leblanc, O., Meunier, A. C., Frouin, J., Taillebois, J., Shankle, K., Khanday, I., Mercier, R., Sundaresan, V., & Guiderdoni, E. (2022). High-frequency synthetic apomixis in hybrid rice. *Nature Communications*, *13*(1), Article 1. https://doi.org/10.1038/s41467-022-35679-3

Vijverberg, K., Milanovic-Ivanovic, S., Bakx-Schotman, T., & van Dijk, P. J. (2010). Genetic fine-mapping of DIPLOSPOROUS in Taraxacum (dandelion; Asteraceae) indicates a duplicated DIP-gene. *BMC Plant Biology*, *10*(1), 154. https://doi.org/10.1186/1471-2229-10-154

Waizenegger, I. C., Hauf, S., Meinke, A., & Peters, J. M. (2000). Two distinct pathways remove mammalian cohesin from chromosome arms in prophase and from centromeres in anaphase. *Cell*, *103*(3), 399–410. https://doi.org/10.1016/s0092-8674(00)00132-x

Wang, C., Liu, Q., Shen, Y., Hua, Y., Wang, J., Lin, J., Wu, M., Sun, T., Cheng, Z., Mercier, R., & Wang, K. (2019). Clonal seeds from hybrid rice by simultaneous genome engineering of meiosis and fertilization genes. *Nature Biotechnology*, *37*(3), 283-+. https://doi.org/10.1038/s41587-018-0003-0

Wang, Y., Fuentes, R. R., van Rengs, W. M. J., Effgen, S., Zaidan, M. W. A. M., Franzen, R., Susanto, T., Fernandes, J. B., Mercier, R., & Underwood, C. J. (2024). Harnessing clonal gametes in hybrid crops to engineer polyploid genomes. *Nature Genetics*, 1–5. https://doi.org/10.1038/s41588-024-01750-6

Ward, J. A., Peiffer, J. A., Bertier, L. D., Mccaw, M. E., Wang, X., & Berube, B. T. (2024). *Polyploid hybrid maize breeding* (United States Patent US20240150778A1). https://patents.google.com/patent/US20240150778A1

Watanabe, Y. (2004). Modifying sister chromatid cohesion for meiosis. *Journal of Cell Science*, *117*(18), Article 18. https://doi.org/10.1242/jcs.01352

Watanabe, Y., & Nurse, P. (1999). Cohesin Rec8 is required for reductional chromosome segregation at meiosis. *Nature*, *400*(6743), 461–464. https://doi.org/10.1038/22774

Weber, E., Engler, C., Gruetzner, R., Werner, S., & Marillonnet, S. (2011). A Modular Cloning System for Standardized Assembly of Multigene Constructs. *PLOS ONE*, *6*(2), e16765. https://doi.org/10.1371/journal.pone.0016765

Whitford, R., Fleury, D., Reif, J. C., Garcia, M., Okada, T., Korzun, V., & Langridge, P. (2013). Hybrid breeding in wheat: Technologies to improve hybrid wheat seed production. *Journal of Experimental Botany*, *64*(18), 5411–5428. https://doi.org/10.1093/jxb/ert333

Yao, L., Zhang, Y., Liu, C., Liu, Y., Wang, Y., Liang, D., Liu, J., Sahoo, G., & Kelliher, T. (2018). OsMATL mutation induces haploid seed formation in indica rice. *Nature Plants*, *4*(8), 530–533. https://doi.org/10.1038/s41477-018-0193-y

Ye, A. A., Cane, S., & Maresca, T. J. (2016). Chromosome biorientation produces hundreds of piconewtons at a metazoan kinetochore. *NATURE COMMUNICATIONS*, *7*, 13221. https://doi.org/10.1038/ncomms13221

Yokobayashi, S., & Watanabe, Y. (2005). The kinetochore protein Moa1 enables cohesion-mediated monopolar attachment at meiosis I. *Cell*, *123*(5), 803–817. https://doi.org/10.1016/j.cell.2005.09.013

Zamariola, L., De Storme, N., Tiang, C. L., Armstrong, S. J., Franklin, F. C. H., & Geelen, D. (2013). SGO1 but not SGO2 is required for maintenance of centromere cohesion in

Arabidopsis thaliana meiosis. *Plant Reproduction*, *26*(3), Article 3. https://doi.org/10.1007/s00497-013-0231-x

Zamariola, L., De Storme, N., Vannerum, K., Vandepoele, K., Armstrong, S. J., Franklin, F. C. H., & Geelen, D. (2014). SHUGOSHINs and PATRONUS protect meiotic centromere cohesion in Arabidopsis thaliana. *Plant Journal*, *77*(5), 782–794. https://doi.org/10.1111/tpj.12432

Zhang, X., Henriques, R., Lin, S.-S., Niu, Q.-W., & Chua, N.-H. (2006). Agrobacterium-mediated transformation of Arabidopsis thaliana using the floral dip method. *Nature Protocols*, *1*(2), 641–646. https://doi.org/10.1038/nprot.2006.97

Zhou, X., Zheng, F., Wang, C., Wu, M., Zhang, X., Wang, Q., Yao, X., Fu, C., Zhang, X., & Zang, J. (2017). Phosphorylation of CENP-C by Aurora B facilitates kinetochore attachment error correction in mitosis. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(50), E10667–E10676. https://doi.org/10.1073/pnas.1710506114