Identification and functional characterization of the meiotic cyclins SOLO DANCERS 1 (SDS1) and SOLO DANCERS 2 (SDS2) in *Zea mays* L.

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Department of Biology
Universität Hamburg

Submitted by

Óscar Sanz Mora

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Supervisor: **Prof. Dr. Arp Schnittger**

1st Examiner: **Prof. Dr. Arp Schnittger**
2nd Examiner: **Dr. Magdalena Weingartner**

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Abstract

In plants, aberrations in meiosis can lead to aneuploid pollen, a decline in fertility, and low yield. Therefore, understanding the mechanisms and regulation of meiosis is critical to plant breeding programs and, by extension, to meeting food needs. Yet, little is known about the control of meiosis in maize.

In the first part of this work, the cyclin SOLO DANCERS (SDS) was characterized in maize. From previous studies in Arabidopsis, it is known that SDS is required by the DNA MEIOTIC RECOMBINASE 1 (DMC1) to mediate double-strand break (DSB) repair. In contrast to *Arabidopsis thaliana*, SDS in rice has a significant function in DSB formation: mutation of the OsSDS gene results in failure to recruit recombination proteins, a complete absence of DSBs, and failure to install the synaptonemal complex (SC).

In my work, I have now shown that *Zea mays* contains two putative *SDS* homologs, *ZmSDS1* and *ZmSDS2*. To study their function, a double mutant was generated in the inbred line A188 using the CRISPR/Cas9 system (Clustered regularly interspaced palindromic repeats). This double mutant was both male and female sterile. Analysis of chromosomes during meiosis showed a lower frequency of synapsis and a low number of bivalent chromosomes. An SDS1-RFP reporter construct in the *sds* double mutant was used to demonstrate the localization of SDS1 in the nuclei of meiocytes in early prophase I. Here, several strong punctate fluorescence signals were observed, indicating localization on chromosomes. Immunolocalization experiments performed in the *sds* double mutant using a RADIATION SENSITIVE 51 (RAD51) antibody showed a significant reduction in the number of RAD51 signals compared with wild type (WT), indicating a decrease in DSBs.

In addition, ENHANCER OF CELL INVASION NO.10 (HEI10) protein is also reduced in the double mutant, also indicating defects in the class I meiotic recombination pathway. However, a direct interaction of SDS1 with HEI10 was not detected. The totality of the data suggests that SDS organizes chromosome formation during meiosis and acts as a local factor for CO formation in maize.

In the second part of the work, the role of SDS introns in the meiosis-specific expression of *ZmSDS1* in Arabidopsis was to be investigated. In initial

experiments, it was found that an intronless *ZmSDS1* cDNA gene driven by the *AtSDS* promoter is unable to complement the mutant. In contrast, the same construct with the genomic open reading frame (ORF) of *AtSDS* does. To investigate this, a series of *AtSDS* and *ZmSDS1* reporter constructs were generated to complement sds mutants in *Arabidopsis thaliana*. Phenotypic characterization of the *sds* mutant plants with the different constructs was performed, including examination of male anthers with meiocytes by confocal laser microscopy. My results showed that the Arabidopsis *SDS* introns are not required for meiosis-specific expression. Moreover, the genomic *ZmSDS1* gene could not be expressed under the control of the *AtSDS* promoter in *A. thaliana*, although a version in which the second intron was exchanged could partially complement the phenotype of the At*sds* mutant. Taking it all together, the *ZmSDS1* introns are not necessary to drive the meiotic-specific expression. I also prove that *ZmSDS1* is the *AtSDS* homolog.

Zusammenfassung

Bei Pflanzen können Aberrationen in der Meiose zu aneuploiden Pollen, einem Rückgang der Fruchtbarkeit und geringem Ertrag führen. Daher ist das Verständnis der Mechanismen und der Regulierung der Meiose von entscheidender Bedeutung für Pflanzenzuchtprogramme und damit auch für die Deckung des Nahrungsmittelbedarfs. Über die Steuerung der Meiose bei Mais ist jedoch nur wenig bekannt.

Im ersten Teil dieser Arbeit wurde das Cyclin SOLO DANCERS (SDS) in Mais charakterisiert. Aus früheren Studien in Arabidopsis ist bekannt, dass SDS von DNA MEIOTIC RECOMBINASE 1 (DMC1) benötigt wird, um die Reparatur von Doppelstrangbrüchen (DSB) zu vermitteln. Im Gegensatz zu *Arabidopsis thaliana* hat SDS in Reis eine wichtige Funktion bei der DSB-Bildung: Eine Mutation des OsSDS-Gens führt dazu, dass keine Rekombinationsproteine rekrutiert werden, keine DSBs entstehen und der synaptonemale Komplex (SC) nicht installiert wird.

In meiner Arbeit habe ich nun gezeigt, dass Zea mays zwei mutmaßliche SDS-Homologe enthält, ZmSDS1 und ZmSDS2. Um ihre Funktion zu untersuchen, wurde in der Inzuchtlinie A188 mit Hilfe des CRISPR/Cas9-Systems (Clustered regularly interspaced palindromic repeats) eine Doppelmutante erzeugt. Diese Doppelmutante war sowohl männlich als auch weiblich steril. Die Analyse der Chromosomen während der Meiose zeigte eine geringere Häufigkeit von Synapsen und eine niedrige Anzahl von bivalenten Chromosomen. Mit einem SDS1-RFP-Reporter-Konstrukt in der *sds*-Doppelmutante konnte die Lokalisierung von SDS1 in den Zellkernen der Meiozyten in der frühen Prophase I gezeigt werden. Hier wurden mehrere starke punktförmige Fluoreszenzsignale beobachtet, die auf eine Lokalisierung auf den Chromosomen hindeuten. Immunlokalisierungsexperimente, die in der sds-Doppelmutante unter Verwendung eines RADIATION SENSITIVE 51 (RAD51)-Antikörpers durchgeführt wurden, zeigten eine signifikante Reduktion der Anzahl der RAD51-Signale im Vergleich zum Wildtyp (WT), was auf eine Abnahme der DSBs hinweist.

Darüber hinaus ist das Protein ENHANCER OF CELL INVASION NO.10 (HEI10) in der Doppelmutante reduziert, was ebenfalls auf Defekte im meiotischen Klasse-I-Rekombinationsweg hinweist. Eine direkte Interaktion von SDS1 mit HEI10 konnte jedoch nicht nachgewiesen werden. Die Gesamtheit der Daten legt nahe, dass SDS die Chromosomenbildung während der Meiose organisiert und als lokaler Faktor für die CO-Bildung in Mais fungiert.

Im zweiten Teil der Arbeit sollte die Rolle der SDS-Introns bei der meiosespezifischen Expression von *ZmSDS1* in Arabidopsis untersucht werden. In ersten Experimenten wurde festgestellt, dass ein intronloses *ZmSDS1* cDNA-Gen, das durch den AtSDS-Promotor angetrieben wird, nicht in der Lage ist, die Mutante zu komplementieren, während das gleiche Konstrukt mit dem genomischen offenen Leserahmen (ORF) von *AtSDS* dies tut. Um dies zu untersuchen, wurde eine Reihe von AtSDS- und ZmSDS1-Reporterkonstrukten zur Komplementierung von *sds*-Mutanten in *Arabidopsis thaliana* erzeugt. Es wurde eine phänotypische Charakterisierung der *sds*-Mutanten mit den verschiedenen Konstrukten durchgeführt, einschließlich der Untersuchung der männlichen Antheren mit Meiozyten durch konfokale Lasermikroskopie. Meine Ergebnisse zeigten, dass die SDS-Introns in Arabidopsis für die Meiose-spezifische Expression nicht erforderlich sind. Außerdem konnte das genomische *ZmSDS1*-Gen in *A. thaliana* unter der Kontrolle des AtSDS-Promotors nicht exprimiert werden, obwohl eine Version, bei der das zweite Intron ausgetauscht wurde, den Phänotyp der Atsds-Mutante teilweise ergänzen konnte. Alles in allem sind die ZmSDS1-Introns nicht notwendig, um die meiotische spezifische Expression zu steuern. Die Ergebnisse meiner Arbeit zeigen, dass *ZmSDS1* das *AtSDS*-Homolog/Ortholog ist.

1. General Introduction

1.1 Meiosis

Sexual reproduction is an important feature in almost all eukaryotic organisms. It comprises several steps in which the DNA content of the parental organisms is halved during meiosis and restored to the original ploidy by fertilization. Meiosis is a highly specialized cell division that establishes a cytological foundation for eukaryotic gametogenesis and inheritance. It ensures DNA exchange between the homologous chromosomes (homologs) as well as their correct segregation. During the meiotic course, the homologs coming from different parental origins are recombined and randomly distributed, giving place to new allelic combinations, and thus, creating more genetic diversity (Ma, 2006; Cai & Xu, 2007).

The meiotic division is preceded by a G_1-phase, followed by a round of DNA replication (S-phase) and a G_2-phase. Later it continues with the two subsequent chromosome-segregation phases: Meiosis I and Meiosis II (Figure 1). The first meiotic division is a reductional phase, during which the homologous chromosomes recombine and segregate. This division is tightly controlled by a large number of proteins to prevent failures along the process (Marston & Amon, 2004). In *Arabidopsis thaliana*, prophase I lasts about 20 h out of the 26h necessary for complete meiosis, which reflects the importance of this first round of chromosome condensation and shuffling (Prusicki et al., 2019). Five substages have been defined, i.e., Leptotene, Zygotene, Pachytene, Diplotene, and Diakinesis, to describe this first part of Meiosis I. During prophase, chromatin condensates, and the homologous chromosomes synapse and recombine forming crossovers (COs), resulting in the formation of bivalent chromosomes, clearly visible in diakinesis. In Metaphase I the synapsed chromosomes align at the equatorial plate and during Anaphase I the homologs separate and are pulled out to each pole of the cell by the action of the spindle machinery. It is then during Telophase I when the chromosomes partially decondense and two well-defined nuclei can be appreciated. Between Meiosis I and Meiosis II there is a resting period called Interkinesis where the cells prepare for the second meiotic division. In Meiosis II these nuclei follow a mitosis-like division in which the sister

chromatids segregate which eventually results in four haploid nuclei that will subsequently develop into sperm or egg cells. It comprises four substages: Prophase II, Metaphase II, Anaphase II and Telophase II. In Prophase II, sister chromatids condensate, and in Metaphase II they align to the equatorial plate, and the spindle, newly formed, attaches to the kinetochores. Later, they are separated and driven to opposite poles of the cells during Anaphase II (Armstrong & Jones, 2003; Mercier et al., 2015). In the final stage Telophase II, four haploid cells will be formed and they will be released as haploid spores in cytokinesis. In Arabidopsis, cytokinesis occurs at the end of the two meiotic divisions, while in monocot plants i.e., maize and rice, it happens before the beginning of meiosis II (Otegui & Staehelin, 2000; Zhang et al., 2018).

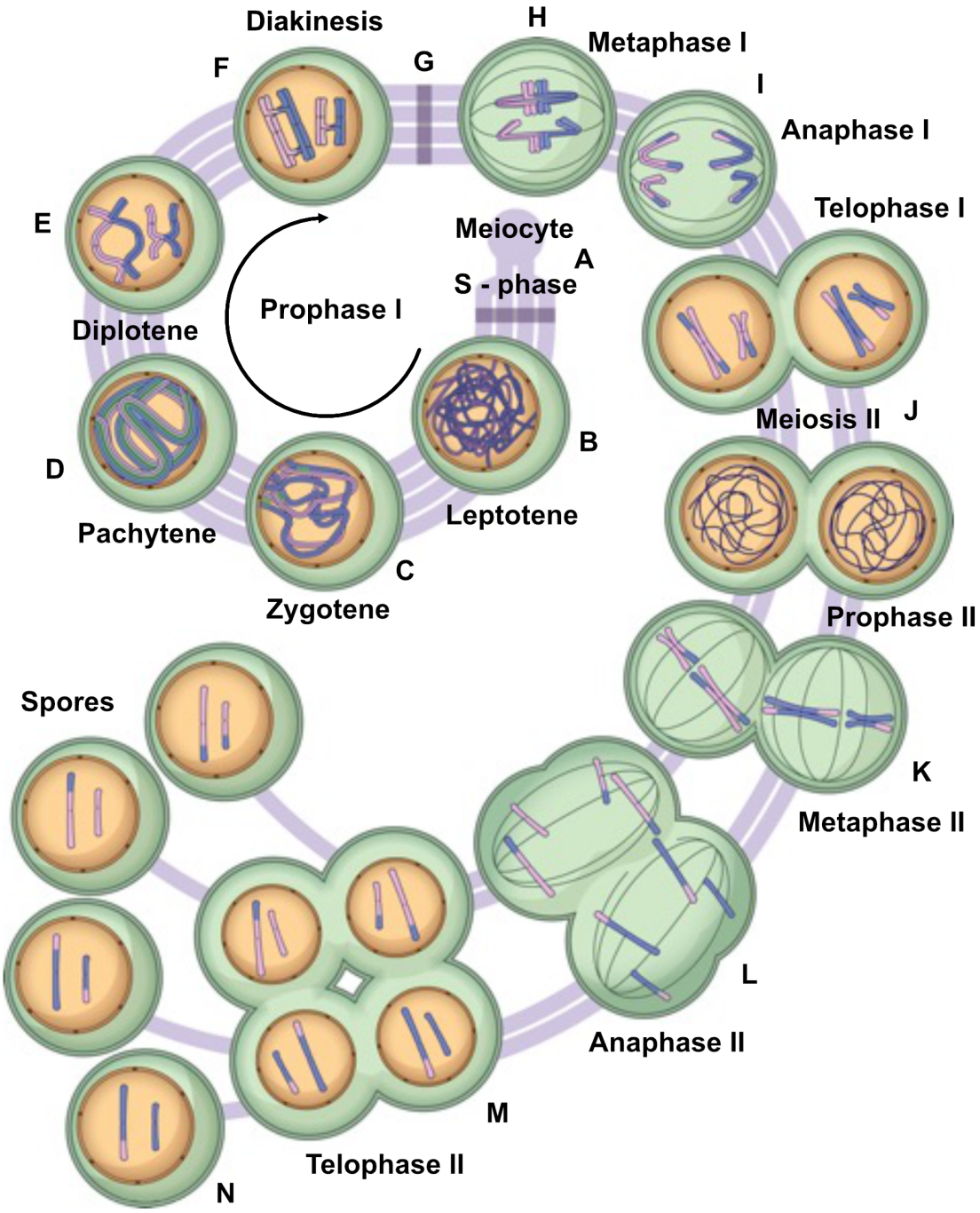

Figure 1. Representation of the Arabidopsis male meiosis. The picture was taken from Mercier et al., 2015.

(A) During the pre-meiotic S-phase, chromosomes replicate forming two identical sister chromatids.

(B) At leptotene, recombination initiates with the formation of double-strand breaks (DSBs), and the chromosome axes are assembled.

(C) At zygotene, synapsis of the homologous chromosomes occurs via synaptonemal complex (SC) assembly, and recombination progresses.

(D) At pachytene, synapsis of homologous chromosomes is complete and recombination keeps going.
(E) At diplotene, the SC disassembles and the homologous are attached to each other through the crossovers.
(F) At diakinesis, chromosomes continue condensing and bivalents can be observed linked by the chiasmata.
(G) Once prophase I is finished, the nuclear envelope breakdown takes place.
(H) At metaphase I, the bivalent chromosomes are aligned on the equatorial plate by the spindle.
(I) At anaphase I, the homologous chromosomes are split and pulled to the two opposite poles after the chromosome-arm chromatid cohesion is released.
(J) In telophase I, two nuclei are produced, and chromosomes decondense.
(K) At metaphase II, the spindle aligns the chromosomes at the equatorial plate.
(L) At anaphase II, the centromeric cohesion release allows the separation of the sister chromatids.
(M) At telophase II, four nuclei are formed.
(N) Cytokinesis is produced and four haploid spores are generated. In monocots, the first cytokinesis occurs before meiosis II separating the two nuclei produced by meiosis I.

1.2 Meiotic recombination

Homologous recombination (HR) is a defining characteristic of meiotic cellular division in the majority of sexually reproducing organisms. It allows the mixing of genetic material to generate new allelic combinations and therefore ensures genetic variability. The process of HR is initiated by the formation of DNA double-strand breaks (DSBs) which eventually may result in crossover (CO) formation, i.e. the exchange of genetic material between homologous chromosomes (Figure 2). This reshuffling of genes becomes visible as chiasmata, which are X-shaped points of attachment between two non-sister chromatids of a homologous pair (Edlinger & Schlögelhofer, 2011; Osman et al., 2011; Hunter, 2015). HR is a conserved process that also secures normal chromosome segregation since at least one CO per homologous pair is needed for the successful alignment of bivalents at the metaphase plate in metaphase I.

The SPORULATION DEFECTIVE 11 (SPO11) endonuclease, is known as the main orchestrator of the DSB formation pathway. It is the homolog of the archaeal topoisomerase VIA protein. It cleaves the DNA strand and catalyzes the process to resect it VIA topoisomerase-like activity (Keeney, 2008). In *Saccharomyces cerevisiae* (*S. cerevisiae*), Spo11 and other five additional meiosis-specific proteins are involved in DSB formation: (Meiosis specific 4 (Mei4), meiotic recombination protein 2/102/104/114 (Mer2, Rec102, Rec104, and Rec114)). A defect in any of the mentioned proteins results in a common mutant phenotype, i.e. the lack of break generation and therefore early problems in the first meiotic division (Keeney et al., 1997; Lambing et al., 2017). In Arabidopsis, three homologs of Spo11 (AtSPO11-1, AtSPO11-2, and AtSPO11-3) have been characterized, and the first two have a function during meiosis (Stacey et al., 2006). There were also found other homologs of Spo11 acting in meiosis in monocot plants like rice (OsSPO11-4) (An et al., 2011) and maize (ZmSPO11-1) (Ku et al., 2020). DSBs are processed to display 3´single-stranded DNA overhangs (ssDNAs), to which RADIATION SENSITIVE 51 (RAD51) and DNA MEIOTIC RECOMBINASE 1 (DMC1) bind. These are two recombinases needed for single-strand invasion into the homologous chromosome in the process called inter-homologous (IH) repair, which is the predominant pathway. Studies in Arabidopsis show that DMC1 is the main player in repairing meiotic DSBs and thus ensuring the inter-homolog crossing-over, while RAD51 only has a supporting role (Ines et al., 2013). In absence of DMC1, RAD51 acts in an alternative pathway using the sister chromatids as a template. Together with the homologous chromosome, the 3´ end-invading strand forms a structure called D-loop (Fig. 2). The formation of this D-loop will trigger the appearance of a great variety of joint molecules, and the processing of the joint molecule intermediates will determine the final outcome of the recombination events. Depending on the pathway by which the recombination intermediates are resolved, the process will end with either the formation of crossovers (CO) or non-crossovers (NCO). Inter-homologous recombination usually follows the ZMM pathway in which an intermediate structure called double holiday junction (dHJ) is formed and resolved to form class I COs. Several proteins have been identified to play a role in this pathway such as ENHANCER OF CELL INVASION NO.10 (HEI10), MUTL-HOMOLOGUE 1/3 (MLH1, MLH3), MUTS HOMOLOG 4/5 (MSH4,

MSH5), and the ZIP family. Joint molecules can also be processed into Class II COs. Less is known about this pathway but some players were identified, such as MMS AND UV SENSITIVE 81 (MUS81). However not all recombination intermediates lead to CO formation as synthesis-dependent strand annealing (SDSA), dHJ dissolution, and additional less defined pathways result in a non-crossover outcome (NCO). Some anti-crossover proteins involved are FIDGETIN-LIKE-1 (FIGL1), FANCONI ANEMIA COMPLEMENTATION GROUP M (FANCM), and the RTR complex composed of RECQ-LIKE HELICASE 4A (RECQ4A), TOPOISOMERASE 3A (TOP3A), and RECQ MEDIATED INSTABILITY 1 (RMI1) (Lam & Keeney, 2014; Mercier et al., 2015; Lambing et al., 2017).

One of the interesting questions to tackle regarding meiotic recombination is how DSB numbers can affect the final number of COs. In Arabidopsis, class I COs are the main type and class II COs only represent a small percentage of the final CO number. Studies performed with the hypomorphic allele *spo11-1*, which shows a reduction in the number of DSBs, resulted in fewer COs. Studies of recombination frequencies using genome-wide approaches showed that not only the CO number was altered in the hypomorphic mutants but also the distribution along the chromosomes (Xue et al., 2018).

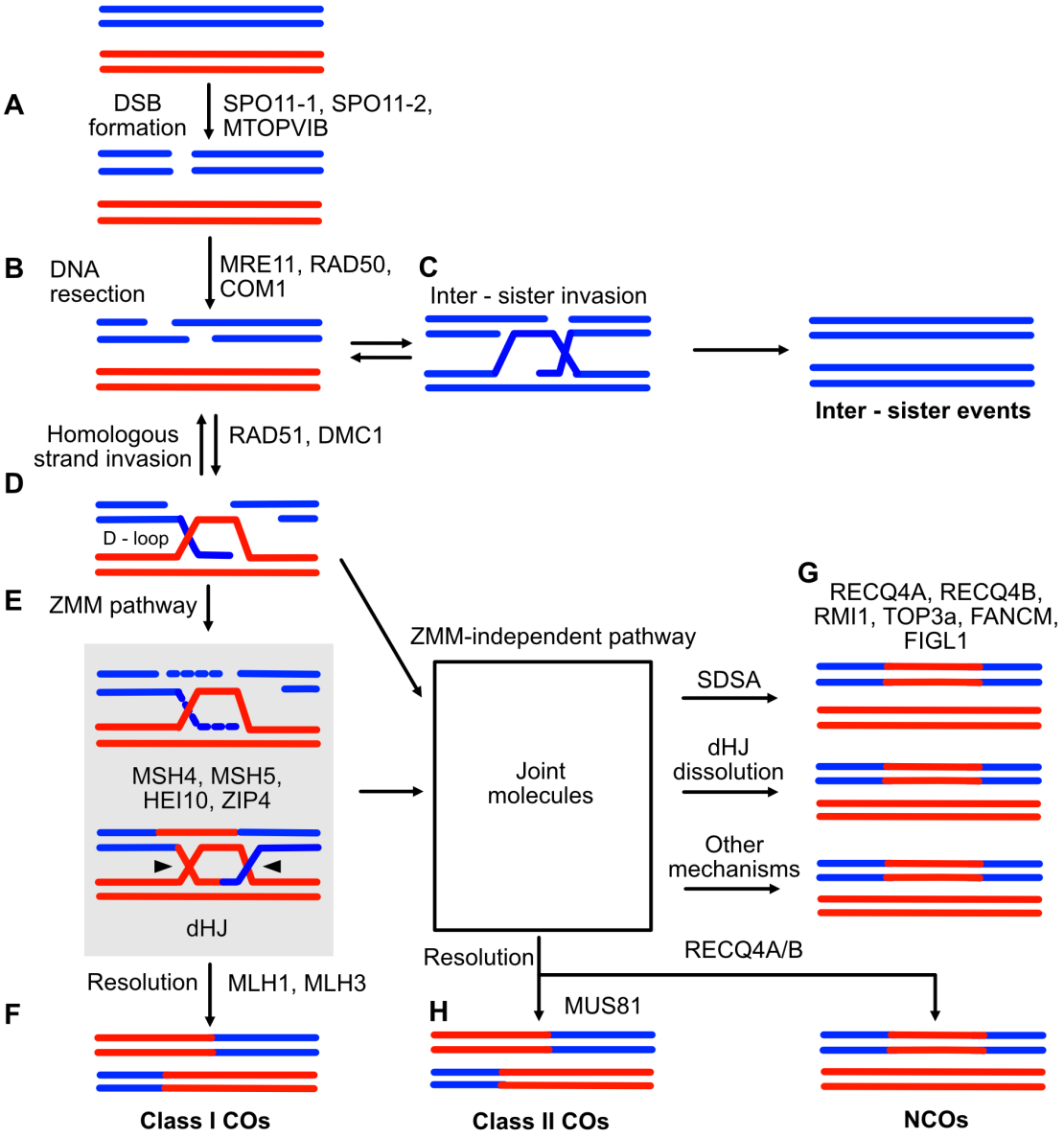

Figure 2. Representation of the meiotic recombination mechanisms.

(A) Meiotic recombination begins with the formation of double-strand breaks (DSBs) by the endonuclease SPO11.

(B) The MRN complex processes the originated break ends into 3´ single-stranded DNA overhangs

(C) The single-stranded ends can either invade the intact sister chromatid or the non-sister chromatid of the homologous chromosome (D).

(D) The homologous strand invasion is carried out by the RAD51 and DMC1 recombinases, and the D-loop is formed.

(E) The DNA intermediate products are protected and processed by the ZMM pathway components.
(F) The ZMM components process and stabilize the DNA intermediates, forming the double Holliday Junction (dHJ) and they are later resolved into class I crossovers (COs).
(G) Recombination intermediates can be alternatively processed into non-crossovers (NCOs). This can happen through different mechanisms (dHJ dissolution, SDSA, and other mechanisms).
(H) Less frequently, a ZMM-independent pathway is chosen, which produces Class II COs.

1.3 The synaptonemal complex

The alignment of homologous chromosomes (pairing) in early meiotic prophase is the most significant event. In the transition from Leptotene to Zygotene, pairing starts and when entering Zygotene, the homologs physically attach (synapsis), which is facilitated by the synaptonemal complex (SC). The SC is a proteinaceous structure evolutionarily well-conserved and is strictly found in meiosis (Fawcett, 1956; Moses, 1956). It comprises a tripartite structure that gets assembled upon synapsis of the homologs and it is composed of lateral elements (LE) (the chromosome axes) and a central element (CE), that is formed by transverse filament proteins (TF) (Figure 3). The chromosome axes (axial elements (AE)) start to be recruited during early prophase I at Leptotene, while synapsis begins at Zygotene and is completed in Pachytene around the time recombination is terminated (Zickler & Kleckner, 1999; Golubovskaya et al., 2011a). Some of the proteins that form part of the chromosome axes and the SC have been characterized in several plant species. In Arabidopsis, the protein Horma-domain proteins ASYNAPTIC 1 (ASY1) and ASYNAPTIC 3 (ASY3) have been identified as AE/LE members. Their mutations result in problems in axis formation and subsequently SC assembly and lead to the formation of univalent chromosomes. Double strand break formation and repair, as well as CO formation, and interhomolog bias are also affected. However, a recent study on the *Arabidopsis* TF proteins ZYP1a and ZYP1b revealed only a minor role of the SC in the formation of crossovers and their distribution along the chromosomes. Curiously, *zyp1a zyp1b* double mutants were analyzed and showed that class I COs

frequency was increased although there was a lack of synapsis (Mercier et al., 2015; Capilla-Pérez et al., 2021).

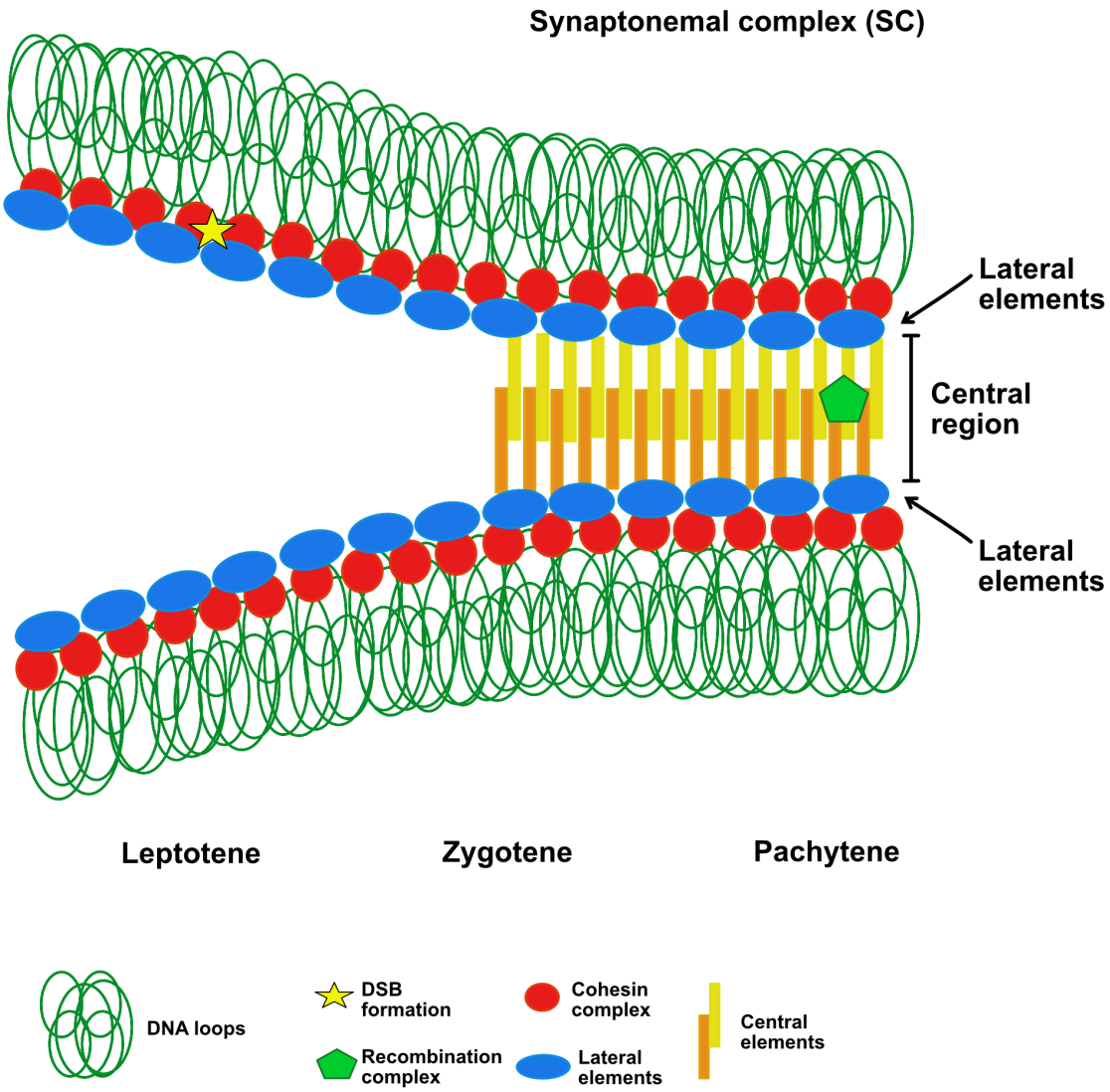

Figure 3. Schematic representation of the synaptonemal complex (SC).
In Arabidopsis, the SC forms a tripartite structure of the lateral elements (including ASY1 and ASY3) and the central elements formed by proteins from the ZYP family. The sister chromatids are held together by the cohesin complex which forms part of the chromosome axes.

1.4 The meiotic cell cycle progression is mediated by CDKs

Progression of meiosis is a very dynamic process, that requires changes in chromosome conformation and localization, and action of the spindle (among other structures), in order to correctly recombine and segregate. This relies on a specialized cell cycle machinery, including the presence of meiotic checkpoints that will control the entry into the subsequent phase. A great number of relevant players (e.g., DNA polymerases, CDK-Cyclin complexes, ubiquitin ligase complexes such as the APC/C as well as the spindle assembly checkpoint (SAC)) are the same as in mitosis. However, how these complexes and activities are reprogrammed and modified in meiosis to favor processes, as well as how meiotic progression is coordinated with recombination and chromosome distribution, are still important open questions (Pesin & Orr-Weaver, 2008; Wijnker & Schnittger, 2013).

The major regulators of the cell cycle are cyclin-dependent kinases (CDKs), which form complexes with cyclins, co-factors necessary for kinase activity and contributing to the substrate specificity (Pagliuca et al., 2011). According to a current model based on studies in other organisms as well as knowledge on the regulation of the mitotic cell cycle, the plant meiotic cycle is driven by oscillating levels of kinase activity (Figure 4). It is assumed that the CDK-Cyclin activity must surpass a certain activity threshold to enter DNA replication. For meiosis I to start, an even higher level of kinase activity is needed which is abruptly lowered at anaphase I onset likely by cyclin degradation via the APC/C. However, in contrast to mitosis, the CDK-activity does not go down to a level that permits a new round of replication but stays elevated. Entering meiosis

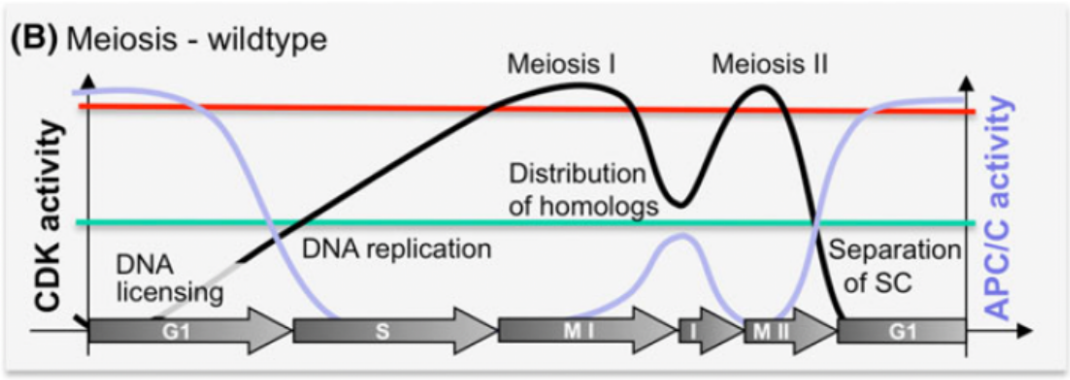

Figure 4. Meiosis undergoes different levels of CDK activity along the process.
Modified from Wjnker and Schnittger, 2013.
During the S-phase, the DNA replicates, and to achieve that, the CDK activity (represented by the black line) surpasses a certain threshold (represented by a light green line). This activity reaches the maximum level to enter both Meiosis I and Meiosis II. Between these two rounds the kinase levels drop without going under the threshold line.

II requires a second boost of activity and again APC/C activity results in cyclin degradation and the onset of anaphase II. Consistent with this model, alterations of this meiotic kinase activity in Arabidopsis have been shown to lead to a premature exit from meiosis, or even the onset of a third meiotic division (Wijnker & Schnittger, 2013).

CDKs are dependent on additional factors to regulate their activity (Figure 5). For full activation, the CDK-cyclin complex needs to be phosphorylated at the CDK's T-loop by a CDK-activating kinase (CAK) (Shimotohno et al., 2003). In addition, CDK inhibitors called ICKs or KRPs modulate CDK activity. They are in charge of negatively regulating CDKs by binding. Although the first studies of these inhibitors were mainly done in mammals and yeast, some of them were later identified in Arabidopsis and crops, such as rice and maize (Barrôco et al., 2006; De Veylder et al., 2001; Coelho et al., 2005; Cheng et al., 2015). CDKs have been organized in subgroups according to their structure and function in the cell cycle. There are eight different classes in *Arabidopsis thaliana* (CDKA to CDKG, and the CDK-like kinases or CKLs; Figure 6). Members of the CDKA and CDKB class have been shown to be directly relevant for mitotic cell-cycle progression, and for CDKA and CDKG type CDKs a direct role in meiosis has been described (De Veylder et al., 2001; Inagaki & Umeda, 2011).

In *Arabidopsis thaliana*, the CYCLIN-DEPENDENT KINASE A;1 (CDKA;1) is the principal CDK in meiosis, regulating a plethora of processes: entry in meiosis, meiosis itself, and gametophyte development. The progression from G1 to S-phase and S-phase includes two regulatory pathways: The RETINOBLASTOMA-RELATED (RBR/E2F) pathway and complexes necessary for the initiation of DNA replication. In the RBR/E2F/DP pathway, the transcription factor E2F activity is regulated by RBR, this last one, needs to be phosphorylated by CDKA;1 (Gutierrez et al., 2002). The Arabidopsis (RBR1) is a key factor to

mediate germline entry. It regulates the stem cell factor WUSCHEL (WUS), which is necessary for plant stem cell fate and also in megaspore mother cell (MMC) differentiation. Several analyses were performed with the *rbr1* mutant, CDKA;1, and several mutated versions of the CDK inhibitors KIP-RELATED PROTEINS (KRP): *krp4*, *krp6*, and *krp7*. They revealed a regulatory cascade to enter into meiosis, where KRPs downregulate CDKA;1 activity in the MMC which permits RBR1 to repress WUS allowing the differentiation of meiocytes (Zhao et al., 2017).

CDKA;1 functions also in later processes after entering meiosis. During chromosome axis formation at early prophase I, ASY1 is recruited and binds to the already installed ASY3. For this to happen a CDKA;1 dependent phosphorylation of ASY1 at a specific phosphorylation site of the HORMA domain is required (Yang et al., 2020). CO formation is one of the most important meiotic steps and CDKA;1, also plays an important role during class I CO formation. In a hypomorphic *CDKA;1* mutant with decreased CDKA;1 activity the number of COs is reduced, while in a booster of CDKA;1 activity resulted in an increase in recombination frequency (Wijnker et al., 2019).

The ordered loss of sister chromatid cohesion prevents missegregation and complex machinery has evolved to ensure the correct chromosome redistribution to the daughter nuclei. The proteins SWITCH1/DYAD (SWI1) and WINGS APART-LIKE1 (WAPL) act antagonistically and play a major role in this process. Upon CDKA;1 phosphorylation the protector of cohesion SWI1 is released from the chromosomes and degraded via 26S proteasome so that WAPL can bind to the cohesin complex and release it from the chromatin (Yang et al., 2019) resulting in the loss of arm cohesion in prophase.

Further roles of CDKA;1 in meiosis have been studied. Microtubule dynamics need also a strong regulation for the correct separation of the chromosomes. It has been shown, that activation of CDKA;1 by CAKs, such as the CDK-ACTIVATING KINASES (CDKDs) is essential for the correct microtubule organization (Sofroni et al., 2020). At last, a final function of CDKA;1 in meiosis reveals its interaction with other CDKD partners to control cytokinesis and posterior entry to meiosis II. Combinations between CDKD;1 and CDKD;3 were produced. Since the double homozygous mutant *cdkd;1/− cdkd;3/−* was gametophytic lethal, at least one heterozygous version was kept in each

combination. The final genotypic variants *cdkd;1/- cdkd;3/+*; and *cdkd;1/+ cdkd;3/-* displayed several meiotic defects, including univalent chromosomes were found, also unbalanced segregation and DNA bridges, pointing out their redundant function in crossover formation and chromosome segregation (Dissmeyer et al., 2007; Sofroni et al., 2020). These CDKDs were lately combined with a CDKA;1 mutated version with reduced activity, VF *cdka;1/-* (Dissmeyer et al., 2009). These combinations resulted in more severe meiotic defects, where meiotic progression is not complete, and very remarkable defects in the progeny ploidy (Sofroni et al., 2020).

A complete loss of function of CDKA;1 causes lethality in the male gametophyte since cell division in the generative cell is lost. When looking at the mature siliques of heterozygous plants, the seed abortion ratio resulted in approximately 50% and the segregation distortion came from the parental inheritance. Besides, pollen analyses in the heterozygous *cdka-1*/*CDKA* revealed that 48.7% of the pollen grains were bicellular and contained only one sperm-like nucleus, the other 52.3% contained tricellular grains. On the other hand, in the WT, the pollen could further develop to a tricellular stage. Al these observations indicate that in the haploid *cdka-1* pollen grains, the first pollen mitotic division (PMI) proceeds normal, but the second division (PMII) is disrupted, therefore, CDKA;1 is necessary for proper PMII cell division. (Iwakawa et al., 2006).

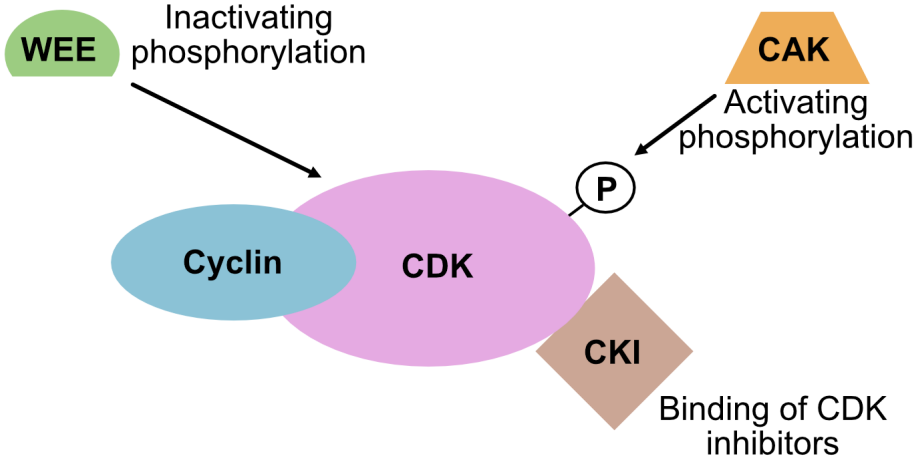

Figure 5. Representation of the CDK-cyclin complexes and their regulators. Cyclin-dependent kinases (CDK) bind distinct cyclins to form complexes and

phosphorylate substrates downstream. These complexes are at the same time highly regulated during the cell cycle, P-loop phosphorylation via WEE (under debate for CDKA;1), CAK (T-loop phosphorylation) or CKI elements (inhibitor binding).

CYCLIN-DEPENDENT KINASE G1 (CDKG1) is a another example of CDK with a role in Arabidopsis male meiosis. It was identified to function at high ambient temperature and to partner with CYCLINL forming a complex involved in chromosome synapsis and recombination. The *cdkg1-1* mutant shows incomplete synapsis and a presence of mixtures between univalents and bivalents observed in diakinesis and metaphase I, that lately missegregate into the daughter cells (Zheng et al., 2014). Genetic analyses revealed the implication of CDKG1 early in the ZMM recombination pathway downstream of the DSB formation (Nibau et al., 2020) however direct targets of CDKG1 have not been identified yet. The Arabidopsis plants in which the CDKG1 function is lost, showed normal recruitment of DMC1 and RAD51. However, the mutant also had a low number of class I CO per cell (2.5 ± 2.4), compared to the WT (9.5 ± 2.1). The class I CO mutant *msh5-2* and the double mutant *cdkg1-1 msh5-2* showed different counts in the number of bivalents, respectively 1.1 ± 0.99 and 2.5 ± 0.95, indicating that the number showed in the double mutant is due to an increase in class II crossovers. Thus, a loss of CDKG1 increases class II CO formation, and

further analyses concluded that CDKG1 is required to stabilize recombination intermediates (Nibau et al., 2020).

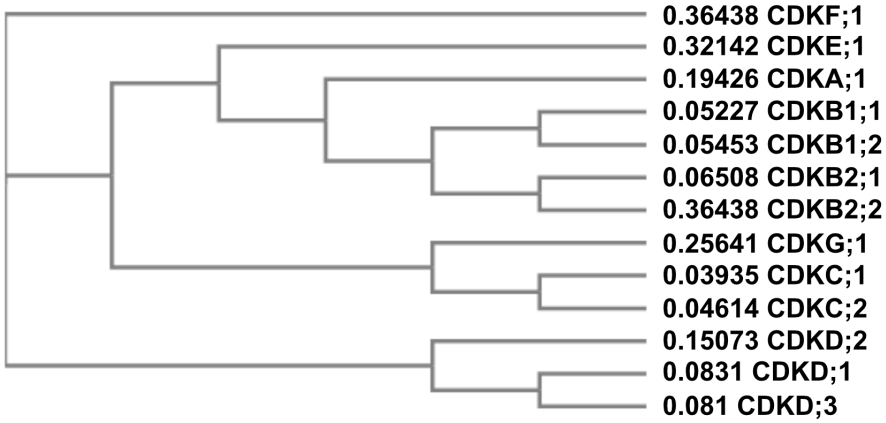

Figure 6. Phylogenetic tree showing the Arabidopsis thaliana CDKs from CDKA to CDKG.

The alignment and phylogenetic tree were generated using the Clustal Omega tool (Madeira et al., 2022), available at: https://www.ebi.ac.uk/Tools/msa/clustalo/. The tree also shows the sequence distance measurements next to the CDK name.

1.5 Meiotic cyclins

Although cyclins have an important role in CDK activation, little is known about their specific roles in Arabidopsis since only some of them have been studied in detail. In *Arabidopsis thaliana*, approximately 50 cyclins have been identified and classified into A, B, C, D, J, H, L, T, U, and SDS-type cyclins (Menges et al., 2005). Only some of them, i.e. 32 cyclins from families A, B, D, and H, have been observed to have a function related to the cell cycle regulation. The D-type cyclin family is necessary for the transition between the G1 and S-phase, while A-type cyclins have been shown to be involved in S-to-M phase progression (Renaudin et al., 1996). B-type cyclins ensure the G2-to-M phase transition and are relevant for the regulation of certain processes within the M phase (Inzé & De Veylder, 2006). Genome-wide studies also detected a large number of cyclins in crop species like rice and maize, indicating the conservation of these regulators between plants. In rice, 9 groups of cyclins were identified, 8 of them were the same as in Arabidopsis, while one of them seems monocot

specific (Hu et al., 2010), the F-type cyclins (CYCF). F-type cyclins seem to have a close relation to A and B cyclins (La et al., 2006; Guo et al., 2007; Hu et al., 2010). A total of 59 cyclins have been identified at sequence level in maize and been grouped into 6 different families (A, B, F, SDS, D, and T). As in Arabidopsis, the A- and B- type cyclins are more closely related than the other groups of cyclins. The D-type cyclins seem to be the group with more cyclins than all the detected ones (21 out of 59). It was previously found that D-type cyclins act like growth sensors for the cell to promote G1 phase initiation and/or establishment (Sherr & Roberts, 1999). Therefore, this high number of D-type cyclins detected in maize might play a role in G1 phase, and also some of them could be tissue-specific (Hu et al., 2010).

The defining structure of cyclins is a region called cyclin core, which is composed of an N- and mostly also a C-terminal five helical cyclin domain (Figure 7). The N-terminal domain contains the CDK-binding region and is therefore required for proper cyclin function while the C-terminal seems to be less conserved among cyclins. A further characteristic of cyclins that in part determines the cyclin type, is the destruction box (D-box). D-boxes can be found in some A- and B-type cyclins. A D-box is a very conserved motif (RxxL) that allows cyclin ubiquitination and subsequent proteolysis via the proteasome pathway and therefore is a key element for cyclin regulation during the cell cycle (Klotzbücher et al., 1996; Morgan, 2013; Wang et al., 2004). Another common feature observed in several cyclins is the PEST region, rich in Pro (P), Glu (E), Ser (S), and Thr (T) residues, which also is a label for rapid protein degradation (Rechsteiner & Rogers, 1996; Rogers et al., 1986). A-type cyclins are characterized by having an LVEVxEEY motif (Chaubet-Gigot, 2000) and according to sequence similarity are further divided into three groups, A1, A2, and A3. B-type cyclins are also subdivided into B1, B2, and B3 classes. In all of them, a common motif (H/Q)x(K/R/Q)(F/L) is present and helps in the identification of this cyclin family. D-type cyclins are subdivided into a broader number of groups ranging from D1 to D7. A motif with the consensus LxCX(D/E) residues can be found at the N terminus of almost all these cyclins and has been shown to be relevant for RBR1 interaction. The other cyclin families (H-, L-, T-, U-, and SDS-type) have been only detected in Arabidopsis and rice, and this possibly means that these cyclins are very low expressed or only in specific

organs. Excepting A-, B-, D-, and SDS cyclins, those other cyclins are shorter in sequence, and some of the domains cannot be easily detectable. Nonetheless, some these cyclin families also possess a common motif that can be used as an identification, for example U-type cyclins have a conserved motif sequence Y(L/A)(E/A)RI(F/A)(R/K)(Y/F), and most of the T-type cyclins contain the (L/I)(Q/R)D(L/V)G(M/I)RL motif (Vandepoele et al., 2002; Wang et al., 2004).

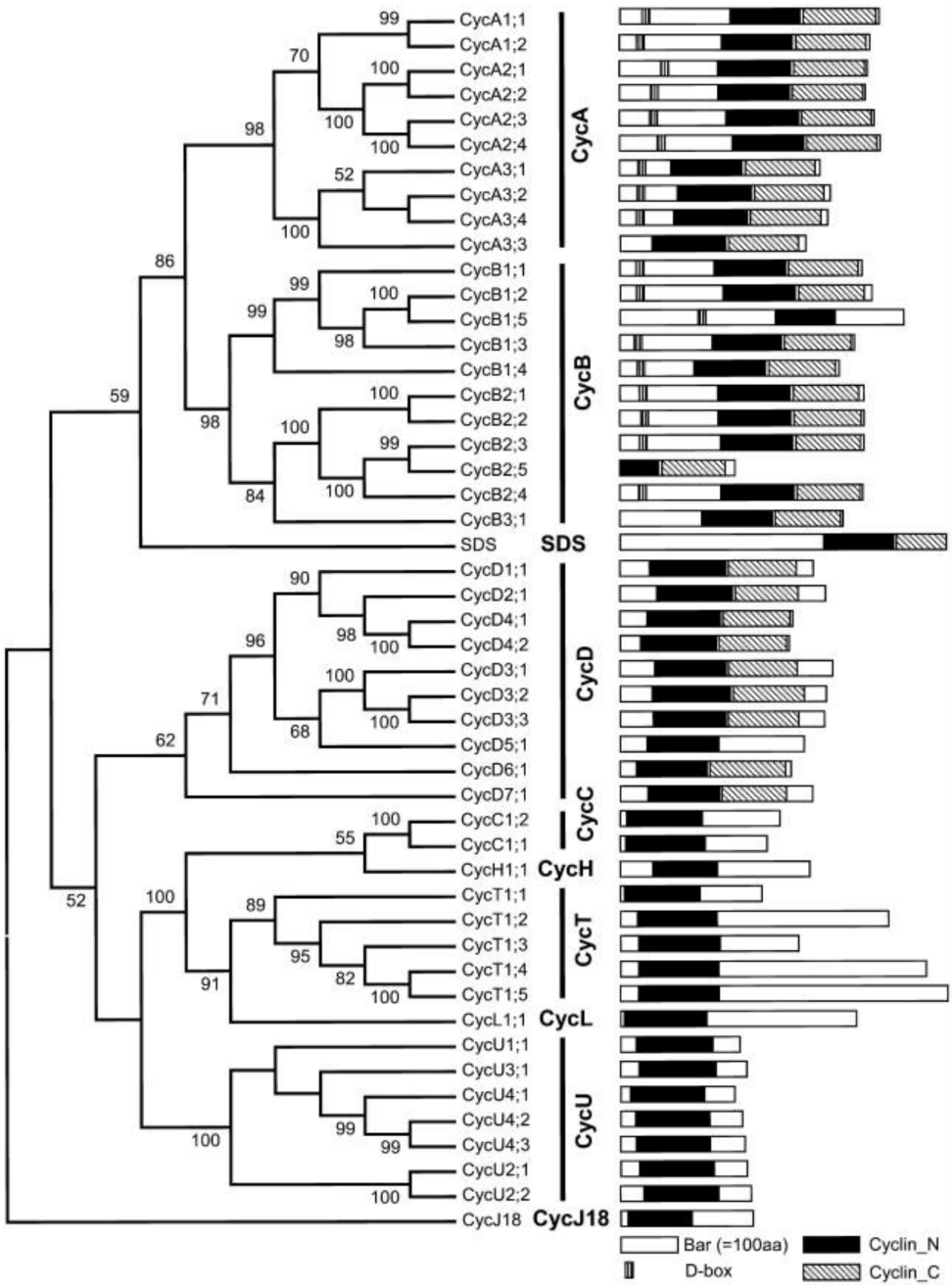

Figure 7. Phylogenetic tree showing 49 Arabidopsis cyclins showing also the domain information. From Wang et al., 2004.

The A-, B-, and D- and SDS cyclin families, share a very similar structure where all of them contain both N and C cyclin domains. Other cyclin families lack the C-terminal domain. A D-box could be identified in nine A- and B-type cyclins.

To date, only eight cyclins have been described to play a role in different meiotic processes in Arabidopsis, i.e., TAM, SDS, and CYCB3;1 as well as the A-type cyclins CYCA2;1, CYCA2;2, CYCA3;2, CYCA3;3 and CYCA3;4 (Figure 8). These A-type cyclins, with the exception of CYCA3;3, are only expressed in very early prophase I. This expression pattern is consistent with their mitotic role, where they function during the S/G2 phase. CYCB3;1, has been shown to associate with the meiotic spindle and to be important for cell wall formation as the analysis of *cycb3;1* mutants showed the presence of ectopic cell wall-like structures in pollen mother cells (PMCs) (Bulankova et al., 2013).

The A-type cyclin CYCA1;2/TAM has been extensively studied. Analyses of *tam* mutants showed failure in entering into meiosis II, generating diploid spores, which resulted in the formation of functional diploid gametes. Further analyses were performed in combination with mutants of the meiotic APC/C inhibitor OSD1 which show a similar mutant phenotype as *tam* mutants. Interestingly, the absence of both TAM and OSD1 provokes different responses in male and female meiosis. The double mutant *tam-2 osd1-1* generated and its fertility studied. The plants produced very few seeds when they were self-pollinated, indicating that it was almost sterile. The double mutant was also reciprocally crossed with WT plants showing that *tam-2 osd1-1* was femalefertile and male sterile. When the double mutant plants were pollinated with WT pollen, most of the resulting plants (99%) were triploid. On the female side, no meiosis II occurs and diploid ovules are formed. On the male side, neither meiosis I nor meiosis II takes place resulting in the production of single spores (monads). Thus it was concluded, that CYCA1;2/TAM in general ensures the transition from meiosis I to meiosis II and on the male side in addition works together with OSD1 to facilitate the correct progression of meiosis I (Cromer et al., 2012; d'Erfurth et al., 2010; Y. Wang et al., 2004).

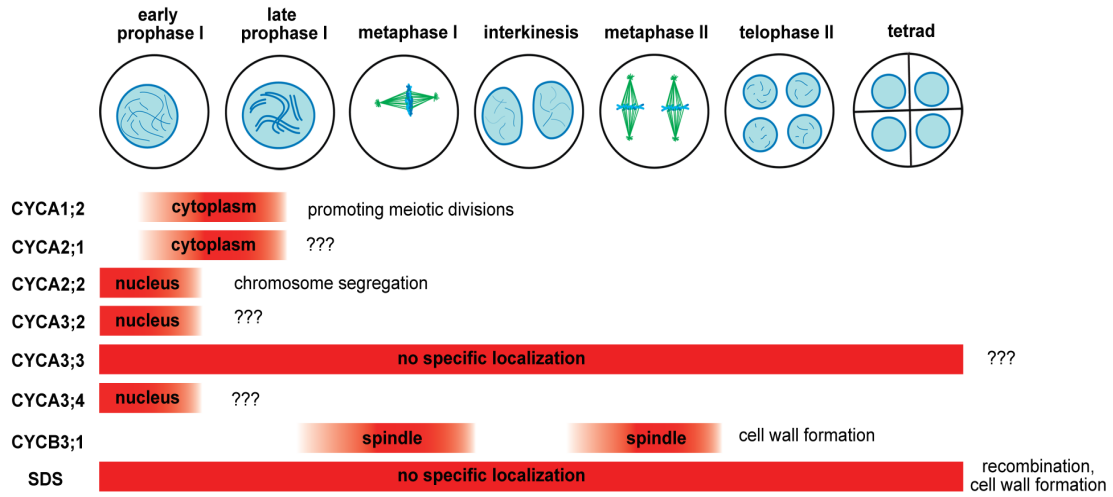

Figure 8. Overview of the Arabidopsis meiotic cyclins. From Bulankova et al., 2013. To date, a total of eight cyclins have been found to act in meiosis of Arabidopsis. Six A-type cyclins, as well as CYCB3;1 and SDS. The red rectangles represent their expression during meiosis.

1.6 The meiosis-specific cyclin SOLO DANCERS (SDS)

Among the mentioned cyclins, SDS is the only one found to be working specifically in meiosis during recombination and cell wall formation, while the others also function in mitosis (Bulankova et al., 2013).

The cyclin SOLO DANCERS (SDS) was first described in Arabidopsis and is the only known meiosis-specific cyclin. It consists of a 578 amino acid protein, and shares very high homology to A- and B-type cyclins. While D-type cyclins have the cyclin core located closer to the N-terminus, in A-, B-type cyclins as well as SDS, the cyclin core is located more to the C-terminus. The N-terminal extension in SDS is much larger than the one in A- and B-type cyclins. SDS is expressed during early prophase I and it is required for homolog synapsis, recombination, and bivalent formation. A mutant *sds* was firstly described and characterized in *Arabidopsis thaliana*. It shows normal vegetative development but has fertility problems as indicated by short siliques and a low number of seeds. Also, a lack of pollen could be observed, as well as abnormal microspore size and a different number of spores (2-8), when in a WT situation a tetrad should contain four spores (Figure 8A). Chromosome spread analysis showed meiotic defects in both male and female reproductive cells. While on the male side no visible defects could be detected during the early meiotic stages, at

diakinesis only univalent chromosomes and DNA fragments were observed, indicating a lack of proper recombination. At metaphase I, not all chromosomes aligned properly at the equatorial plate which subsequently caused chromosome missegregation. The *sds* mutants are also characterized by strong female sterility, however, chromosome spreads showed a milder meiotic mutant phenotype than the observed in the male meiocytes. Approximately 48% of the analyzed nuclei contained only univalents, while, the rest of the cells had a mixture of bivalent and univalent chromosomes. At metaphase I, there was also a lack of proper chromosome alignment on the female side, although some chromosome pairs were still observed and segregated normally. In total, 8% of all meiocytes lead to fertile spores resulting in a few seeds after pollination of an *sds* mutant with WT pollen. (Azumi et al., 2002a).

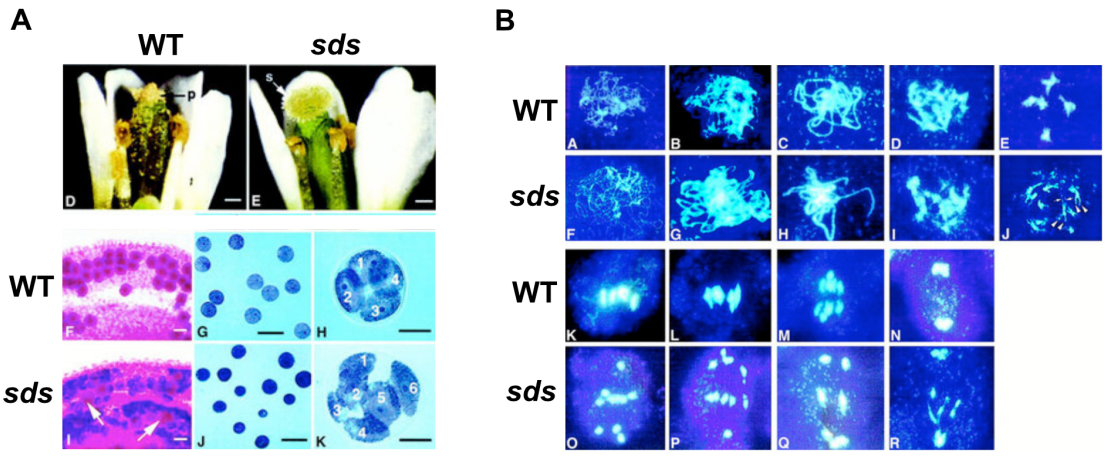

Figure 9. Images of the Arabidopsis sds mutant compared to the WT (Col-0). Modified from Azumi et al., 2002.

(A) The sds mutant shows a lack of pollen and aborted pollen grains. Pollen also differs in size compared to wildtype and instead of the four expected spores at tetrad stage, the sds mutant shows a variable number.

(B) The sds mutant displays fragmented and univalent chromosomes at the end of prophase I during diakinesis. In later meiotic stages, some chromosomes cannot align to the metaphase plate, provoking a failure in segregation that will end with the observed pollen defects.

Little is known about the CDK partners of SDS or their targets. It has been hypothesized that SDS controls the timing of sister chromatid cohesion, via direct

regulation of SWI1 (Azumi et al., 2002). According to a current model, in late prophase I, CDKA;1 together with a cyclin, possibly SDS, phosphorylates SWI1 for degradation via the 26S proteasome, allowing WAPL to bind PDS5 and release the cohesin proteins from chromatin (Yang et al., 2019).

The *sds* mutant is capable of producing DSBs, however, it ends up with univalents and chromosome fragmentation, meaning that the breaks cannot be repaired the same way as in the wildtype. RAD51 and DMC1, two proteins necessary for homologous chromosome recombination, act after DSB formation. The *Atdmc1* mutant has a phenotype similar to *sds*. Immunolocalization experiments using antibodies against DMC1 and RAD51 showed a mislocalization of DMC1 foci but not of RAD51 in *sds* mutants. Therefore, SDS is probably necessary for DSB repair via homologous recombination in a DMC1-dependent manner (Muyt et al., 2009). With respect to cross-over formation, the AAA-ATPase FIDGETIN-LIKE 1 (FIGL1) and SDS were found to have antagonistic roles. While FIGL1 limits the actions of DMC1 and RAD51 during DMC1/RAD51 inter-homolog strand invasion, SDS does the opposite, i.e. SDS promotes single-strand invasion and repair between homologs but not sister chromatids (Bouyer et al., 2018; Girard et al., 2015).

An additional meiotic function of the Arabidopsis *SDS* was found, independent of its role in meiotic recombination and homolog synapsis. SDS acts in late meiosis together with CYCB3;1 to avoid the formation of ectopic cell walls in PMCs. In both *sds* and *cycb3;1* single mutants only some PMCs displayed aberrant cell wall formation, while in the *cycb3;1 sds* double mutant, the number of meiocytes with such deformations increased up to 40%. Interestingly, SDS and CYCB3;1 both lack a D-box, and it was speculated that this might prevent their destruction via APC and therefore allow them to act post anaphase onset. This was tested by adding a D-box to the SDS N-terminal domain, which restricted its expression in meiosis to a single flower bud that corresponded to a meiotic stage, unlike the SDS without D-box, that was expressed in anthers undergoing meiosis and in postmeiotic floral buds. To sum up, all these data brought up the conclusion, that SDS has two independent meiotic functions, i.e., it acts in early meiosis to ensure chromosome pairing, and in late meiosis copes with CYCB3;1 to avoid cell wall aberrations (Bulankova et al., 2013).

A recent study in Arabidopsis tried to give hints on how gene expression to specify the fate of the male germline is regulated, for example. The genes that are activated in the male germline and how they are coordinated. Because SDS was seen to act in the late stages of anther development (Bulankova et al., 2013), it was also interesting to see whether it had a role in the male germline. As reported, SDS was weakly expressed during this specification pathway. Also, a reporter construct with the SDS promoter and CDKA1 (*ProSDS:CDKA1-GFP*) was generated, and the GFP expression was high and could be detected in sperm cells in mature pollen. Previous microarray analyses could not detect SDS in the germline due to its low expression level (Borges et al., 2011). In this recent study (Zhen et al., 2020), the construct *ProSDS:CDKA1-GFP* could enhance this expression pattern in sperm cells. This indicates a possible new function of *SDS* in the male germline. Up to now, there is a transcription factor known as an activator of male germline genes, DUO1 POLLEN1 (DUO1) (Borg et al., 2011; Peters et al., 2017), and the analyses show that SDS might act independently from this pathway. However, the molecular mechanisms by which SDS acts here are still unknown (Zhen et al., 2020).

SDS was also identified and described in rice (*Oryza sativa L.*), it shares a 30.6% of sequence identity on the C-terminal domain with AtSDS. In addition, its role in male meiosis seems different from that of its homolog in Arabidopsis. While AtSDS earliest function seems in DSB repair, OsSDS is required already before DSB formation. As the *Atsds* mutant, the *Ossds* mutant displays normal vegetative growth, but when looking at the male inflorescences only empty and shrunken pollen grains were found, indicating inviable spores. The mutant was also pollinated with WT, and no seeds were found, which meant a certain degree of sterility in the female gametes. Some chromosome spreads assays were performed in the male meiocytes of the mutant to check for meiotic alterations. No obvious defects were observed between Leptotene and Zygotene, but at the Pachytene stage, severe defects in pairing and synapsis of homologs could be detected. In fact, no full homolog synapsis was seen. Later at Diakinesis 24 univalents occurred with no sign of fragmentation. At later stages, there was evidence for chromosome misalignment and missegregation, which led to malformed Dyads and Tetrads, that produced sterile pollen. At a molecular level, the mutant SDS could not produce DSBs and while the chromosome axes were

formed, proper SC formation failed. Regarding the recombination machinery, i.e., OsMSH5, OsMER3, and OsZEP4 of the ZMM pathway were not recruited (Wu et al., 2015). A second study analyzes the expression of OsSDS creating RNAi-mediated lines. SDS was mainly expressed in flowers and also transcribed in other tissues. Since expression of the OsSDS was also highly observed in the shoot apex, it might not only function in meiosis but also in mitosis. Phylogenetic analyses demonstrated that SDS is a very conserved protein in flowering plants and its clade became different from other cyclin families. As only one *SDS* copy has been detected in Arabidopsis and rice, it was possible that these species underwent at least one, if not more genome duplications and that several additional copies of SDS were lost unlike with other cyclins (i.e., A- and B-type cyclins). Thus, maybe several copies of SDS are not beneficial and/or it has a different evolution pattern. (Chang et al., 2009). The phenotypical differences detected between *Atsds* and *Ossds* mutants likely reflect the differentiation of protein function between monocots and dicots (Wu et al., 2015).

1.7 Maize as a model organism

Maize (*Zea mays L ssp. mays*) is a plant of the family Gramineae (Poaceae) and is nowadays one of the most important crops in the world in terms of the agricultural industry and biological research (Strable & Scanlon, 2009). The origin of grasses dates back to 70-55 million years ago (Kellogg, 2001).

Maize was generally thought to be of tetraploid origin, although no indication of the presence of the two progenitor genomes could ever be found in the genomic sequence of modern maize. In a new approach, the genomic fragments from the regions surrounding five duplicated loci from the maize genome and their orthologous loci in sorghum were isolated and sequenced. Afterwards, the results were compared to the orthologous regions contained in the rice genome. The phylogenetic and distance analyses determined that maize certainly had a tetraploid origin. Furthermore, the genomes of the two maize progenitors and sorghum diverged 11.9 million years ago, and the event of tetraploidization happened 4.8 million years ago (Swigonová et al., 2004). Sequence analyses revealed that the maize genome is 2.3-gigabases in size, and approximately 85% of it is composed of transposable elements (TE), of which

one of the most studied families is the *Mutator* (Mu) family. Maize is diploid (2n = 20) and a total of 32,000 genes have been predicted. The maize inbred line B73 is the main genome reference, and a great amount of bacterial artificial chromosomes (BACs) and fosmid clones have been generated for genomic studies (Schnable et al., 2009). In addition to B73 recently a high number of additional inbred lines have been sequenced and sequence information is available at the Maize Genetics and Genomics Database (MaizeGDB; Woodhouse et al., 2021).

Maize has been an object of study in biology for the last 100 years. Cytological studies performed on this plant showed that the organization of its genome is very complex (McClintock, 1950). During maize meiosis, the association of non-homologous chromosomes was observed, despite the fact that maize is diploid (Figure 10). Also, chromosome assays in maize haploids revealed the generation of both bivalents and multivalents. All this demonstrates the presence of large regions rich in homology and most likely, due to the chromosomal duplications previously mentioned (Gaut et al., 2000; McClintock, 1950; Rhoades, 1951).

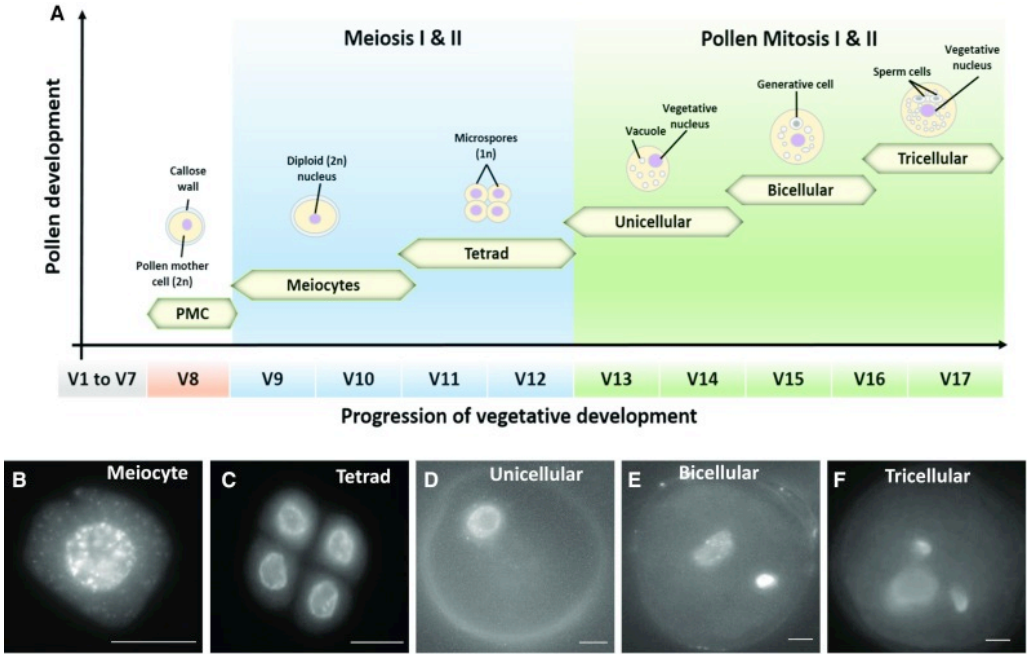

Figure 8. Representation of the stages of pollen development of the maize inbred line B73. Image from (Begcy & Dresselhaus, 2017).

(A) The pollen develops between the vegetative stages (V1-17).

(B-F) Pictures of the different pollen developmental stages: meiocyte (B), tetrad (C), unicellular (D), bicellular (E), and tricellular (F).

Maize is widely used in biology as a crop model system. It is easy to grow and pollinate under certain conditions. The male and female inflorescences are located in different areas of the plant (Figure 11). The male inflorescence (tassel) is located on top of the plant, and it is structured in spikelets that harbor the anthers containing the pollen grains. It is in the anthers therefore where the male meiocytes (microsporocytes) will develop. Each tassel will harbour hundreds of anthers, which will lead to hundreds of meiocytes which will undergo simultaneous meiotic stages. The female inflorescences (ears), contain a large number of ovules and are located in several nodes under the tassel, originating from axillary bud apices at the side of the branch. The female meiocytes (megasporocytes) are located in the ovules (Cande et al., 2009; Cheng et al., 1983).

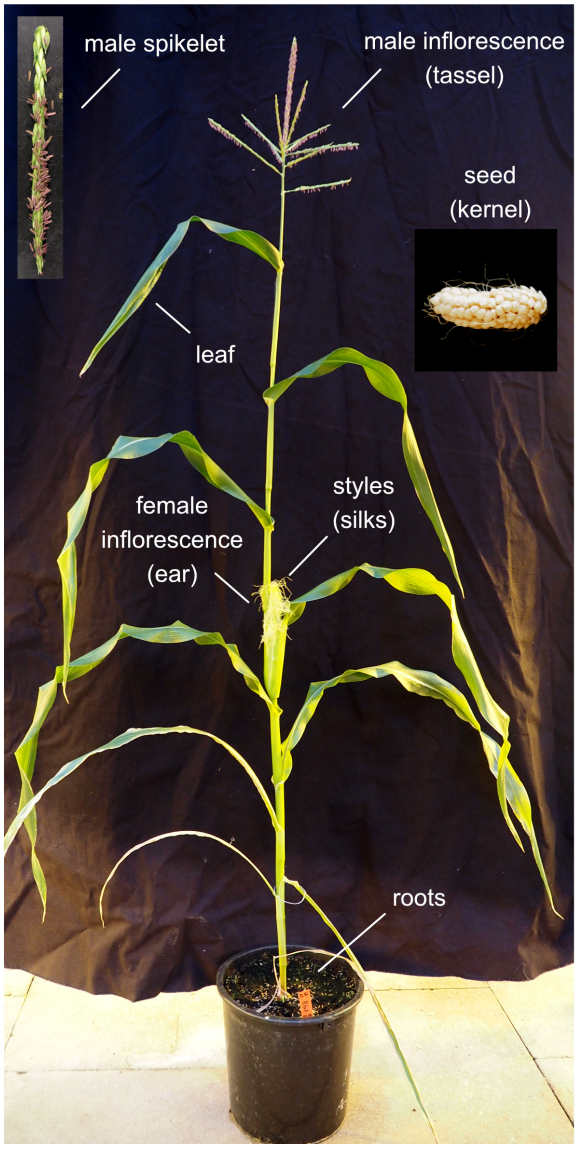

Figure 11. The architecture of a maize plant.
During the reproductive stage, the male and female inflorescences develop showing respectively the tassel and the ears. The anthers harbored in the tassel, contain the meiotic cells that undergo microsporogenesis. Megasporogenesis happens in the ovules, that are contained in the ears. After fertilization, mature cobs have several seeds.

As meiosis in maize is quite synchronized within one spikelet, and the chromosomes are large in size compared to other monocot crops (rice, sorghum, wheat, and barley), maize has become a renowned plant model to study meiosis, especially in cytogenetically (Strable & Scanlon, 2009). New techniques are

constantly developed or adapted from other plant species (like Arabidopsis) to unravel different aspects of maize meiosis. One approach is the tagging of meiotic proteins of interest with fluorescent proteins (FP) to watch their localization and dynamics, using microscopy methods, such as live-cell imaging and super-resolution microscopy. Proteomic approaches have been also used to identify proteins expressed during meiosis, and also to track posttranslational protein modifications (Phosphorylation, SUMOylation, Ubiquitinoylation, etc...). Another interesting aspect is the use of transcriptomics, using tools like microarrays and RNA-seq. One of the major questions to tackle in meiotic research is the control of meiotic homologous recombination, for which techniques like crossover mapping, i.e., by genetically linked genes expressing fluorescent proteins in pollen or seeds. Also, genotyping-by-sequencing is a technique that covers sequences of F2 populations derived from F1 hybrids and gives a genome-wide crossover distribution (Arbeithuber et al., 2015; Yelina et al., 2015). Chromatin immunoprecipitation sequencing (ChIP-seq) to determine how DSBs get repaired and follow a CO pathway, so it would be possible to manipulate the CO ratio using genes of interests (He et al., 2017). Engineering plants to generate mutants is also a very used way to identify meiotic elements. A big effort is put into new methods to enlarge the available mutant collection by reverse genetics (TILLING, insertion lines such as *Ac/Ds* transposons and the *Mutator* (*Mu*) transposable element system, CRISPR/Cas9, RNAi-based gene silencing, etc.) in order to knock down and out homologs of known meiotic genes for the functional characterization in maize (Lambing & Heckmann, 2018).

1.8 Studying meiosis in maize by forward genetics

Maize is a magnificent plant for forward genetics. With this approach, screening plants with male sterility helped to find 50 meiotic mutants, which represent approximately 35 genes. These mutants mostly affect male and female meiosis and 10% of them cause both male and female sterility. Mutants displaying defects in homologous pairing, recombination, synapsis, or in the meiotic chromatin structure, are also showing problems in bivalent formation. Taking all this into account, maize meiotic mutants could be classified according to the observed phenotype: meiotic commitment mutants, desynaptic mutants, sister chromatid

cohesion, and chromosome segregation mutants, and meiotic exit mutants (Cande et al., 2009).

Some of the meiotic genes have been thoroughly characterized. For example, in DSB formation there is *SPO11-1* (Ku et al., 2020), *SPO11-2*, and *SPO11-4*. The maize *spo11-1* mutant demonstrated that DSB and bivalent formation are defective and it also formed an aberrant AE structure. Confocal microscopy approaches showed that during recombination initiation, SPO11-1 has a very dynamic localization. Further experiments point out the relationship between SPO11-1 being loaded o AEs and that the AEs are remodeled during the beginning of recombination (Ku et al., 2020). Two maize homologs of the AtRAD51 were also described (RAD51A1 and RAD51A2). Some microscope analyses showed that the maize *rad51* mutants have reduced homologous pairing, synapsis of nonhomologous chromosomes, and reduced bivalents, as well as some chromosome breaks observed in anaphase I. This subsequently led to an approximate 33% of aneuploid daughter cells. This reveals that RAD51 is required for the correct chromosome pairing and in absence of it there non-homologous pairing and synapsis will occur (Franklin et al., 1999; Li et al., 2007).

A third RAD51 was found in maize (RAD51C). Its mutant showed complete male and female sterility, and experiments with the DNA damage compound mitomycin, revealed that homologous chromosome pairing was inhibited, and afterwards, the meiotic chromosomes were consistently entangled between diakinesis and anaphase I, as well as chromosome fragmentation. Immunolocalization experiments of *rad51c* also showed the proper localization of the axial elements absence of first division 1 (ZmAFD1/AtREC8) and ASY1 but failed to localize ZYP1. Interestingly, DSB formation was normal, as indicated by the γH2AX (sensitive molecular marker for DNA damage) signals, but DMC1 was not present in the early prophase. Taken all together, the maize *RAD51C* is necessary for both DSB repair and homologous recombination (Jing et al., 2019).

An interesting fact to point out, is that the meiotic recombination genes are highly conserved in eukaryotes, but in the case of the crop plant maize, some of these genes have high levels of sequence polymorphisms and are duplicated in the genome (*MRE11*, *RAD51A*, *SPO11*, etc...), indicating some level of adaptative evolution. Some of them were suggested to have redundant functions (Sidhu et al., 2017).

Several maize meiotic genes which their mutants lead to defective synapsis or non-homologous synapsis were also studied (i.e., *ZYP1*, *ASY1*, *AFD1*, and *desynaptic 2* (*DSY2*/*AtASY3*; Lee et al., 2015)). The results explained that *zyp1*, *asy1,* and *afd1* displayed a normal SC but there were discernible defects in pairing. Furthermore, only *dsy2* was seen to undergo ordinary pairing and the SC cannot be kept (Golubovskaya et al., 2011b).

To sum up, maize is a leading cytological model to investigate meiosis, since it has very large and well-defined chromosomes and the meiotic stages can be easily identified. Furthermore, there is a great number of genetics resources available., i.e., mutant collections.

All the current information about maize is available in the Maize Genome and Genetics Database (https://www.maizegdb.org).

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Research aim

The cell cycle progress depends on cyclin-dependent kinases (CDKs), which function in conjunction with cyclin co-factors. In plants, ten different cyclin groups have already been defined. SDS (SOLO DANCERS) is a novel meiosis-specific cyclin previously described in *Arabidopsis thaliana* (Azumi et al., 2002). It was found to have an essential role in the correct interaction between the homologous chromosomes during prophase I and ensuring the proper synapsis and recombination. However, the SDS function seems to vary between plant species, i.e., rice (OsSDS).

The primary aim of my thesis was to identify and characterize the Arabidopsis *SDS* homologs in the maize (*Zea mays L.*) model plant. The second idea was to generate a mutated version of *SDS* to study in detail its molecular function, whether it also acts in meiosis, and how much this meiotic role has varied from the ones described in both Arabidopsis and rice.

In a second approach, I wanted to tackle the question of how *SDS* is expressed in Arabidopsis and if its introns somehow regulate this expression. In a further procedure, I wanted to confirm the maize *ZmSDS1* as the *AtSDS* ortholog gene and if it could rescue the *SDS* function in the mutated version *sds* in *Arabidopsis thaliana*.

Chapter I

Functional characterization of the meiotic cyclin SDS in maize

1. Introduction

Meiosis is essential for sexual reproduction in eukaryotes since it halves the DNA content found in the parental organisms in two rounds of chromosome segregation events known as meiosis I and meiosis II. This reduction assures that the genome size of a species remains constant after fertilization. Moreover, meiosis is key to genetic diversity through the generation of newly assorted, yet complete sets of chromosomes and the exchange of DNA segments between homologous chromosomes (homologs) through cross-overs (COs). To accomplish both tasks, the reduction of DNA content and the generation of genetic diversity, meiocytes follow an elaborate program that requires tight regulation (Mercier & Grelon, 2008; Mercier et al., 2015).

Meiotic recombination starts with DNA double-strand break formation (DSBs) that is executed by the endonuclease SPO11 (Grelon et al., 2001; Jing et al., 2019; Keeney et al., 1997). Following this, the recombinases DMC1 and RAD51 mediate single-strand invasion into the homologous chromatids or the sister chromatid based on the sequences similarity (Lambing et al., 2017). The resulting DNA structures can give rise to crossovers (COs) or non-crossovers (NCOs).

In case of COs, DNA double Holliday junction (dHJ) are formed between homologous chromosomes and can be resolved as Class I COs, which are interference sensitive, i.e. they never occur close to each other. This procedure is responsible for generating the majority of the crossovers in plants. A second and less frequent pathway forms class II COs, which do not show interference (Mercier et al., 2005, 2015).

The synaptonemal complex (SC) facilitates synapsis and recombination between homologous chromosomes. It forms a tripartite structure in which the chromatids are bound to the axial elements (AE), where the proteins ASY3 and the Horma-domain ASY1 are installed. When a disruption in any of these two proteins occurs, several recombination defects, such as the formation or repair of DSBs or CO reduction. There is also a central region containing the central-element proteins ZYP1. Some studies were carried out in Arabidopsis with ZYP1 mutants, and they show that pairing occurs between homologous but they do not

manage to synapse, also CO interference is abolished. On the other hand, they also point out that ZYP1 avoids non-homologous recombination instead of limiting CO formation between homologs. Interestingly, this contrasts with what it was observed in the ZYP1 homologs from other plant species. The rice ZEP1 is thought to limit CO formation, while the barley ZYP1 promotes COs (Capilla-Pérez et al., 2021; Mercier et al., 2015).

A key regulatory force in meiosis, well known from mitosis, are cyclin-dependent kinases (CDKs) that control a plethora of substrates by phosphorylation and by that promoting the progression through the cell division program. CDKs usually require a cyclin partner for their activity and correct localization. In contrast to animals and yeast, plants such as the reference plant Arabidopsis thaliana, have a large number of CDKs and cyclins. However, their specific function is often not clear, especially not in meiosis.

Out of the eight classes of CDKs found in Arabidopsis, so far only CDKA;1 and CDKG1 have been implicated in meiosis with CDKA;1 being the major regulator involved in several aspects of meiosis. Initially, CDKA;1 activity needs to be low to allow the differentiation of meiocytes mediated by the plant Retinoblastoma homolog RBR1 (Zhao et al. 2017). Upon entry into meiosis, CDKA;1 is required for several aspects of meiosis including the formation of the chromosome axis (Yang et al., 2020), number and positioning of COs (Wijnker et al., 2019), regulation of sister chromatid cohesion (Yang et al., 2019), the regulation of the microtubule cytoskeleton (Sofroni et al., 2020), and the entry into meiosis 2 (Dissmeyer et al., 2007; Sofroni et al., 2020).

CDKG1 is in charge of stabilizing early prophase I, of several elements of synapsis and CO formation. Studies with the *cdkg1-1*, *msh5-2,* and *fancm-1* mutants were performed. The *cdkg1;1* single mutant was highly affected in synapsis. However, in the *cdkg1-1fancm-1* double mutant, this synapsis was restored and subsequently, the CO elements went back to wild type levels, leading to the regular numbers in class I CO formation. In absence of CDKG;1 or MSH5-2 the class I COs are reduced, but in the *cdkg;1msh5-2* double mutant the number of bivalent chromosomes is increased compared to the wild type. This suggested that a lack of CDKG;1 implies a raise of class II COs. To sum up,

CDKG;1 acts downstream the DSBs and controls the proteins involved in the progression of the ZMM pathway (Nibau et al., 2020).

In contrast, much less is known about the cyclin partners of CDKA;1 and CDKG;1. Out of the more than 30 cyclins present in the Arabidopsis genome, so far seven cyclins have been found to be required for different aspects of meiosis in Arabidopsis TAM, SDS, and CYCB3;1 as well as the four redundantly acting A2-type cyclins CYCA2;1, CYCA2;2, CYCA2;3 and CYCA2;4 (d'Erfurth et al., 2010; Bulankova et al., 2013). Among these, only SDS has been found to be meiosis-specific while the other genes also act in mitosis.

The SOLO DANCERS (SDS), acts in prophase I and is critical for the correct homolog synapsis, recombination, and bivalent formation. This protein is very similar to the A and B type cyclins and shares with them 31% of amino acid sequence identity in the C-terminal half. Although some of the meiotic pathways where SDS is involved have been described, the molecular mechanisms and its targets are still unknown. One hypothesis is that SDS might regulate the timing of sister chromatid separation, via controlling SWI1 actions (Azumi et al., 2002; Yang et al., 2019). A closer view into the recombination machinery shows how SDS is necessary for the formation of DMC1 foci and counteracts the actions of FIGL1 and FLIP has been also identified as a new negative regulator factor that reduces CO formation, via controlling the dynamics of DMC1 and RAD51 during strand invasion (Girard et al., 2015; Bouyer et al., 2018).

A second study determined that CYCB3;1 plays a role together with SDS to control cell wall formation (Bulankova et al., 2013). SDS is also expressed in the male germline, indicating a putative function specific to this pathway, although the molecular mechanisms remain unknown (Zhen et al., 2020).

SDS was also characterized in rice (*Oryza sativa*) and showed some differences in meiosis with the Arabidopsis one. While in the *Atsds* mutant it was observed some degree of female fertility, the *Ossds* was both male and female sterile. Regarding protein function, At*sds* displayed chromosome fragmentation, which suggests that it has a role in DSB repair. On the other hand, the OsSDS seemed to be involved in the DSB formation but its specific mechanism is still unknown. These different observations of SDS between Arabidopsis and rice suggest an evolutionary distinction in their role in meiosis (Wu et al., 2015a).

The knowledge of molecular control of meiosis and especially meiotic recombination in crops, more concretely in maize is still limited, despite of the great potential for application in plant breeding. Here, I have characterized the SDS function in maize that is represented, as we show, by two redundantly acting genes. My results show, that both SDS are necessary for CO formation, proper synapsis, and bivalent formation

2. Results

2.2 Identification of two maize *SDS* homologous genes

Two maize genes, named in the following *SDS1* (Zm00001d048026) and *SDS2* (Zm00001d028274), were found by BLAST searches to be homologous to SDS from Arabidopsis (*AtSDS*) and rice (*OsSDS*, Supplemental Figure 1). Both predicted maize SDS proteins share a high degree of similarity (85%) with each other including a highly conserved cyclin domain at the C-terminus. With OsSDS (469aa), ZmSDS1 (455 aa) and ZmSDS2 (456 aa) share 69% and 71% identity, respectively. With Arabidopsis SDS (578 aa), ZmSDS1 is 45% and ZmSDS2 40% identical.

Zm*SDS1* (located on chromosome 9 and 3682 bp long) and Zm*SDS2* (located on chromosome 1 with a length of 4743 bp) have a similar gene structure as At*SDS*, with seven exons and 6 introns of which the second and third intron is the largest, this second intron contains a 290 bp transposable element (GRMZM5G802038) inside its sequence. Interestingly, the analysis of the *SDS* genes in various inbred lines of maize showed that *SDS1* in line A188 contains an insertion of 3188 bp in the first exon, 351 bp after the ATG (Figure 1A). Because of the formation of a stop codon, this insertion is predicted to result in a severely truncated open reading frame of only 390 bp with a predicted protein length of 130 aa. However, A188 plants are fully fertile and do not show any obvious meiotic defects suggesting that either SDS2 is of key importance in maize or that SDS1 and SDS2 are highly redundant.

To first address this point, the expression of SDS1 and SDS2 in meiosis was analyzed in A188 and B73 inbred lines (data not shown). In B73, the longest

SDS1 transcript seems to be highly expressed from pre-meiotic stages until it disappears after late meiosis. Of the four SDS1 transcripts identified in B73, is the longest one thought to be the active one since it codifies to the annotated functional protein. In contrast, a full-length transcript of *SDS1* in A188 could not be found likely due to the large insertion in exon 1. However, several short splice variants could be detected indicating that the promoter of *SDS1* is still functional. In contrast, while *SDS2* was found to be only poorly expressed in B73, we observed that *SDS2* in A188 is strongly active consistent with it being the only functional *SDS* gene in this inbred line.

As it seems in the A188 and B73 inbred lines, when one SDS is missing, the other takes over its function showing some level of redundancy. To study this further, a cross between these two inbred lines was made and studied at a transcriptional level. When both maize lines were crossed (A188 x B73) both *SDS* genes were expressed. These expression analyses were performed by Dr. Reinhold Brettschneider (University of Hamburg).

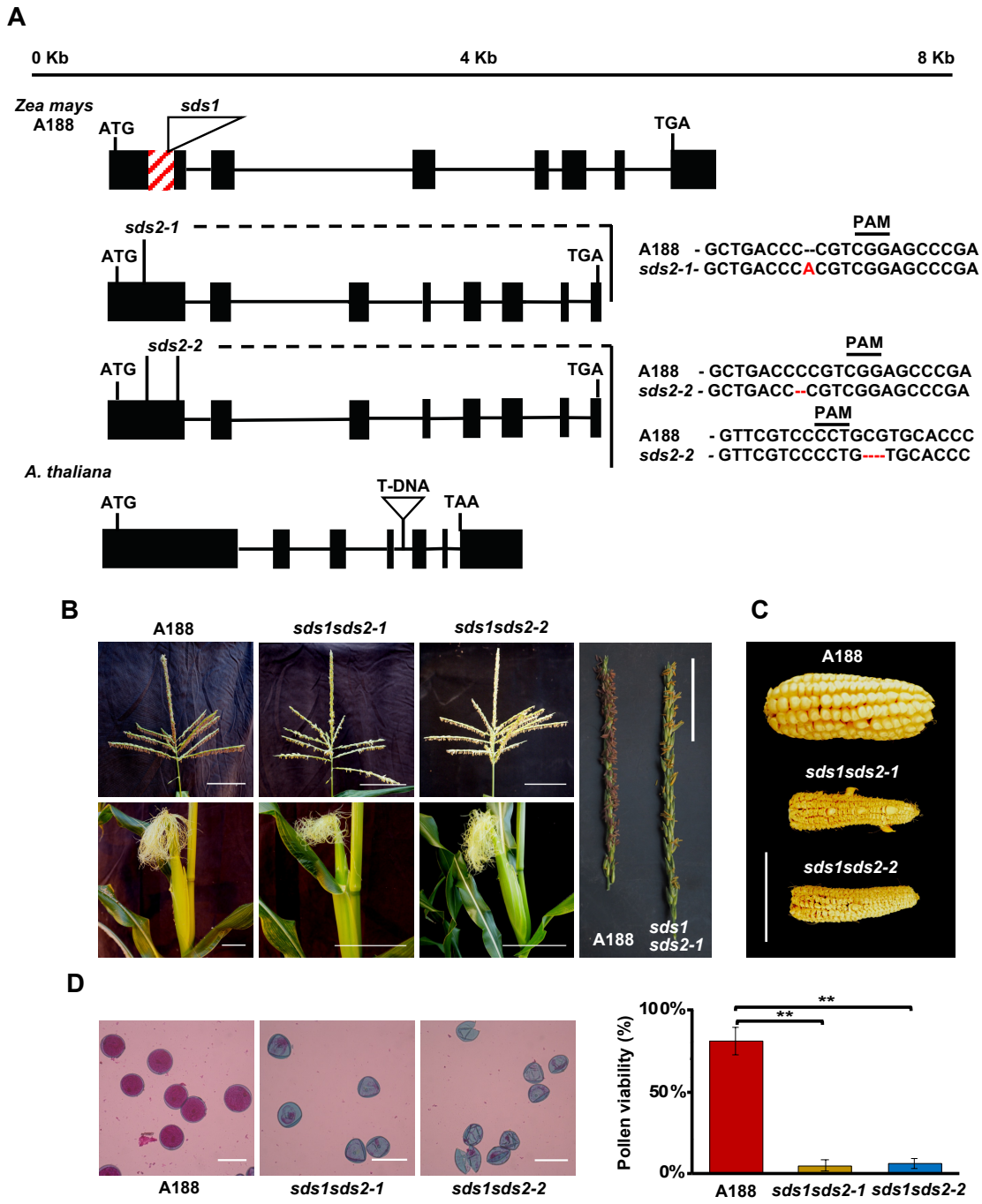

Figure 9. Characterization and mutation of the maize *SDS* genes.

(A) Gene model of *SDS57* and the double mutant alleles of SDS57 SDS16 generated by CRISPR/Cas9 in the inbred line A188. Red gridlines represent the 3.1 Kb insertion contained in *SDS57,* present in nature in the A188 inbred line. The red letters and dashes show the different mutations generated by CRISPR/Cas9 in *sds1 sds2-1* and *sds1 sds1-2* mutant alleles.

(B) The *sds1 sds2-1* and *sds1 sds2-2* double mutants show defects in male and female inflorescences. Bars = 10 cm.

(C) The *sds1 sds2-1* double mutant can generate some seeds when it is self-pollinated. Bars = 5 cm.

(D) Comparison of a tassel of an A188 plant with the sds1sds2-1 double mutant, showing the second yellow anthers due to the aborted pollen. Bars = 5 cm.

(E) Quantification of the pollen viability of A188 and the two double mutants. The asterisks indicate a significant difference (one-way ANOVA, followed by Tukey's test, $p < 0.05$). Error bars represent ± SD.

2.3 The *sds* double mutants are female and male sterile

To explore the function of *SDS2*, we mutated this gene by CRISPR/Cas9 in A188 and created a plant with presumably no SDS activity (Figure 1A). Four different CRISPR/cas9 targets were designed and two of them, both in the first exon, gave rise to mutations. The first mutation (mutant allele named *sds2-1*) causes an A insertion 427 bp after the ATG, which leads to a shift of the original Open Reading Frame (ORF), resulting in a predicted truncation of the original protein (146 aa). The second mutant allele *sds2-2* contains a C deletion at position 427 bp plus a CG deletion at 589 bp and is predicted to produce a shortened and inactive protein of also 146 aa.

The offspring of both heterozygous *sds2* mutants in the *sds1* mutant background was grown and phenotypically analyzed. All the double homozygous mutant plants exhibited normal vegetative growth and there was no difference observed compared to A188 matching rice and Arabidopsis *sds* mutant plants (Supplemental Figure 2A). A segregation analysis for both mutant alleles was carried out, to determine if it follows Mendelian segregation regarding its fertile/infertile phenotype. 33 plants were studied for the *sds1sds2-1* segregating population and 11 of them were double homozygous mutants ($\chi 2 = 1.22; P > 0.05$). Regarding the *sds1 sds2-2*, a total of 65 plants were taken into consideration for the segregation analysis. In this case, 13 were double mutants ($\chi 2 = 0.86; P > 0.05$). Taken together, these results suggest that *sds2-1* and *sds2-2* are recessive and indicate that *SDS2* is not needed for gametophyte and/or embryo development (Supplemental Figure 2B).

In contrast to the unperturbed sporophytic growth, both double mutants were almost fully sterile. While the A188 builds maroon-colored anthers, which indicates a mature pollen content, the tassels of the double-mutant plants

displayed yellow anthers, often reduced in number, which is an indication of anther abortion (Figure 1 B-C). Consistently, a strong reduction of the viable pollen was observed in *sds1 sds2-1* and *sds1 sds2-2* with 5% and 6%, respectively, while A188 has 81% of pollen viability (Figure 1D). In addition, many of the viable pollen grains were different in size in comparison with pollen from A188 and hence, are likely aneuploid since the size of pollen usually correlates very well with its DNA content.

In the case of the female inflorescence, the ears of *sds1 sds2-1* and *sds1 sds2-2* grew normal silks but had a high number of aborted seeds on the cobs (Figure 1B-C). When the mutant plants were self-pollinated, some of the mutants were capable of producing a few viable seeds. However, these seeds had different sizes, again indicating ploidy defects.

2.4 Both *sds* double mutants have defects in male meiosis

The unequal pollen sizes of *sds1 sds2* double mutants suggested missegregation of chromosomes in meiosis consistent with meiotic defects reported for mutants in *SDS* in Arabidopsis and rice (Azumi et al., 2002; Wu et al., 2015b). To explore a possible role of SDS in maize meiosis, we performed chromosome spreads of male meiocytes in the inbred line A188 (Figures 2A-H) and the *sds1 sds2-1* (Figures 2I-P) double mutant. In A188, chromosomes condensate in leptotene and become visible as a thin thread-like structure (Figure 2A). Next, chromosomes become paired and continue to condense, indicative for zygotene (Figure 2B). The appearance of thick, thread-like chromosomes defines pachytene (Figure 2C). During diakinesis, 10 distinct bivalent chromosomes become visible (Figure 2D). During metaphase I, homologous chromosomes align at the equatorial plate and often display a diamond-like shape (Figure 2E). Chromosomes are then pulled apart and have an elongated shape at anaphase I (Figure 2F). At the end of prophase I, homologous chromosomes have been separated to opposite poles of the meiocytes resulting in a dyad configuration (Figure 2G). Meiosis II follows with the separation of sister chromatids resulting in the formation of tetrads (Figure 2H).

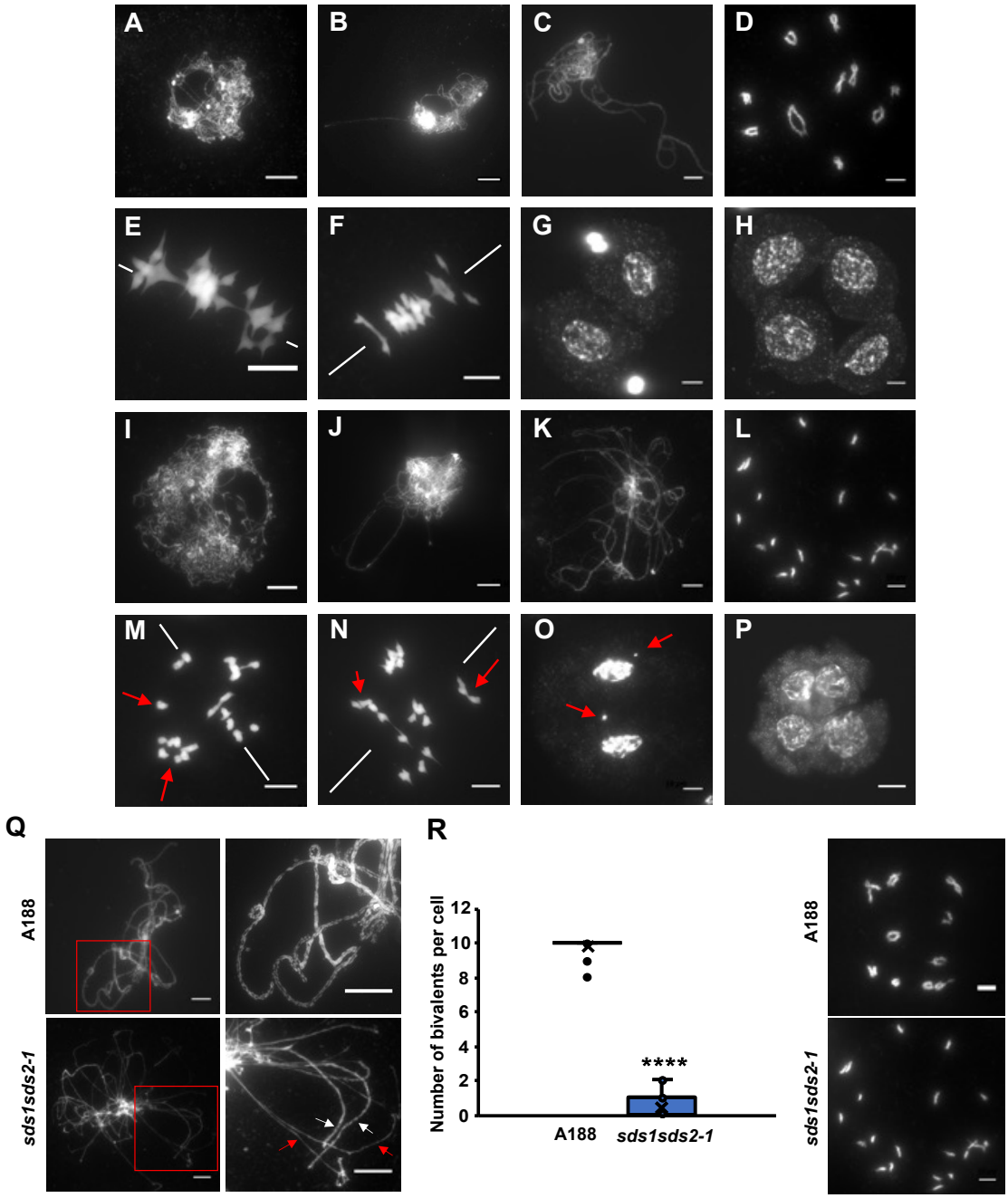

Figure 2. Male meiosis in A188 and *sds1 sds2-1*. The *sds1 sds2-1* double mutant is defective in synapsis and crossover formation.

(A-H) Meiosis in A188. (A) Leptotene, (B) Zygotene, (C) Pachytene, (D) Diakinesis, (E) Metaphase I, (F) Anaphase I, (G) Dyad, (H) Tetrad.

(I-P) Meiosis in *sds1 sds2-1*. (I) Leptotene, (J) Zygotene, (K) Pachytene, (L) Diakinesis, (M) Metaphase I, (N) Anaphase I, (O) Dyads, (P) Tetrads. The white lines represent the orientation on the equatorial plate. The red arrows show micronuclei, laggards, and multivalents. Bars = 10μm.

(Q) Comparison between A188 and the double mutant at pachytene stage. The double mutant shows some synapsed regions (white arrows) as well as some desynapsed (red arrows). Bars = 10 μm.
(R) Quantification of bivalents in A188 and *sds1 sds2-1*. The *sds* double mutant presents a significantly reduced number of bivalents. The red arrow indicates the formed multivalents. Bars = 10 μm.

The double mutant *sds1 sds2-1* showed several deviations in meiosis from A188 after zygotene. In pachytene-like stages, chromosomes were not paired and remained visible as thin thread-like structures (Figures 2I-P). The mutant phenotype showed univalent at diakinesis stage, but it was also very frequent to see some bivalents or multivalents (showed by a red arrow). A quantification of the number of bivalents per cell was performed (Figure 2R). The counting was best on cells during diakinesis stage. For A188 a total number of 78 cells were counted, and as expected an average of 10 ± 0.4 bivalents per cell could be observed. Interestingly in the *sds1 sds2-1* (n=56), the number of bivalents was significantly reduced (two-tailed t-test, $P < 0.05$) but not completely, showing 0.4 ± 0.7 bivalents, reaching up to 2 bivalents in some cells. All these observations from the double mutant *sds1 sds2-1* brought together, confirmed the aborted phenotype observed in the tassels and pollen grains. In metaphase I and anaphase I, laggards can be observed misaligned out of the axis of the spindle and chromosome bridges (Figure 2M, N). At the dyad stage, several micronuclei were found underlining the segregation defects observed in anaphase I (Figure 2O). Correspondingly, tetrads with different-sized nuclei are found at the end of meiosis II (Figure 2P).

A closer look into the chromosomes highlighted more differences in between A188 and the *sds1 sds2-1* double mutant (Figure 2Q). The zoomed image of A188 shows the synapsed chromosomes. On the other hand, the *sds1 sds2-1* double mutant presents fewer threads with the described structure (showed with the white arrows), whereas the unsynapsed regions are very well defined showing individual threads (pointed with the red arrows).

The second double mutant *sds1 sds2-2* was also analyzed and showed the same phenotypical defects as in *sds1 sds2-1* (Supplemental Figure 3G-M).

2.5 Expression of a genomic ZmSDS1 construct largely restores the mutant phenotype of the *sds1sds2-1* double mutant

Since A188 is naturally mutated for *SDS1*, we could not determine up to this point what the role of *SDS1* is in the absence of *SDS2*. Therefore, a genomic *SDS1* reporter construct was generated based on the *SDS1* sequence of the B73 inbred line. In this reporter line, the ORF for RFP was introduced before the stop codon at the C-terminus to allow the localization of SDS1 *in vivo*. This construct was then transformed in *sds1 sds2-2* mutants (Figure 3A).

The resulting *sds1 sds2-2* mutants containing the *SDS1:RFP* reporter were then analyzed and did not show any somatic alterations from A188 plants indicating that this construct did not have a dominant negative effect on plant growth. Importantly, complemented homozygous mutant plants had fertile anthers and showed a similar viable pollen rate (65%) as A188 (76%), and clearly different from the *sds1 sds2-1* and the *sds1 sdds2-2* double mutant plants, which both only produce approximately 6% of viable pollen (Figure 3D). Similarly, the *sds1 sds2-2* plants containing the *SDS1:RFP* genomic construct reached the same seed set as A188 (Figure 3C).

The chromosome spreads pictures taken from the double mutant with the transgene, partially complements the mutant phenotype (Figure 5B; Supplemental Figure 4). At pachytene stage, the chromosomes looked fully synapsed and no evidence of other cells with desynaptic problems was found. Taking a look into the diakinesis stage, 71 cells where analyzed and most of them 10 bivalents were appreciated, however in some cases there were also cases of cells with univalents or outnumbered sets of chromosomes resulting in a total count of 9,2 ± 0,9 bivalents. During metaphase I, almost in all the cells (n=13), the chromosomes are correctly localizing on the equatorial plate, but still, 1,9 ± 1,6 laggards are visible.

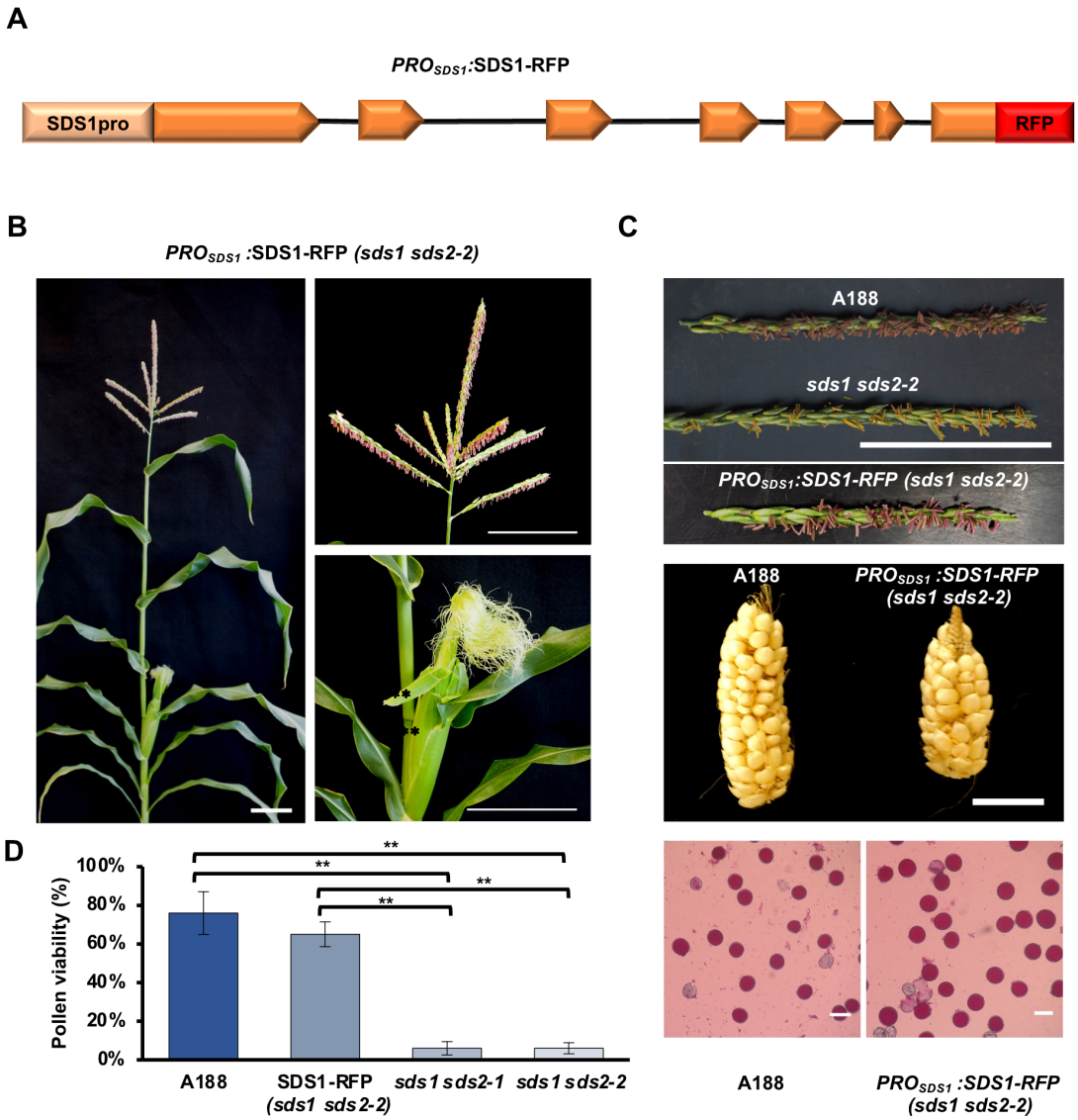

Figure 3. Characterization of the SDS1-RFP in *sds1 sds2-2* background.

(A) Representation of the SDS1 reporter construct (*PRO$_{SDS1}$:SDS1-RFP*).

(B) Phenotype of the SDS1-RFP in *sds1 sds2-2*. The images show a whole plant displaying A188 vegetative growth, as well as fertile male inflorescences and normal silks. Bars = 10 cm.

(C) Comparison of the SDS1-RFP in *sds1 sds2-2* tassels with A188 and *sds1 sds2-2*. The SDS1-RFP in *sds1 sds2-2* generates also a great number of seeds. Bars = 5 cm.

(D) The SDS1-RFP in *sds1 sds2-2* shows viable pollen grains. The bar graph compares the percentage of pollen viability of A188, SDS1-RFP in *sds1 sds2-2*, *sds1 sds2-1*, and *sds1 sds2-2*. The asterisks represent significance ($P < 0,05$) determined by the ANOVA test, followed by Tukey´s test. Bars = 50 µm.

2.6 Loss of SDS function results in a reduced number of DSBs

So far, SDS function has only been studied in rice and Arabidopsis leading to the conclusion that its role is apparently different in these two species (Azumi et al., 2002; Muyt et al., 2009; Wu et al., 2015b). While SDS was found to be required for DSBs in rice, this was not the case in Arabidopsis. Furthermore, SDS was shown to be needed for DMC1 loading in Arabidopsis, a function that could not be assessed in rice due to the absence of DSBs. Hence, a key question at the beginning of this study was what the general pattern of SDS function is and whether maize *sds* mutants would resemble rice or Arabidopsis.

To address this point, we performed immunolocalization experiments in A188 and both in *sds* double mutants using an antibody against RAD51 (Figure 6A). To faithfully quantify RAD51 foci numbers, we developed an application that automatized the analysis of consecutive z-stacks spanning the entire nucleus (see Material and Methods section, Supplemental Figure 8). This revealed that A188 had on average 201 ± 28.6 (n = 39) RAD51 foci consistent with previous reports in A344 inbred line (Supplemental Figure 6A). In the case of both *sds* double mutants, there was a significant reduction of foci in comparison to the wildtype with *sds1 sds2-1* displaying 41.9 ± 9.0 (n = 28) foci and *sds1 sds2-2* (n = 33) with 51.3 ± 14.9. Thus, in contrast to both rice and Arabidopsis, loss of SDS in maize does not lead to a complete absence of DSBs while at the same time, the number of DSBs is severely reduced.

One possible difference between rice and Arabidopsis with respect to the *sds* mutant phenotype could be that chromosome axis assembly and DSB formation are differently regulated, i.e., DSB formation might require the assembly of the meiotic axis whereas in Arabidopsis DSB formation might be independent of axis formation (Yang et al., 2020).

To assess possible axis defects in *sds* mutants in maize, we studied the localization of DSY2, the maize homolog of the axis protein ASY3 in Arabidopsis and Red1 in yeast, by immunolocalization in male meiocytes. As shown in Supplemental Figure 5, no obvious defects in DSY2 positioning on the axis in *sds1 sds2-1* could be observed. Thus, we conclude that at least up to the point of DSY2 recruitment, axis formation functions independent of SDS in maize. However, we cannot exclude that other axis or axis-associated proteins such as ASY1 fail to or are not properly localized in maize *sds* mutants. To further explore

this point, a co-localization assay was performed to study ASY1 and SDS1 (Supplemental Figure 5B). For this, an antibody against ZmASY1 and the reporter SDS1-RFP (A188) were used together in maize meiocytes during zygotene stage and analyzed under the confocal microscope. While ASY1 was localizing along the chromosome axis showing a strong signal, SDS1 was expressed as a dotty signal all over the nuclear region, but not specifically along the axis. Also, some stronger spots can be distinguished where the SDS1 seems to accumulate more. However, this does not match the chromatin knobs observed in the DNA or any other particular location either. Therefore, SDS1 is expressed in the same stage as ASY1, but it does not interact with it, or at least not directly. An explanation for this could be that SDS1 does not act in the axis and regulates other recombination elements, for example, the ZMM class I CO pathway. It would be also interesting to study whether is SDS2 the homolog that has a role with the axis proteins.

2.7 Expression of ZmSDS1 can partially complement the *sds* mutant in Arabidopsis

Given the different mutant phenotypes of SDS in rice, Arabidopsis, and maize, we next asked whether this difference was due to SDS function itself versus a different functional context, e.g., a difference in chromosome axis organization. To address this question, we aimed for a heterologous complementation assay of Arabidopsis *sds* mutants with the maize SDS gene.

To approach this, we first generated a genomic reporter line for Arabidopsis *SDS* (*PRO_{AtSDS}:AtSDS-GFP*). Indeed, this construct could fully complement the Arabidopsis *sds* mutant phenotype (Supplemental Figure 7). We also found a clear SDS:GFP signal in the nuclei of male meiocytes. This signal was homogenously distributed in the nucleoplasm (Supplemental Figure 7A). Next, we generated a genomic ZmSDS1 construct (from the B73 inbred line, *PRO_{AtSDS}:gZmSDS1-GFP*) and transformed this into Arabidopsis plants. However, neither the *sds* mutant phenotype was rescued in 16 plants nor could we detect a GFP signal in male meiocytes (Supplemental Figure 7A).

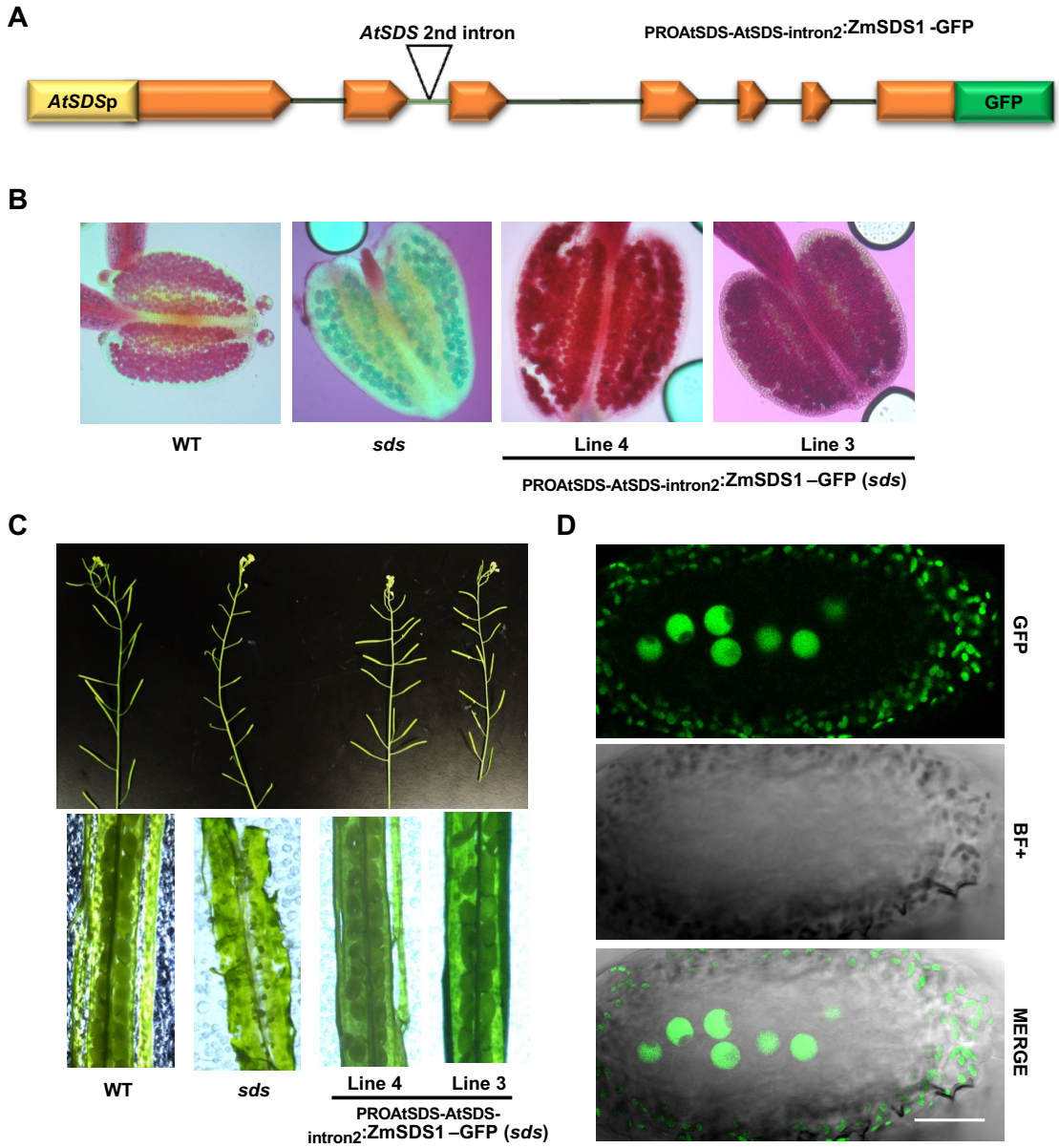

Figure 4. The genomic *PRO*$_{AtSDS\text{-}AtSDS\text{-}intron2}$*:ZmSDS1–GFP*, rescues partially the *Arabidopsis sds* mutant phenotype.

(A) A model of the *PRO*$_{AtSDS\text{-}AtSDS\text{-}intron2}$*:ZmSDS1–GFP* construct, where the second intron was replaced with the second one from *AtSDS*.

(B) Pollen viability of Arabidopsis anthers of WT, *PRO*$_{AtSDS\text{-}AtSDS\text{-}intron2}$*:ZmSDS1–GFP*.

(C) Comparison of silique length and seed viability of WT, *atsds* mutant, and two lines containing the genomic *PRO*$_{AtSDS\text{-}AtSDS\text{-}intron2}$*:ZmSDS1–GFP*.

(D) Nuclear GFP localization of the *PRO*$_{AtSDS\text{-}AtSDS\text{-}intron2}$*:ZmSDS1–GFP* in anthers in male meiocytes. Bar = 10 μm.

One of the obvious differences between the genomic region from Arabidopsis versus maize is the presence of a transposable element in the second intron of maize *SDS1* raising the hypothesis that this transposon might have a negative effect on the expression of ZmSDS1:GFP in Arabidopsis. Therefore, another genomic *ZmSDS1* construct was generated in which the endogenous second intron was exchanged with the second intron from *AtSDS* (Figure 4A). This construct was called $PRO_{AtSDS\text{-}AtSDS\text{-}intron2}$*:ZmSDS1-GFP*. Notably, a GFP signal was now found to accumulate in male meiocytes and this construct could largely rescue the Arabidopsis *sds* mutant phenotype as assessed by pollen viability (Figure 4B) and seed set, and silique length (Figure 4C). WT plants presented 66 ± 5,1 seeds per silique in contrast to *sds* (1,75 ± 1,45). Notably, the *sds* lines harboring the transgene showed an increase in both line 3 and line 4 (38,3 ± 3,3 and 46,5 ± 4,9 respectively) although still did not reach WT levels (Supplemental Figure 7B). Thus, we conclude that *SDS* functionality is largely conserved among flowering plants and the differences in the mutant phenotypes are most likely due to different chromosome/chromatin configurations. Moreover, meiosis-specific expression appears to be largely conserved as well with the exception that Arabidopsis transcriptional control machinery is not compatible with the intron present in the maize *SDS1* gene.

2.8 ZmSDS1 accumulates in foci in male meiosis

By virtue of the fluorescent tags of our genomic SDS reporter lines, we next studied the localization patterns of Arabidopsis and maize SDS proteins in detail. First, anthers of the Arabidopsis plants that incorporated the genomic PRO_{AtSDS}*:AtSDS-GFP* construct were checked (Supplemental Figure 7A). A strong signal was specifically covering the whole nuclei of all the meiocytes. No signs of GFP expression were observed in other areas of the cell or anthers, as well as in other organs of the plants. This nuclear pattern was detected from the G2/Leptotene stages and prolonged through zygotene and pachytene. The same observation was perceived when analyzing male meiocytes with the construct $PRO_{AtSDS\text{-}AtSDS\text{-}intron2}$:ZmSDS1-GFP.

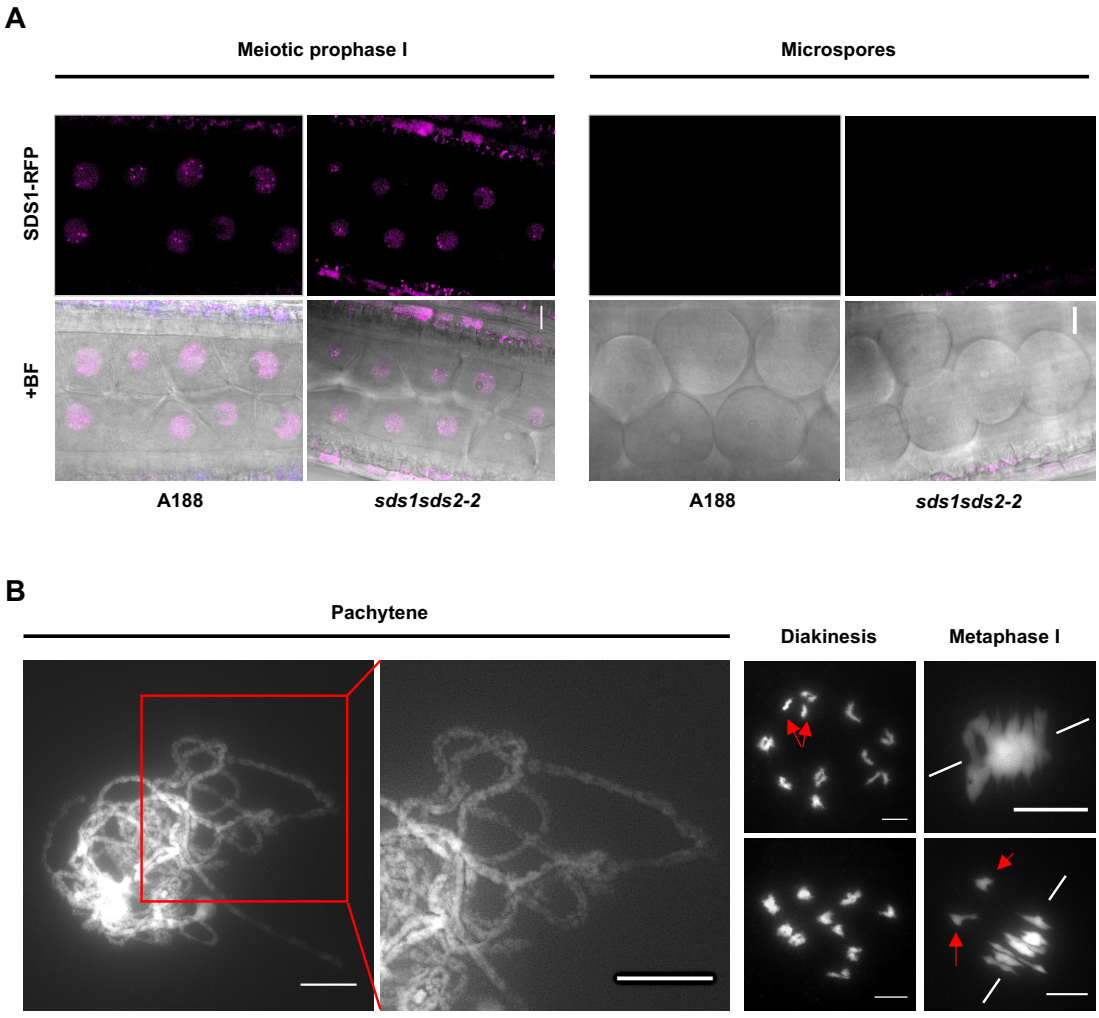

Figure 5. Phenotypical characterization of SDS1-RFP in *sds1 sds2-2*.

(A) Confocal images of SDS1-RFP during meiotic prophase I and microspores in both A188 and *sds1 sds2-2*. Bars = 20 µm.

(B) Images of chromosome spreads at different meiotic stages of SDS57-RFP in *sds1 sds2-2*. The white lines represent the orientation on the equatorial plate. The red arrows show univalents and laggards. Bars = 10 µm.

Next, we followed the accumulation and localization pattern of SDS1:RFP in A188 and *sds1 sds2-2* mutant plants by confocal laser-scanning microscopy (Figure 5A). In early meiotic prophase I in the A188 meiocytes, the signal starts to appear specifically in the nucleus. This signal looks fuzzy and also it becomes strong in form of several dots (up to 10) can be observed. The signal continues to increase during mid-prophase I until late prophase I, when it decreases until it´s totally gone during the formation of microspores. A similar pattern was

detected with the reporter in the double mutant background (*sds1 sds2-2*). During early prophase I, the blurry signal shows up again and the same number of dots can be counted. This signal will continue until late prophase I when it ceases and is no longer seen in microspores. It is reasonable to assume, that those dots observed up to 10 could correspond to the SDS1 highly localizing to the 10 bivalent chromosomes.

2.9 The number of HEI10 foci is strongly reduced in *sds1 sds2-1*

The localization of SDS to distinct foci that match the number of cross-overs in male maize meiocytes, suggested that SDS could play a local, i.e., chromatin-associated role in controlling meiotic recombination. Due to the complete absence of DSBs in rice, a role for SDS in recombination in crops could so far not be assessed. However, since maize *sds* double mutants still generate approximately 20% of DSBs seen in wildtype inbred line control, it became possible to assess the role of SDS on cross-over formation in this crop. Theoretically, the 40-50 DSBs in *sds* double mutants should be sufficient to allow the generation of the typically observed ~ 20 cross-overs per chromosome in maize male meiocytes (He et al., 2017). However, our chromosome spreads already indicated that *sds* double mutants produce a great number of univalents indicating that cross-over levels are severely reduced. To study this aspect in more detail, we performed immunolocalization assays with a HEI10 antibody in A188 and *sds1 sds2-1* (Figure 6B). Notably, we found a significant reduction of HEI10 foci in *sds* mutant in pachytene in comparison to the wildtype (Mann-Whitney U Test, $p<0.0001$) (Supplemental Figure 6B): In A188 11.5 ± 5.2 (n = 40) were detected, whereas in *sds1 sds2-1* most of the cells would generate either 1 focus of none, 0.68 ± 1.0 (n = 50). Thus, the reduction of HEI10 foci is clearly much stronger than expected by the number of DSB, even if one would assume that DSBs and cross-over number linearly correlate with each other.

The localization of HEI10 and SDS1-RFP was also assessed in A188 at zygotene (Figure 6C). HEI10 was detected as multiple foci along the chromosomes. On the other hand, SDS1 could be seen as in Supplemental Figure 5B, in the nuclear region and accumulates in big dots. These SDS1 clusters do not seem to be associated with HEI10. A study performed in

Arabidopsis (Morgan et al., 2021), suggests a coarsening model where CO position can be predicted where HEI10 is accumulating during pachytene. Thus, either SDS1 acts towards other elements of the Zmm class I CO pathway, such as MLH1 or MLH3 or is in later meiotic stages (Pachytene - Diplotene) where HEI10 and SDS1 will localize together. It would be also important to see the role of SDS2 during recombination.

Taken together, we conclude that SDS in maize is an important regulator not only of DSB formation but also of recombination.

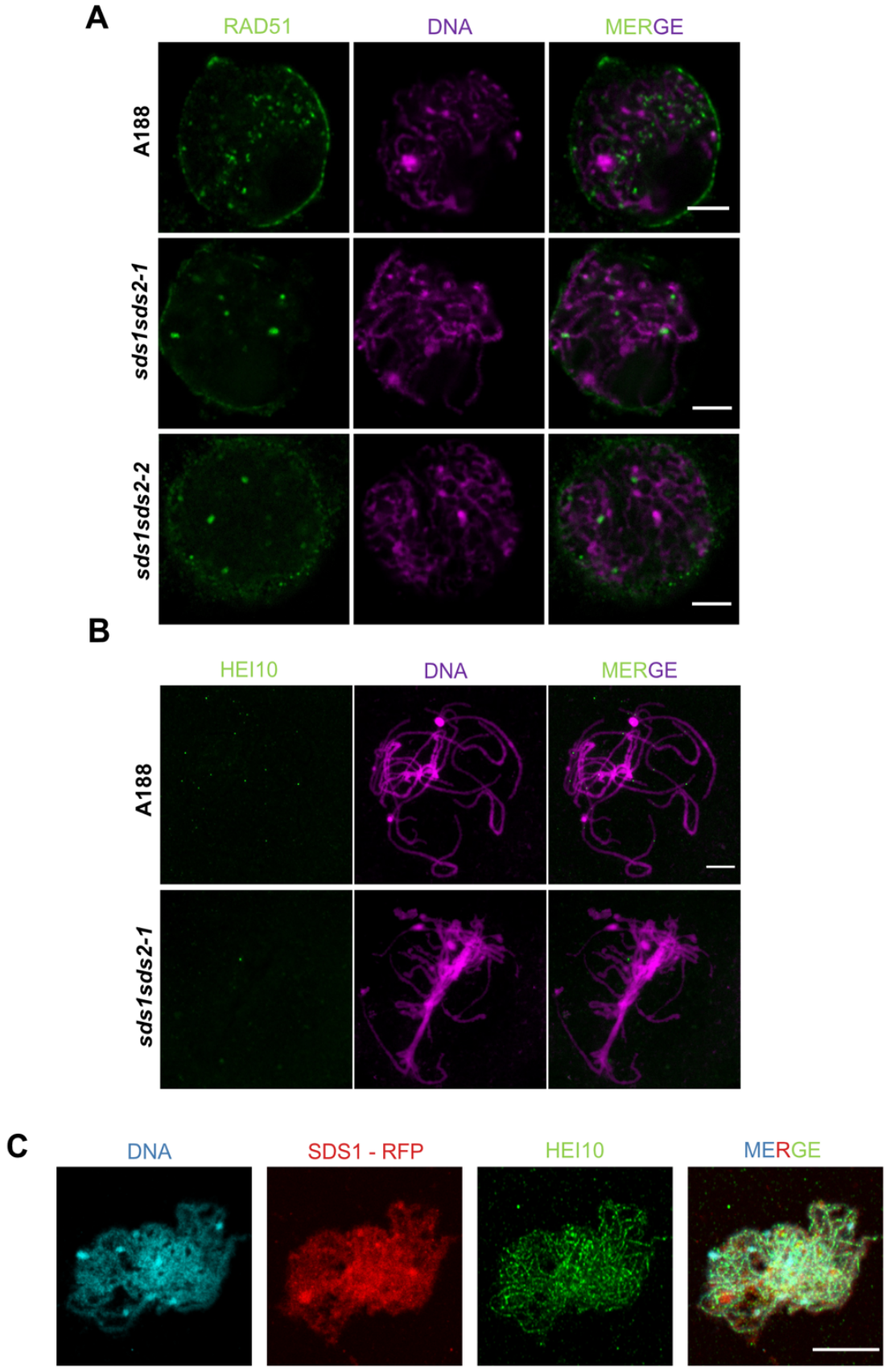
A
RAD51
DNA
MERGE
A188
sds1sds2-1
sds1sds2-2
B
HEI10
DNA
MERGE
A188
sds1sds2-1
C
DNA
SDS1 - RFP
HEI10
MERGE

Figure 6. Localization of RAD51, SDS1-RFP, and HEI10 in A188 and the *sds* double mutants.

(A) Comparison of RAD51 foci in A188, *sds1sds2-1,* and *sds1sds2-2*. Bars = 10 µm.

(B) Comparison of HEI10 foci in A188 and *sds1sds2-1*. Bars = 10 µm.

(C) Co-localization of SDS1-RFP with HEI10 and DNA in A188. Bars = 10 µm.

3. Discussion

3.1 Maize has two *SDS* genes that act redundantly

In this study, we have characterized the function of the meiotic cyclin SDS which is covered in maize by two redundantly acting genes, *SDS1* and *SDS2*.

SDS is an atypical cyclin that bears similarities with A- and B-type cyclin (Azumi et al., 2002). Currently, the function of SDS is not very well understood, although it seems to be necessary for the recombination pathway. Our analyses performed in the double mutants and the reporter line suggest that SDS is necessary to reach the wildtype levels of DSBs as well as COs. As exposed in Figure 1, the double mutants had visible fertility defects when looking at the tassel and the Peterson staining confirmed the high degree of pollen abortion. The cobs contained in the ears could not develop viable seeds, neither when it was pollinated with a WT plant nor by self-pollination, indicating a problem in female meiosis as well. Therefore, SDS acts in both male and female meiosis, more concretely in the meiocytes. However, cytological assays must be performed in female meiocytes to confirm this.

The RNA-seq expression analysis reported on the MaizeGDB in the inbred line B73, *SDS1* is expressed in anthers while there was no transcript detected for *SDS2*. In our results described in A188, *SDS1* is naturally mutated and *SDS2* gets upregulated and there is no observation of a mutant phenotype. Consistent with this, the SDS1 reporter in the *sds1 sds2-2* mutant background complements at least partially the mutant phenotype, demonstrating a high redundancy amongst both genes.

3.2 SDS1 and SDS2 are both encoding meiotic cyclins

Cdk-cyclin complexes are acting during the cell cycle at different stages and they are necessary for the correct function/progression of it.

The complementation experiments carried out in Arabidopsis show how SDS1 rescues the same mutant phenotype as the AtSDS does. The GFP expression is localized in the male meiocyte nuclei and rescues pollen fertility.

In Arabidopsis, SDS acts in early prophase I during recombination and bivalent formation. It also acts together with CYCB3;1 to ensure a correct cell wall formation. The single mutants *sds* and *cycb3;1* exhibited ectopic parts of cell walls around the cells in pollen mother cells (PMCs), a defect that became more noticeable when generating the *sds cycb3;1* double mutant (Bulankova et al., 2013).

CDKs are in charge of the correct cell cycle progression. Mutations in one of the *CDKA* or *CDKB* gene, generate a mutated version of a protein that provoke subsequent strong growth defects. CDKA;1 is thought to be the main Arabidopsis CDK involved in cell-cycle progression. The *cdka;1* mutant homozygous plants are lethal in their growth and development (Iwakawa et al., 2006; Nowack et al., 2007). To sum up, the reduction of CDKA;1 activity by itself results in a much more severe phenotype than in any of the single and even double mutants of the above-mentioned cyclins suggesting an even higher level of redundancy. Our results showed that the maize *SDS* double mutants had no visible defects in vegetative growth. The chromosome spreads reflected the role of both SDS1 and SDS2 proteins for proper chromosome segregation. Clear cell wall deformities were not seen. As our results show, no complete failure of the meiotic recombination was found in the maize *sds* double mutants since some cells could still form some bivalents, so no complete loss of function mutant phenotype was detected. There is no evidence of a third *SDS* maize gene after all the analysis, there is a possibility of another maize cyclin taking over and partially restoring the recombination machinery in the *sds* double mutant. As the recombination pathway has several pathways and requires the action of multiple elements, another explanation could be that SDS acts only in one of the pathways and the others are not SDS-dependent. For example, SDS could be acting in the class I CO crossover pathway. Another possibility is that SDS has a function in an upstream process, i.e., SC or DSB formation. Taking all these data together, we

can therefore demonstrate that ZmSDS1 and ZmSDS2 are meiotic cyclins and that both are homologs of the Arabidopsis SDS.

3.3 The function of SDS1 and SDS2 in meiosis

The *sds* double mutants had remarkable defects in male meiosis. The confocal images performed with the SDS1-RFP reporter showed that SDS activity occurs during prophase I as described in rice. The chromosome spreads revealed the lack of pairing in some chromosome areas, as well as a great number of univalents that led to chromosome missegregation. Nonetheless, some bivalents could be visible sometimes including chromosome bridges during the segregation in Anaphase I. This mutant phenotype differs from the OsSDS where only the presence of univalents was found (Wu et al., 2015b). These data suggest that the maize SDS1 and SDS2 are necessary for recombination, synapsis, and bivalent formation, although their function might be a bit different from what was observed in rice and Arabidopsis.

That the maize *sds* double mutant was capable of generating some bivalents and the presence of chromosome bridges, suggests that at least a reduced activity of DSBs might happen. The immunolocalization experiments performed for RAD51, showed a foci reduction but not an absence of them in the double mutants, compared to A188. RAD51 could be not loaded properly, although it is more likely that a reduced number of DSBs are formed and RAD51 is only recruited to the few generated breaks. Nonetheless, the molecular mechanisms of how both SDS proteins function during DSB formation are still unknown.

A study carried out in AtSPO11-1, shows a relationship between DSBs numbers and the COs final number. The observations in the *spo11-1* hypomorphic alleles suggest that a decrease of DSBs leads to a lower CO number (Xue et al., 2018). However, the reduction of cross-overs in *sds1 sds2* double mutants is much stronger (only one-tenth of the wild-type HEI10 foci were found and most of the homologs appear as univalent) than could be easily explained by a reduction in DSBs (one-quarter reduction). Thus, SDS in maize appears to influence both DSB formation and cross-over formation.

ZmSDS1-RFP also had an increase of bivalents, but sometimes the presence of a few univalents could be observed and the confocal pictures showed up to 10 strong dots, which could be its localization in the chromosomes. Because of the size and number of the dots seen, and that maize has a maximum of ten bivalents, it could be that the localization of SDS in those stages is centromeric and it could be involved in chromosome separation. This could explain, why in stages later than prophase I some laggards were observed and ended up not segregating during the rest of the meiosis process. The difference in bivalent numbers between the double mutants and the reporter indicates an increase in CO, making it possible to be involved in CO formation or in the process leading up to COs. Some in vitro kinase assays performed in Arabidopsis with the CDKA;1-SDS complex, revealed that some of the class I CO elements contained CDKA;1 consensus phosphorylation site, like MSH5. In addition, the protein MLH1 was found to be a target of this CDK-cyclin complex and in a lower degree of CDKA;1-TAM (Wijnker et al., 2019).

Both maize SDS could be necessary for the phosphorylation of some of the recombination elements and their recruitment to the chromosomes. As the class I CO formation is the one observed in a higher frequency, it would be more likely that SDS1 and SDS2 load elements from that pathway and in the mutated version only partially. This is consistent with what is seen in OsSDS, where there is an absence of some of the ZMM proteins involved in the class I CO formation, such as MSH5, MER3, and ZEP4 (Wu et al., 2015b).

The maize *sds* double mutants also exhibited at least partial desynapsis of chromosomes at pachytene stage (Figure 2Q, Supplemental Figure 3M), which was restored in presence of one of the SDS copies (SDS1-RFP). It has been already reported in Arabidopsis and rice that a decrease in DSBs also affects the loading/installation of certain elements of the SC (Grelon et al., 2001; Yu et al., 2010). OsSDS fails in the localization of the central element ZEP1, but it can perfectly localize PAIR2 (ASY1) and PAIR3 (ASY3). Maize DSB-defective mutants, such as *spo11-1*, showed aberrations in the axial elements and the absence of the central element ZYP1 (Golubovskaya et al., 2006). The maize immunolocalization assays against DSY2, show no difference between A188 and *sds1 sds2-1*, with an elongated signal along the chromosome axis, that correlates with the OsSDS case. However, higher resolution microscopy would be needed

to determine if there is really the presence of patchy signals in the double mutant chromosomes. SDS1 also does not look like it localizes with ASY1, according to the colocalization experiments carried out with the SDS1-RFP reporter (Figure 6C). Thus, a possible function of SDS1 and SDS2 could be the installation of some central elements of the SC, i.e., ZYP1, but neither ASY1 nor DSY2 axial elements.

The number of HEI10 foci is highly reduced or even inhibited in *sds1 sds2-1*. HEI10 is known to be a primary protein for CO positioning and crossover interference. A coarsening model was described in Arabidopsis, where HEI10 is localized along the chromosomes during early pachytene and clusters into big foci in late pachytene (Morgan et al., 2021). The same kind of localization was found for the rice HEI10, however, HEI10 has not been characterized yet in maize and its localization remains unknown. In the *sds1 sds2-1* double mutant, when a CO is formed, a HEI10 focus is visible, which means it is mediated by the class I CO pathway. Whether SDS is directly acting on HEI10 cannot be clarified. The co-localization assays between SDS1-RFP and HEI10 during zygotene do not show a physical interaction between the SDS1 signal and the HEI10 foci, although the maize HEI10 was predicted to contain phosphorylation sites. A coarsening model was suggested, where in late pachytene, HEI10 foci will accumulate in specific places promoting COs (Morgan et al., 2021). Therefore, I would speculate with SDS loading HEI10 to those positions and/or designating the COs. In this study, I also conclude that SDS could act in the recombination pathway, maybe recruiting some other element of the class I CO machinery, or it works more upstream during the DSB formation and the rest is a downstream effect. CO homeostasis has been identified in maize as well as in some other organisms. It ensures that at least one CO per bivalent is formed so there is balanced segregation. This is strongly controlled and after reaching this limit, a proportional relation between DSBs and CO number was found. The CO number also seems to vary between the different maize inbred lines (Sidhu et al., 2015). Since both SDS are involved in the recombination pathway, one of our hypotheses is that they could also regulate the amount of COs in different inbred lines. Future studies will approach the molecular mechanisms by which the maize SDS1 and SDS2 proteins act in the meiotic recombination pathway.

Cyclins are very dynamic proteins that form complexes with CDKs to regulate a plethora of processes, including timing. It was proposed that AtSDS could regulate the timing of sister chromatid separation via acting over SWI1, as well as its involvement in other meiotic processes like recombination and cell-wall formation (Azumi et al., 2002; Bulankova et al., 2013). Therefore, the maize SDS could also have more functions apart from recombination is a possibility, including timing regulation of some phases. Live imaging experiments could be carried out to study the dynamics of SDS.

Taken all together, here I show that *AtSDS* has two maize homologs (*SDS1* and *SDS2*) that could have a redundant function. My data suggests that it could act in several stages organizing the meiotic chromosomes and loading proteins necessary for CO formation. However, the molecular mechanisms by which SDS regulates these mechanisms still need to be investigated. Furthermore, future experiments should focus on the genetic diversity of SDS in different maize inbred lines and how its meiotic role in recombination could be related to crossover homeostasis.

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Chapter II

Assessing the role of introns in the expression of *SOLO DANCERS (SDS)*

1. Introduction

1.1 Gene expression can be regulated by introns

Gene expression is imperative to the genotype-phenotype relationship in all organisms, and it also plays a significant role in the genetic bases of evolution in a variety of phenotypic aspects. A gene encoding a protein *per se* is not enough to drive its own transcription. Instead, some specific sequence motifs and other elements will be required and will manage and regulate the genetic expression at different levels (Wray et al., 2003). For many years, the goal of many studies was to find those key factors driving the expression of individual genes. In the beginning, the cis-elements harbored in the promoter regions of the genes were thought to be the responsible sequences. Yet, other genetic regulators can also control expression, among them, intronic sequences (Gallegos & Rose, 2018).

Introns are DNA sequences considered as “junk DNA” for many years, however now we know that they can be important for gene regulation (Gallegos & Rose, 2018; Sambrook, 1977). Introns are also relevant for exon shuffling and alternative splicing, processes that lead to broader protein diversity. In eukaryotes, a functional mRNA transcript is formed by the accurate removal of all introns from the pre-mRNA transcript. A failure or the partial removal of these sequences will result in mutated and/or non-functional transcripts, and might also influence the expression level (Long et al., 1995; Maniatis & Tasic, 2002). In addition, very complex machinery is necessary for intron maintenance and posterior elimination. Therefore, the fact that introns are still part of the genomic sequences in all the eukaryotes although they imply a high cost of energy, indicates their importance. The possibility of exon shuffling and the generation of new proteins, implicate a great evolutionary advantage. In addition, the effect that introns have on gene expression is sometimes ignored. If the gene introns are removed, i.e., using the cDNA of genes, even including native promoters and terminators, often leads to a drop in gene expression or special misexpression. However, although most regulatory introns can boost the expression levels, usually at the mRNA accumulation level, there are others that have either a negative or no effect. Yet, the degree of enhancement will be different and dependent on various factors, i.e., not only introns but also the promoter, gene,

flanking sequences, and the kind of cell or the tissue where the expression occurs (Rose, 2008).

Although our knowledge of intron-mediated gene expression regulation is still limited, some mechanisms could be deduced, such as the presence of enhancers or alternative promoters within intronic sequences, the intron-mediated enhancement of expression (IME) and the nonsense-mediated decay pathway (NMD, Figure 1), which eliminates defective RNA molecules (Le Hir et al., 2003; Rose, 2008). NMD is triggered when a premature stop codon (PTC) (derived from an intron or an exon) is detected and leads to fast degradation of the faulty mRNA. Furthermore, there is evidence showing a relation between the intron position downstream of a PTC and the likelihood of this stop codon triggering NMD. As a general rule, introns placed at least 50-55 nt downstream of a stop codon will lead to NMD (Cheng & Maquat, 1993; Maquat, 2004; Kertész et al., 2006). To trigger the NMD by the relative intron positions and stop codons, communication between nuclear splicing and the cytoplasmic translation must be achieved. This connection is performed by the exon Junction complex (EJC). (Maquat, 2004; Rose, 2008). The EJC binds approximately 20-24 nt upstream of the exon-exon junctions in the spliced mRNA (these junctions are the former intron locations) and recruits proteins necessary for the NMD activation (Behm-Ansmant & Izaurralde, 2006).

Introns located upstream of the open reading frame (ORF) sequences of a gene can also boost expression. This fact led to the hypothesis that some of these introns might contain enhancers bound by regular transcription factors. Such an intron-located enhancer can be found in the *Arabidopsis agamous* (*AG*) gene, which contains a second intron (2.999 bp large) that can be used in both orientations to regulate the expression of a reporter gene (Deyholos & Sieburth, 2000).

It has been found that some introns can drive gene expression of a gene without a promoter. These introns can contain as well promoting sequences that are in part responsible for an increase in the expression. This is the example of the first intron found in both rice genes: *RICE BETA-TUBULIN ISOTYPE* 16 (*Ostub16)* and *PHOSPHATE DIKINASE 2* (*OsCPDK2* ; Kim et al., 2006; Morello et al., 2006).

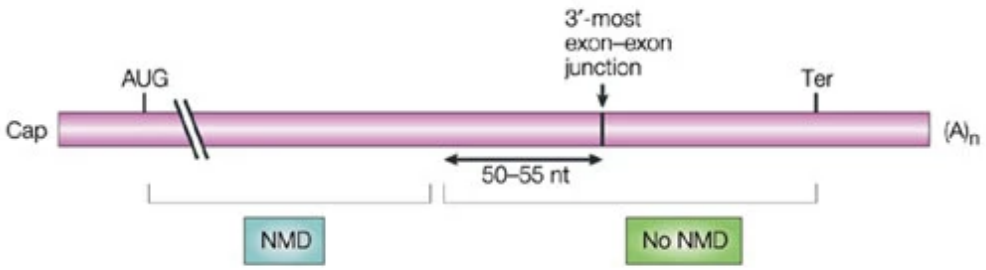

Figure 10. Representation of NMD and intron position. A PTC (premature termination codon) in the area indicated in blue, with an intron located at least 50-55 nt downstream, will trigger NMD. In the case of this PTC in the green region, NMD will not occur. (Image from Maquat, 2004).

The IME is a less defined, complex phenomenon that occurs at different levels of gene expression, i.e., at the level of transcription as well as on the post-transcriptional level, and has been described in mammals, yeast, plants, and other organisms (Figure 2). A study performed in maize protoplast, using the maize (*ALCOHOL DEHYDROGENASE- 1*) *ADH1* introns 2 and 6 gave the first hints on how IME affects the expression (Mascarenhas et al., 1990).

Comparisons with an intronless version showed that the sequence containing introns can boost the expression of the transcript between -2 and -10-fold. For IME to occur the introns need to be in the correct orientation and to be near the transcription initiation start (TIS), which is different from elevated expression mediated by enhancers. Also, some sequences rich in C/T-stretches and specific motifs, such as TTNGATYTG, and CGATT, have been directly related to the IME (Laxa, 2017). An example of IME in *Arabidopsis thaliana* concerns the (*PHORIBOSYLANTRHANILATE TRANSFERASE 1*) *PAT1* gene, where the first two introns can increase the accumulation of mRNA from the PAT1:GUS reporter construct without affecting the rate of transcription (Rose & Beliakoff, 2000; Rose, 2002).

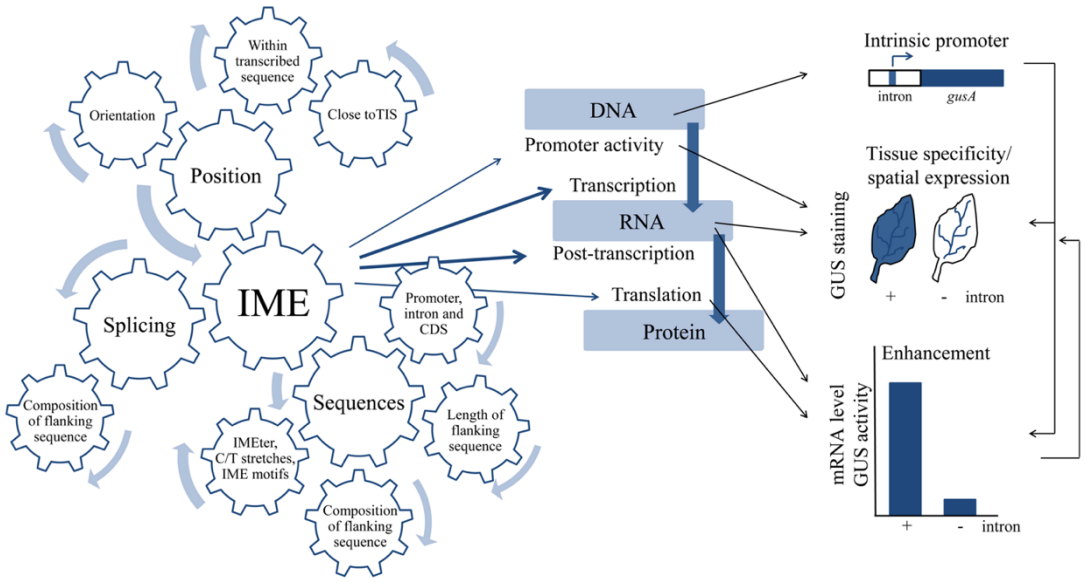

Figure 11. **Scheme of the Intron Mediated Enhancement (IME).** IME is a complex mechanism that acts at different levels of gene expression. The gears show the parameters *that* influence IME, and the arrow thickness is related to the importance of this parameter for IME. Image from Laxa, 2017.

1.2 Intron-mediated gene regulation as a biotechnological tool

The biotechnological industry is putting efforts into applying intron-mediated regulation to boost the expression of heterologous genes. As an example, the transgene for Bt-toxin needs to be highly expressed in maize roots, to reach a high resistance towards the western corn rootworm. However, Bt-toxin expression should be lowered in other plant areas, such as the kernels, since they will be used for food consumption (Saxena & Stotzky, 2001; Emami et al., 2013). The first experiments in this direction were performed in Arabidopsis, where an intron of the *UBIQUITIN 10* (*UBQ10*) gene was fused to different genes that were originally expressed constitutively, tissue-specific, or regulated by light. For example, the gene *CYCLIC NUCLEOTIDE GATED CHANNEL 2* (*CNGC2*) is known to be constitutive in leaves, *YABBY 3* (*YAB3*), which is tissue-specific and expressed in the abaxial surfaces of the leaves. At last, the *UV-B LIGHT INSENSITIVE 3* (*ULI3*) and *MEMBRANE STEROID BINDING PROTEIN 1* (*MSBP*1), are two genes that express stronger in the light than in the dark. The results suggested that this intron changed the expression amount and spatial patterns of *CNGC2* and *YAB3*. Not only there was a higher accumulation in the

leaves but it was expressed also in the roots. In the case of *ULI3* and *MSBP1 genes, their regulation* was preserved (Emami et al., 2013).

An indication, that introns are relevant for tissue-specific expression was found in research concerning the SDS gene of Arabidopsis which is only expressed in meiocytes. It has been published that a 1.5 kb SDS promoter fragment alone cannot drive this meiocyte-specific expression, but if this promoter is combined with the rest of the *SDS* gene, WT-like expression occurs (Huang et al., 2016). Elements responsible for correct spatial and temporal gene expression were also identified in the introns of other plant genes like TubA1 (Jeon et al., 2000), AG1 (Sieburth & Meyerowitz, 1997), ACT1 (Y. Q. An et al., 1996), and PRF1 (Jeong et al., 2006).

2. Results

2.1 Analysis of the role *SDS* introns in gene expression

Following the hypothesis that SDS introns are needed for meiocyte specific expression (Huang et al., 2016), I studied the role introns of the AtSDS and its homologous genes in maize, *ZmSDS1,* and *ZmSDS2* (see Chapter I). A bioinformatical approach was used to pinpoint those introns with the highest likelihood of influencing SDS expression by IME. The three SDS genes have similar genomic structures, having six introns in the case of AtSDS and seven in the case of ZmSDS2 (Table 1). Also, the genomic and the protein sequences have practically similar lengths and approximately a 30% of identity in the cyclin C-terminal domain.

Gene ID	Chromosome Location	Genomic Size (bp)	cDNA Size (bp)	Exon/ Intron Number	Protein length (aa)
AtSDS (Col-0) (AT1G2450)	Chr. 1	3114	1737	7/6	578
ZmSDS1 (B73) (GRMZM2G093157)	Chr. 9	4743	1368	7/6	455
ZmSDS2 (B73) (GRMZM2G344416)	Chr. 1	3682	1481	8/7	464

Table 1. SDS sequences in A. thaliana and Zea mays. The sequences were downloaded from TAIR (Berardini et al., 2015) and MaizeGDB (Woodhouse et al., 2021b).

The SDS sequences were visualized using the Unipro UGENE software (Okonechnikov et al., 2012). Four different splice variants have been annotated for *AtSDS* and *ZmSDS1*, whereas *ZmSDS2* only has one known transcript (Figure 1). The mRNAs likely giving rise to the full-length proteins are indicated with red arrowheads. In the maize SDS genes, the second and third introns are the longest, with the second introns being about 1,5 kb in size. Alternative splice variants of AtSDS and ZmSDS1 harbor additional introns (marked by black asterisks). The most prominent one, longer than 1 kb, is found in *AtSDS* near the 5´end of the gene.

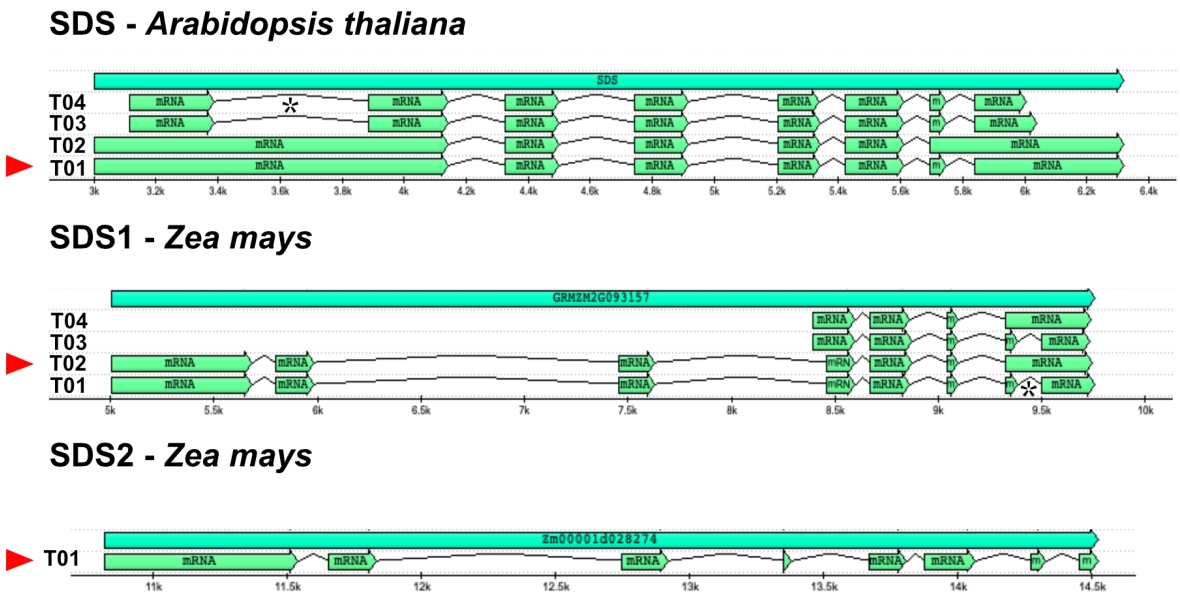

Figure 1. Representation of the SDS gene models and their splice variants. For AtSDS and ZmSDS1, four different splice variants are known *(T01-4)*, while ZmSDS2 has one transcript annotated *(T01)*. The red arrowheads indicate the most prominent transcript according to *TAIR (*https://www.arabidopsis.org/index.jsp*) and MaizeGDB. (*https://www.maizegdb.org/*) databases.* The asterisks indicate alternative introns found in other splice variants.

To predict the likelihood of an intron contributing to IME, a tool called IMEter was used, and all the intron sequences were studied (Parra et al., 2011; Rose et al., 2008). The scores as calculated by the current version (v2.1) and an older version (v1) are given. The higher the score, the more likely an intron is to enhance gene expression. As previously described by Laxa in 2017, introns closer to the promoter/transcription start site (TSS) are more likely to enhance expression, however, to have a complete view, all the introns of each gene were analyzed (Table 2). Interestingly, for Arabidopsis, the highest values were calculated for the first intron of the alternative splice variant (T03; 33.92 score).

Sequence identifier	**v1 score**	**v2.1 score**	**Percentile**
SDS A. thaliana			
Intron 1	6.93	5.10	80
Intron 2	-8.57	3.13	64
Intron 3	-12.13	3.65	69
Intron 4	-8.83	0.00	26
Intron 5	-3.09	2.15	51
Intron 6	-6.68	0.00	26
Intron 1 (2^{nd} splice variant)	66.58	33.92	99
SDS1 Zea mays			
Intron 1	5.74	6.44	79
Intron 2	-33.35	11.76	90
Intron 3	-49.89	1.03	30
Intron 4	-3.91	0.00	26
Intron 5	-11.14	0.00	26
Intron 6	-11.14	0.00	26
Intron 7 (2^{nd} splice variant)	-2.57	0.94	29
SDS2 Zea mays			
Intron 1	3.89	5.08	73
Intron 2	-30.66	6.93	81
Intron 3	-15.72	4.14	68
Intron 4	-19.75	0.00	26
Intron 5	-2.55	0.00	26
Intron 6	-6.24	2.49	53
Intron 7 (2^{nd} splice variant)	-3.54	0.00	26

Table 2. Scores showing the probability of the AtSDS, ZmSDS1, and ZmSDS2 introns of enhancing gene expression using the IMEter algorithm. The table shows the scores given for the first version (v1) and the second (v2.1). The more positive the score, the higher the likelihood that the intron will boost gene expression.

2.2 Assessing the relevance of introns for the meiosis-specific expression of the *Arabidopsis* SDS

In a first approach, the role of the 1st and 2nd intron of AtSDS was analyzed. A complementation assay was performed, for which several reporter constructs with SDS versions differing in intron number were cloned and transformed into the *Arabidopsis sds* homozygous mutant (Azumi et al., 2002c)Figure 2). In all constructs, SDS was C-terminally fused to an enhanced green fluorescent protein (EGFP) tag (Figure 3). Transgenic plants were scored for complementation of the fertility defects characteristic of *sds* mutants and analyzed for GFP expression in meiocytes by confocal microscopy. The 2.1 kb Arabidopsis promoter (PRO_{AtSDS}) including the 5´ AtSDS untranslated region (UTR) was also added to each construct. All the constructs cloned also contained the *Agrobacterium tumefaciens* nopaline synthase terminator (T-NOS; (F. Zhang et al., 2014).

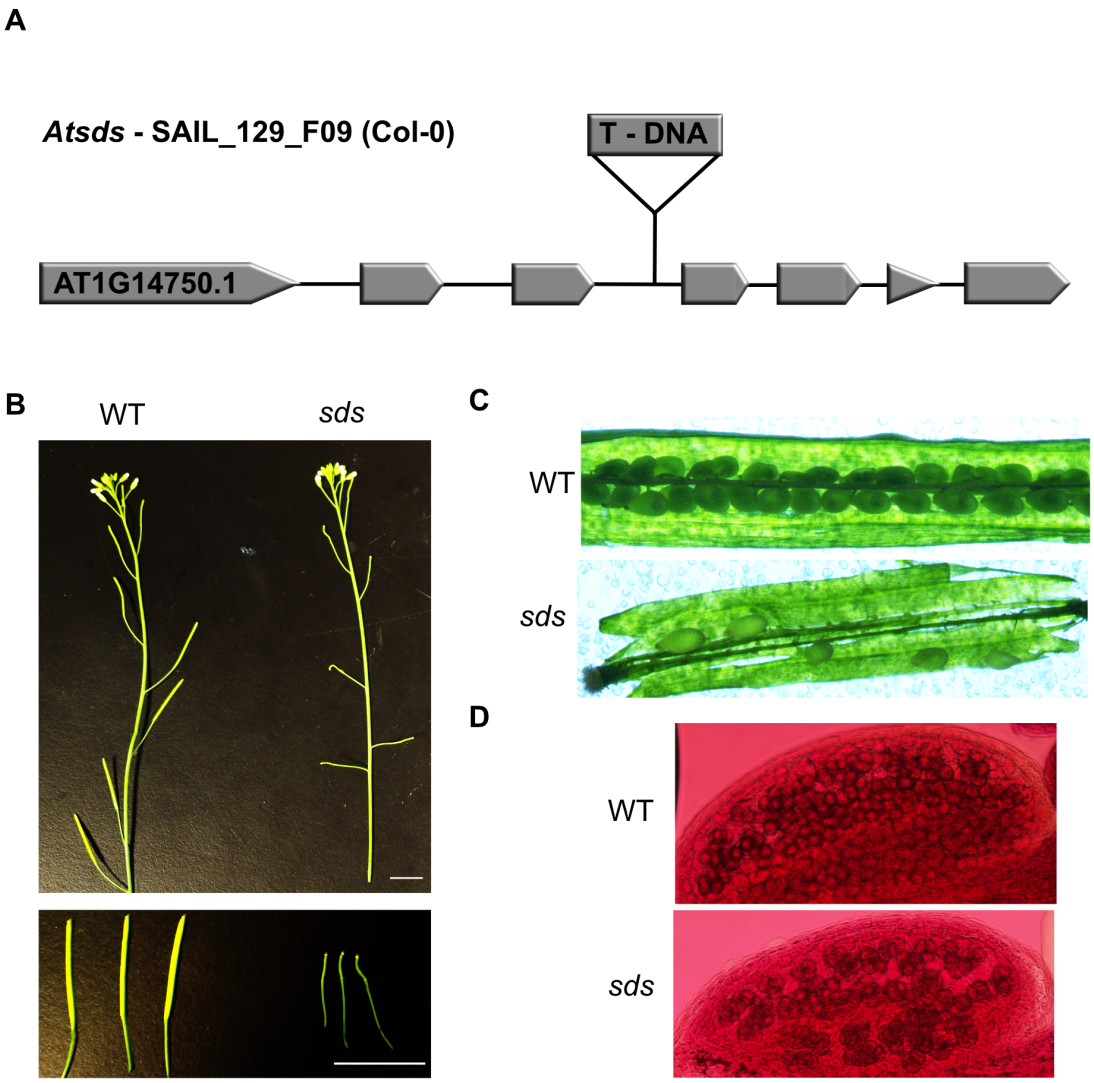

Figure 2. Overview of the Arabidopsis sds mutant.

(A) Scheme of the genomic region of sds. The rectangle represents the T-DNA insertion, located in the third intron of the gene.

(B) Comparison of wildtype and sds siliques. The mutant siliques are shorter than those of the wild type. Bars = 1 cm.

(C) Seed set analysis of the wildtype and sds mutants. The mutant only generates a few viable seeds.

(D) Peterson staining of wildtype and sds anthers. The mutant generates a few abnormal pollen grains.

The following Arabidopsis reporter constructs were generated and analyzed: the whole genomic sequence of SDS (*PRO$_{AtSDS}$:gAtSDS-GFP*), the cDNA of *SDS* from (*PRO$_{AtSDS}$:cAtSDS-GFP*), the genomic SDS with the first intron deleted (*PRO$_{AtSDS}$:gAtSDS$^{\Delta i1}$-GFP*), and the genomic SDS where its second intron was removed (*PRO$_{AtSDS}$:gAtSDS$^{\Delta i2}$-GFP*) (Figure 3). Four lines were analyzed per construct, pictures from representative lines are shown in figure 3. The gAtSDS and the cAtSDS constructs showed full rescue of the mutant phenotype on the female side as scored by silique length (non quantitatively, Figure 4A) and seed set (Figure 4B and C) with 60 ± 9.1 seeds per silique for gAtSDS-GFP and 57.7 ± 8.4 seeds per silique for cAtSDS-GFP compared to 61.6 ± 4.4 seeds per silique in the wildtype. In the case of gAtSDS$^{\Delta i1}$-GFP, and gAtSDS$^{\Delta i2}$-GFP, an incomplete rescue of the seed set phenotype was observed with 42.8 ± 4.1 and 42.2 ± 2.8 seeds per silique, respectively compared to 1.9 ± 1.7 seeds per silique in *sds* mutants. However, the results shown for these last two constructs are preliminary, since only a few lines were checked and need further analysis.

Confocal analysis of anthers from gAtSDS-GFP and cAtSDS-GFP expressing plants showed a very strong signal in male meiocytes but not in other organs. The GFP was localized specifically in the whole cell nucleus during early meiosis (Figure 4D), i.e. from G2/Leptotene until Pachytene according to the cell shape, nucleus position, and nucleolus position described in the previous established landmark system (Prusicki et al., 2019). The complete analysis of gAtSDS-GFP is described in Chapter I.

The pollen viability was only analyzed qualitatively (Figure 4E). However, in none of the lines, I found evidence for aneuploidy as seen in *sds*, i.e., no big amounts of dead or differently sized pollen. indicating a rescue, at least partially, of the mutant phenotype for all constructs. However, in the future, a quantitative analysis is required to fully confirm this.

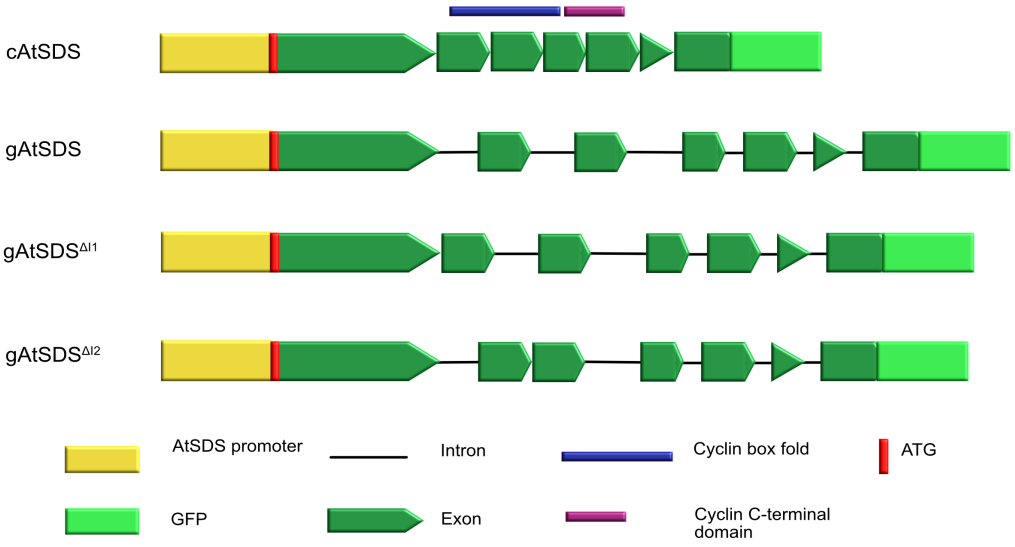

Figure 12. Schematic representation of the AtSDS constructs generated and analyzed in this thesis.

The 2.1 kb Arabidopsis promoter *(*PRO_{AtSDS}*)* including the 5´ AtSDS untranslated region (UTR) were added to each construct: gAtSDS-GFP, cAtSDS-GFP, $gAtSDS^{\Delta i1}$-GFP, and $gAtSDS^{\Delta i2}$-GFP. A NOS terminator (T-NOS) is included downstream of the GFP in each construct.

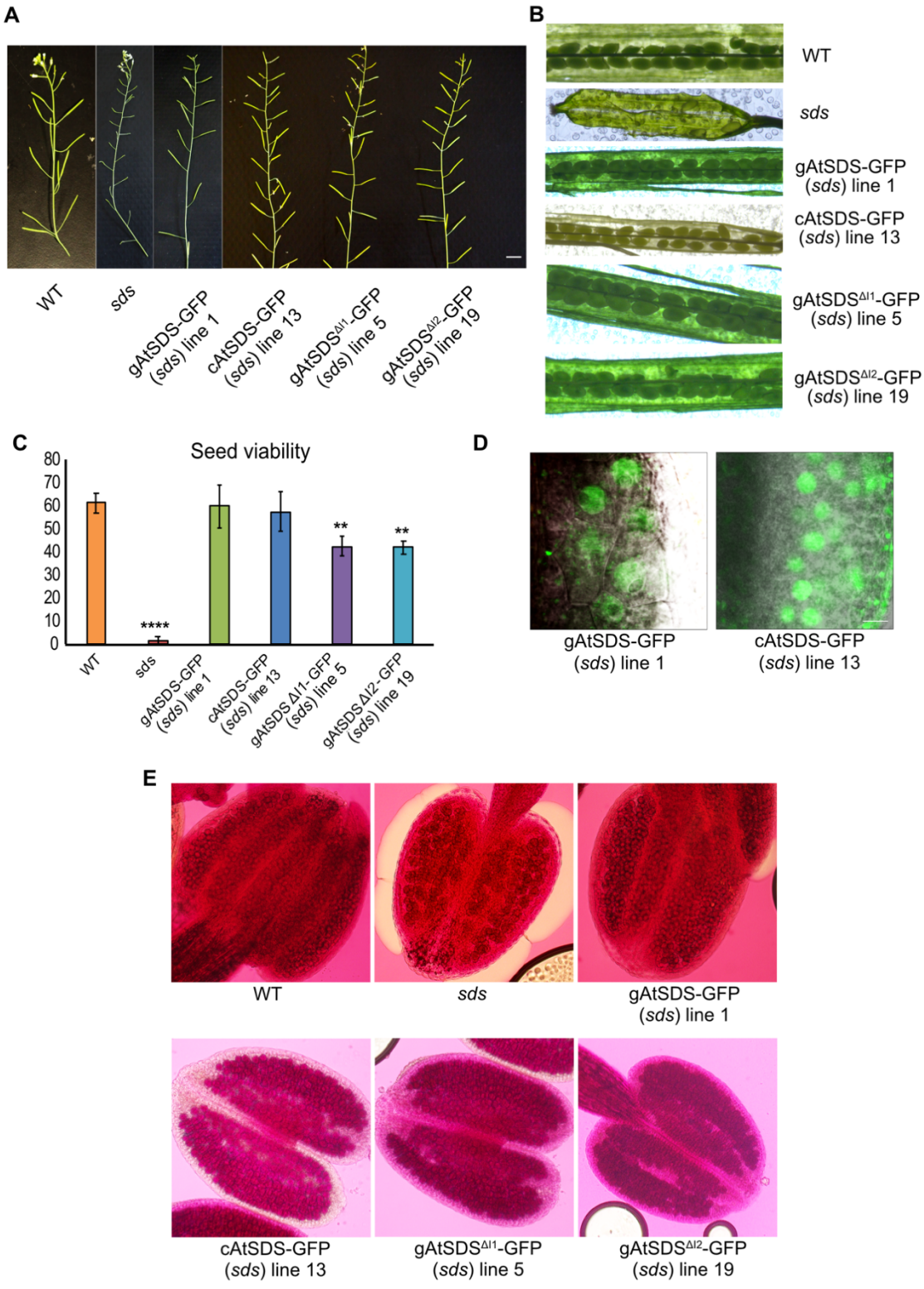

Figure 13. Complementation analysis of AtSDS constructs in *sds*.

(A) Main branches of plant lines expressing gAtSDS-GFP, cAtSDS-GFP, gAtSDS$^{\Delta i1}$-GFP, and gAtSDS$^{\Delta i2}$-GFP showing fully grown siliques. Bars = 1 cm.

(B) Seed set of plant lines expressing gAtSDS-GFP, cAtSDS-GFP, gAtSDS$^{\Delta i1}$-GFP, and gAtSDS$^{\Delta i2}$-GFP in *sds* background.
(C) Peterson staining of the anthers of gAtSDS-GFP, cAtSDS-GFP, and gAtSDS$^{\Delta i1}$-GFP, and gAtSDS$^{\Delta i2}$-GFP expressing plants.
(D) Seed quantification of the seed sets shown in (C). The seeds from 10 siliques per plant were analyzed. Asterisks represent significant differences from wildtype as determined by ANOVA test ($P < 0.05$).
(E) Pictures of GFP expression in Arabidopsis male meiocytes of gAtSDS-GFP, and cAtSDS-GFP expressing plants Bars = 10 µm.

2.3 The second intron of ZmSDS1 needs to be removed for SDS1 to be expressed in *Arabidopsis thaliana*

In the second set of experiments, I addressed the questions if maize SDS1 (ZmSDS1) could complement the Arabidopsis *sds* mutant phenotype and if introns 1 and 2 of *ZmSDS1* would influence SDS1 expression. Therefore I generated the following constructs (Figure 5):

A) *PRO$_{AtSDS}$:gZmSDS1-GFP* which contained the genomic region of ZmSDS1 starting at the START codon combined with the same SDS promoter/5'UTR fragment from Arabidopsis. The T-NOS terminator was again included after the GFP.
B) *PRO$_{AtSDS}$:cZmSDS1-GFP* which contained the cDNA of ZmSDS1 corresponding to the second longest transcript (T02) starting at START codon, combined with a promoter/5' UTR fragment from Arabidopsis. The T-NOS terminator was again added. The protein generated from this transcript lacks the three last amino acids of the cyclin C-terminal domain. This is the only transcript I could amplify by PCR.
C) *PRO$_{AtSDS\text{-}AtSDS\text{-}intron1}$:gZmSDS1-GFP* which corresponds to construct A, except that the first intron was replaced by the first intron of Arabidopsis splice variant T01.
D) *PRO$_{AtSDS\text{-}AtSDS\text{-}intron2}$:gZmSDS1-GFP* which coincides with construct A, although the second intron was replaced by the second intron of *Arabidopsis thaliana* splice variants T01.

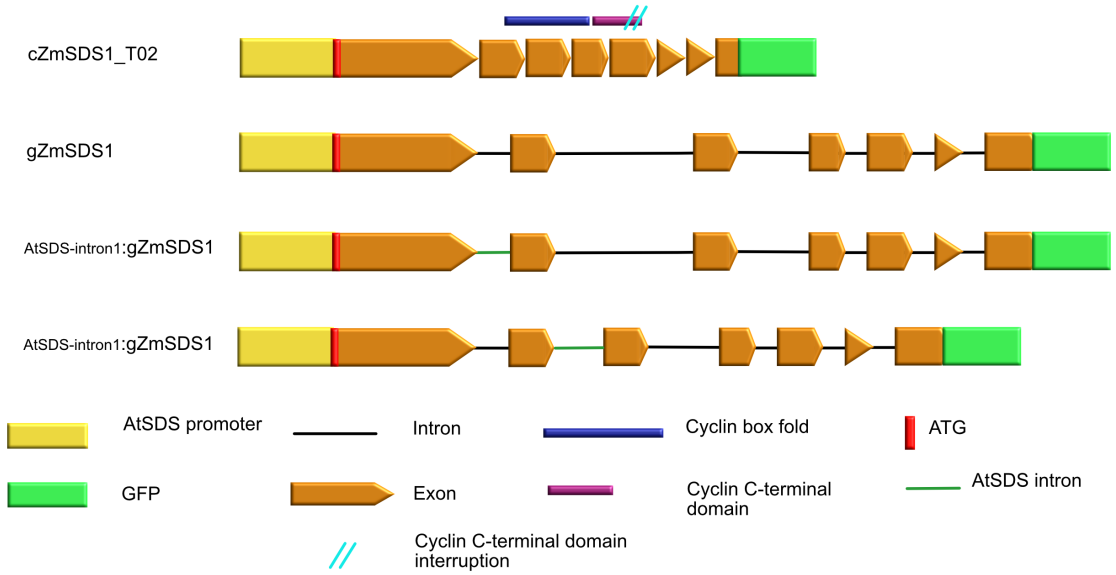

Figure 14. Phenotypic analysis of ZmSDS1 reporter constructs.
Models of the ZmSDS1 constructs that were generated in this dissertation.

Neither the genomic (A) nor the cDNA construct (B) of ZmSDS1 showed any rescue of the Arabidopsis *sds* mutant phenotype (Figure 6A-E). The results for the genomic construct are described in detail in Chapter I and match the results for the cDNA construct except for the level of GFP expression. A faint signal could be detected in the male meiocytes of plants harboring the cDNA construct. The cZmSDS1-GFP transgenic plants had siliques that were shorter than the WT (Figure 6A) and the number of viable seeds per silique was 1,2 ± 0,7 (Figure 6B-C) similar to the *sds* mutant in contrast to the genomic construct where no expression was seen, a weak GFP signal could be detected in meiocytes of plants expressing the cDNA construct (Figure 6D). The anthers also displayed uneven-sized pollen grains (Figure 6E).

Like the gZmSDS1-GFP and de cZmSDS1-GFP transgenics, also the plants containing the AtSDS-intron1:gZmSDS1-GFP construct did not show *sds* phenotypic rescue. Neither the anthers nor any other organs were found to express GFP, the silique length was as short as in the mutant and I counted 2.1 ± 1.7 seeds per silique, which corresponded to the *sds* mutant level. The pollen staining displayed the same malformed pollen as the observed in the *sds* mutant (Figure 6A-E).

However, plants expressing the construct *PRO$_{AtSDS\text{-}AtSDS\text{-}intron2}$:gZmSDS1-GFP* showed partial rescue. The complete dataset is presented in Chapter I. To summarize, the siliques of plants expressing construct D were shorter than the wildtype but notably longer than the *sds* mutant without the transgene. The seed sets were also partially restored, i.e. I counted 34.4 ± 6.1 seeds per silique compared to 64.1 ± 8.4 in the wildtype. The anthers contained qualitatively, viable pollen grains as also seen in the wildtype plants. A quantification is still needed to assess whether a similar number of pollen grains is observed in all the plants. The GFP signal could be found in the nuclei of the meiocytes, similar to the genomic *AtSDS* rescue construct. To see if this SDS1 construct was expressed in other organs, samples from the true leaves and the roots from Arabidopsis seedlings were examined under the confocal microscope. There was no signal found in any of the samples, therefore we concluded that this SDS1 construct is expressed only in anthers.

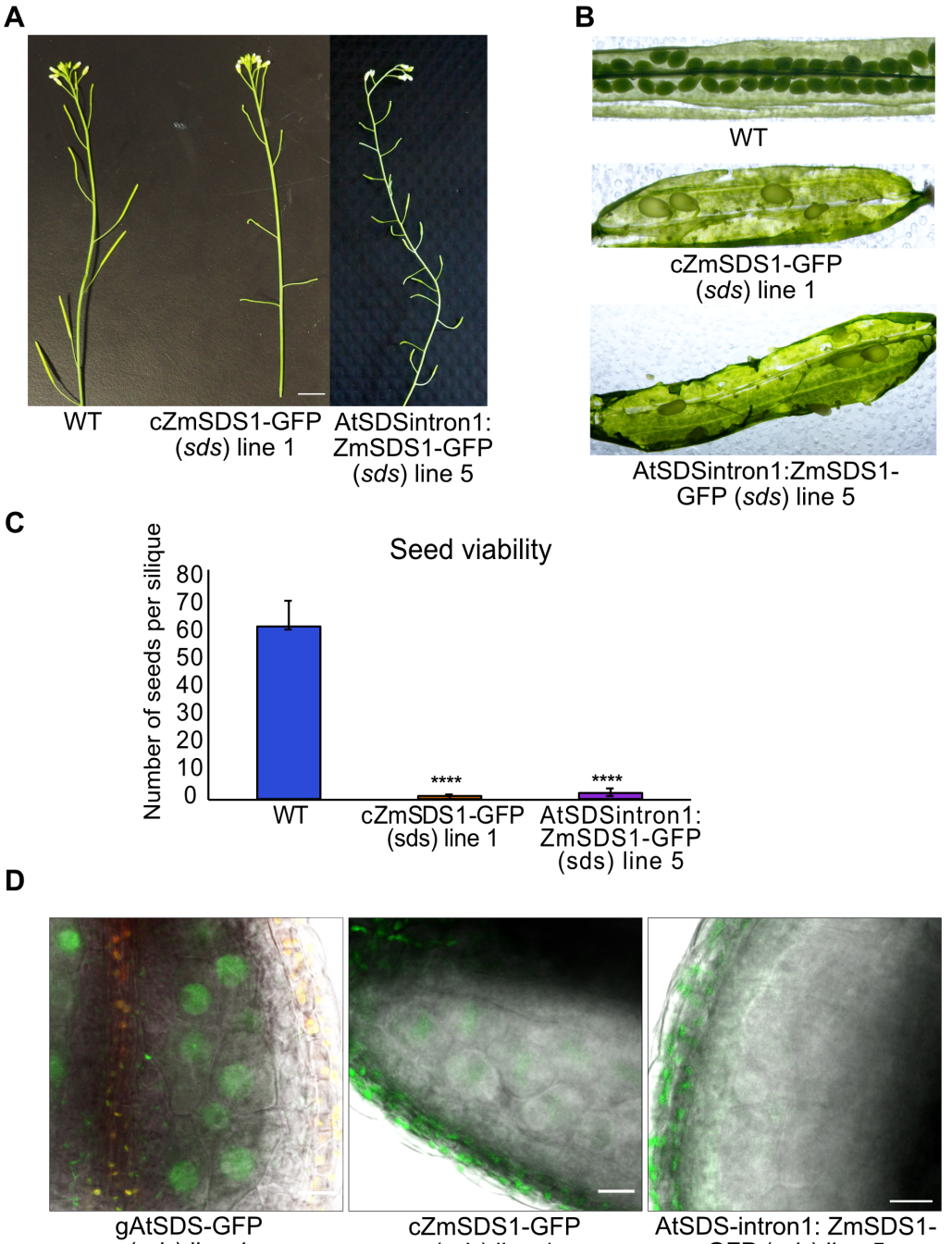
A
WT
cZmSDS1-GFP (sds) line 1
AtSDSintron1: ZmSDS1-GFP (sds) line 5
B
WT
cZmSDS1-GFP (sds) line 1
AtSDSintron1:ZmSDS1-GFP (sds) line 5
C
Seed viability
Number of seeds per silique
80
70
60
50
40
30
20
10
0
****
****
WT
cZmSDS1-GFP (sds) line 1
AtSDSintron1: ZmSDS1-GFP (sds) line 5
D
gAtSDS-GFP (sds) line 1
cZmSDS1-GFP (sds) line 1
AtSDS-intron1: ZmSDS1-GFP (sds) line 5
E
WT
cZmSDS1-GFP (sds) line 1
AtSDS-intron1: ZmSDS1-GFP (sds) line 5

Figure 15. Complementation analysis of ZmSDS1 constructs in sds (Arabidopsis).

(A) Main branches of WT (Col-0) and plant lines expressing *cZmSDS1-GFP*, and *gZmSDS1*^{AtSDSi1}*-GFP*.

(B) Open siliques of WT (Col-0) and plants expressing cZmSDS1-GFP (T02) and AtSDS-intron1:gZmSDS1-GFP.

(C) Quantification of viable seeds in the WT (Col-0) and plants expressing cZmSDS1-GFP (T02), and AtSDS-intron1:gZmSDS1-GFP lines. Asterisks represent significant differences from WT as determined by ANOVA test ($P < 0.001$).

(D) Confocal pictures of Arabidopsis anthers of the transgenic lines expressing gAtSDS-GFP, cZmSDS1-GFP (T02), and AtSDS-intron1:gZmSDS1-GFP. Bars = 10 µm.

(E) Peterson staining of anthers of WT (Col-0) and plants expressing cZmSDS1-GFP (T02), and AtSDS-intron1:gZmSDS1-GFP.

3 Discussion

3.1 The first two introns of *AtSDS* are not necessary for the meiosis-specific expression of SDS

Here, I wanted to investigate the influence of *SDS* introns in the regulation of gene expression, since a previous publication had suggested that the AtSDS introns could possibly contain regulatory motifs that led to its spatial and temporal expression. I used the IMEter algorithm to test the likelihood of an intron boosting gene expression (Parra et al., 2011). This tool could give a hint of which introns should be investigated first (Morello et al., 2011). Almost all the introns gave a positive score, but the first two introns had the highest score, therefore introns 1 and 2 were the first to be analyzed experimentally. However, in contrast to what was concluded previously (Huang et al., 2016), my cDNA version of *AtSDS* (*PRO*$_{AtSDS}$*:cAtSDS-GFP*) could complement the *sds* mutant, showing that neither the first two introns nor any other intron are essential for *SDS* expression. This analysis was complemented by deleting intron 1 and intron 2 from the genomic version (*PRO*$_{AtSDS}$*:gSDS*$^{\Delta i1}$*-GFP*; *PRO*$_{AtSDS}$*:SDS*$^{\Delta i2}$*-GFP*), which also led to fully functional constructs. The experiments performed by Huang in 2016 showed that their *SDS* promoter itself could not achieve the meiotic-specific expression, and

the signal could be found in other parts of the plant like the cotyledons, true leaves, and shoot apical meristem. On the other hand, the *SDS* promoter fused together with the genomic *SDS* gene, was expressed specifically in microspore and megaspore mother cells. As there was evidence of other gene introns regulating spatial expression (Fu et al., 1995a, 1995b; Jeong et al., 2006), they concluded that the same could happen in *AtSDS*. However, my results differ at least for the first two introns. There is still the possibility that other *SDS* introns harbor some regulatory sequences. Another possibility is the difference between the constructs I designed and the ones used by Huang et al., 2016. For example, the promoter they used was 1.5 kb long and it comprised from upstream the SDS coding region to the 3´non-coding regions of the *SDS* adjacent gene. In my case, I used a 2.1 kb *SDS* promoter that reaches the whole adjacent gene and the first exon of the next adjacent one. Also, my constructs had the 1 kb sequence amplified downstream of the SDS genomic region, which could have some regulatory motifs. Therefore, these discrepancies in the amplified sequences could make the difference in why my constructs led to different results than those explained by Huang et al., 2016.

As the Arabidopsis *SDS* plays also a role in female meiosis, it would be worth it to check its expression in female meiocytes, however, that was not performed in this study.

3.2 The maize SDS1 can be expressed in *Arabidopsis* when its second intron is removed

None of the maize constructs but *PRO*$_{AtSDS\text{-}AtSDS\text{-}intron2}$*:gZmSDS1-GFP* could at least partially rescue the *Atsds* phenotype. Since the Arabidopsis cDNA rescues the *sds* mutant phenotype (see above) we can conclude that Arabidopsis introns are not required for *SDS* expression. So it is more likely that the removal of the second maize intron, and not the addition of the equivalent exon from Arabidopsis, is the reason for the partial rescue seen with the *PRO*$_{AtSDS\text{-}AtSDS\text{-}intron2}$*:gZmSDS1-GFP* construct. The intron 2 of *ZmSDS1* is 1,5 kb long and according to information found in the maize database, there is a transposable element present within this second intron. Thus, maybe the heterologous TE

causes problems in the expression of *ZmSDS* in Arabidopsis, possibly due to heterochromatinization of the respective region.

In summary, these experiments show that *ZmSDS1* can be considered orthologous to *AtSDS1* since it at least partially complements the sds mutant phenotype. However, it would be interesting to investigate if the partial rescue is due to lower levels of expression, an overall less functional protein due to structural diversity or if only a subset of SDS function is rescued.

While the inhibitory intron 2 of maize can explain the non-rescue seen in plants carrying the genomic *ZmSDS1* construct, the failure of the cDNA construct must be due to other problems.

In the case of *ZmSDS1*, in the database, there are four splice variants annotated (Figure 1). At the beginning of this thesis, it was not clear which splice variant is the prominent one and therefore T02 was chosen for the here described experiments, as it could be easily amplified from cDNA made from the maize B73 inbred line. However, this second transcript is lacking 12 bp compared to T01 therefore the corresponding protein has 4 aa less, 3 of which are located at the end of the cyclin C-terminal domain. So possibly a non-functional protein is generated by this splice variant.

When analyzed by confocal, a weak GFP signal could be detected in meiocytes of plants carrying the cDNA construct. This could indicate rapid degradation of a non-functional protein, or hampered expression of the construct. Additional qPCR experiments could be done to test for the amount of mRNA produced, compared to the wildtype.

However, with respect to more recent RNAseq expression data T01 can be considered the primary splice variant and therefore should be tested in Arabidopsis instead of T02. Little is known about *ZmSDS1* or its different transcripts, therefore the option of having another transcript working instead of the one that is considered to be the active one is still a matter of discussion.

In summary, I could show that while *AtSDS* introns are not relevant for sufficient *AtSDS* expression in meiocytes, intron 2 of *ZmSDS1* exerts a negative effect on expression when used in a heterologous system, i.e., *Arabidopsis thaliana*. It was suggested in metazoan genes, that a large-sequence intron could lead to a delay of the transcription and thus, affect its dynamics and timing mechanisms (Swinburne et al., 2008).

With the generation of this genomic *ZmSDS1* variant (*PRO*$_{AtSDS\text{-}AtSDS\text{-}intron2}$*:gZmSDS1-GFP*), we could successfully generate a maize reporter gene that can be used to study the meiotic dynamics in other organisms such as *Arabidopsis* and can drive meiotic-specific expression.

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Materials and methods

1. Plant material and growth conditions

In this study, the *Zea mays* inbred lines A188 and B73, were obtained from Maize Genetics Cooperation Stock Center (http://maizecoop.cropsci.uiuc.edu). The inbred line A188 was backcrossed with B73 for the expression analysis. The maize plants were grown under greenhouse conditions with a 17h light/7 dark photoperiod, at 24°C day and night at 18°C.

The *Arabidopsis thaliana* ecotype Columbia (Col-0) was used in this study. The T-DNA insertion line *sds* (SAIL_129_F09) was obtained from the Salk Institute Genomics Analysis Laboratory (SIGnAL, http://signal.salk.edu/cgibin/tdnaexpress). All plants were grown in growth chambers under short-day conditions: 16h day/8h night at 21°C/18°C and 60% of humidity.

For more information about plant materials see Appendix **Table 2**.

2. Generation of the SDS double mutant by CRISPR/Cas9 genome editing

The *SDS* double mutant was generated in the maize A188 inbred line, where *SDS1* was already naturally mutated. Therefore, the genome-editing tool was used as a new approach to mutate *SDS2*. To achieve this, several sgRNAs were designed, 2 to target exon 1, a third for exon 4, and another one for exon 5. First, the generated oligos to target the first exon were cloned using the Gateway system into the cloning vector pENTRY4 sgRNA4, and the other two oligos for exons 4 and 5 were inserted in pENTRY4 sgRNA5 (Mini). The vectors were previously digested with Bsa1 and afterwards, the sgRNAs with the Bsa1 overhangs were ligated to the linearized vectors. The ligated products were transformed into *E. Coli* and selected in LB medium plates supplemented with Kanamycin. Some positive colonies were selected and in a second round of cloning like previously done, the vectors were digested but this time with BtgZ1, and the primers with BtgZ1 overhangs were inserted. To check that the clones were correctly inserted, they were sequenced by Sanger sequencing (Eurofins Genomics). To fuse the two targets in the pENTRY4 sgRNA vector with the two targets subcloned in pENTRY4 sgRNA5, the HindIII fragment of pENTRY4

sgRNA4 was cut out and ligated with the HindIII digested pENTRY4 sgRNA5. All the generated constructs were transferred to the p7oM-LH-GW destination vector. Afterwards, they were transformed into Agrobacterium tumefaciens by electroporation and transformed into maize. The generation of this double mutant and the expression analyses explained in Chapter I, were performed by Dr. Reinhold Brettschneider (University of Hamburg). All the primers and vectors used for the cloning are described in Appendix **Tables 1**, **7** and **9**.

3 Cloning of DNA constructs for complementation experiments

The ZmSDS1 reporter was generated by Dr. Martina Balboni (University of Hamburg). To sum up, the genomic sequence of SDS1 was amplified from the BAC clone chromosome 9 (AC205249.4) from the B73 inbred line by PCR. The sequence also included a 2 kb upstream sequence prior to the start codon and 550 bp downstream the stop codon. The amplified DNA was then inserted by SLiCE (Y. Zhang et al., 2014) into the entry vector pENTR2B and a *SmaI* restriction site was added by PCR before the stop codon. The construct was afterwards tagged with mRFP1. When the construct was complete and sequenced, it was transferred to the destination vector p7oM-LH-GW and transformed into Agrobacterium (LBA4404 strain).

For the complementation experiments in Arabidopsis, all the genes were amplified by PCR and cloned using the site-specific recognition Gateway system into pDONR221. Prior to transformation, each construct was subcloned into the destination vector R4pGWB504 (Nakagawa et al., 2008), which has already integrated the reporter gene enhanced green fluorescent protein (eGFP). The PRO_{AtSDS} was previously cloned into the pDONR-P4P1r plasmid and then included in the final vector. The *gZmSDS1* and the *cZmSDS1* of the B73 inbred line, were amplified by PCR with the attb overhangs and cloned into the pDONR221. To create the g*ZmSDS1* with the *AtSDS* second intron, such intron was amplified by PCR and inserted in the *gZmSDS1* (with its second intron deleted) by SLiCE reaction. For the construct $PRO_{AtSDS\text{-}AtSDS\text{-}intron1}$*:gZmSDS1-GFP*, the first intron of the g*ZmSDS1* was deleted and replaced by the first intron of *AtSDS,* and the cloning was continued as described before. The PRO_{AtSDS}*:gZmSDS1*$^{\Delta i2}$*-GFP* was generated by deleting the second intron of the

genomic *ZmSDS1* using a PCR approach. The *PRO$_{AtSDS}$:gAtSDS$^{\Delta i1}$*-GFP and *PRO$_{AtSDS}$:gAtSDS$^{\Delta i2}$-GFP* constructs were cloned by Gateway cloning as described before. The first intron and second intron were deleted by PCR in each respective genomic construct. *PRO$_{AtSDS}$:gAtSDS-GFP* and *PRO$_{AtSDS}$:cAtSDS-GFP* were amplified by PCR and subcloned by Gateway. All the primers and vectors used for the cloning are described in Appendix **Tables 1** and **9**.

4. Plant transformation

The *PRO$_{ZmSDS1}$:ZmSDS1-mRFP/p7oM-LH-GW* construct was transformed in the maize A188 background. To achieve this, the construct was firstly transformed in *Agrobacterium tumefaciens* (LBA4404) using the electroporation method and cultivated for three days onto YEB selective medium with 100 mg/L Spectinomycin and 10 mg/L Tetracycline. The positive bacteria were driven to a tube containing 5 mL of liquid infection medium (Appendix **Table 5**). The tube was incubated with shaking at 100 rpm for up to 4 h at RT. To pick up the immature embryos, the maize plants were firstly grown in a greenhouse under the conditions described before. The ears were harvested 10 days after self-pollination and stored in a cold room at 4 °C. Afterwards, immature embryos of 1.8 – 2.1 mm size were selected and dissected. To carry out the dissection, the ears were split in two parts and sterilized for 25 minutes in a 3% NaOCl + 0.1% Labosol, which was later rinsed in 1.5 L of sterile water. The dissected embryos were dipped in 2 mL of infection medium and washed twice with a fresh infection medium. The final wash was removed and the embryos were suspended in 1 mL of *Agrobacterium* (OD600 = 0.4) suspension liquid. To improve the embryo infection, the tubes were inverted 20 times and let it stand for five minutes at RT. Embryos were put onto a plate with the scutellum positioned face up, and the plate was filled up with co-cultivation medium. The excess of Agrobacterium was removed from the medium surface by pipetting. The plates with the embryos were stored in dark conditions for 3 days at 22 °C. The embryos were later transferred to the following media: resting medium (7 days at 28°C, in the dark), selection medium I (14 days at 28°C, in the dark), selection medium II (14 days at 28°C, in the dark), selection medium III (14 days at 28°C, in the dark, regeneration medium I (21 days at 26°C, in the dark), regeneration medium II (21 days at 26°C,

in the light). After all these incubations, the regenerated plantlets were put into magenta boxes full of regeneration medium II and left for one week. Once this process was done, the plantlets were moved to the greenhouse. The maize transformations were performed by Dr. Reinhold Brettschneider (University of Hamburg) and Dagmar Stang (University of Hamburg).

The constructs studied in *Arabidopsis thaliana* were transformed with the floral dip method (Clough & Bent, 1998). To sum up, the constructs were transformed into *Agrobacterium tumefaciens* (MP90) by heat shock method and selected in both 30 mg/L Gentamycin and 100 mg/L Spectinomycin. A positive colony was picked and resuspended in a liquid selective medium and incubated O/N in darkness at 28°C. Afterwards, the sample was centrifuged at 4000 rpm for 5 minutes and the supernatant was discarded. The pellet was resuspended in a 3 mL transformation medium. Flowering plants were used and prepared, cutting the siliques and opening flowers. The transformation liquid was sucked with a pipette and impregnated the closed flower buds with it. After completing the coating, the plants were put inside plastic bags and left for 48 hours in darkness. Then the plants were taken to a dry chamber with long-day conditions, where the plants would grow new siliques and left to dry. T1 seedlings were selected under half-strength MS agar plates with Hygromycin b (50 mg/L), and the T2 seedlings were selected in ½ MS agar plates with Hygromycin B 25 mg/L. For the buffers and solutions, as well as bacteria strains see Appendix **Tables 5** and **7**.

5. gDNA extraction and plant genotyping

For genotyping maize plants, first, a piece of leave was taken as a sample from each plant and frozen in liquid nitrogen, and ground. Then, the genomic DNA was extracted using a Genomic DNA Mini Kit-Plant (Geneaid). To assess the zygosity of *SDS2*, A 674 bp fragment comprising part of the first exon and first intron of *SDS2*, was generated by PCR using Terra PCR Direct Polymerase Mix (Takara Bio). The subsequent PCR product was run in a 1% agarose gel and the band was extracted and purified using a DNA purification kit (MACHEREI-NAGEL). The purified samples were sent for sequencing (Eurofins Genomics) and the results were analyzed to detect the mutation of *SDS2*.

The maize A188 lines containing the *PRO$_{ZmSDS1}$:gZmSDS1-RFP* construct were previously selected by spraying BASTA solution (250 mg/L BASTA (BASF) and 0.1% Tween 20 (Sigma)). The gDNA extraction and SDS2 genotyping procedure was done as described before.

In the case of Arabidopsis, the first true leaves from 8/12-day-old seedlings were ground and 250 µl of Magic buffer (Appendix 5.1 Table 5) were added. To genotype the *sds* plants a PCR with Dream Taq PCR Master Mix (Thermo Fisher) was performed, and three different primers were used to assess the presence of the T-DNA insertion (Appendix **Table 3-4**).

6. Staging of the maize anthers

All the anthers were collected as described by He in 2016. To obtain the right stages, one anther was smashed on a slide with a drop of Acetocarmine (Appendix **Table 5**) solution and afterwards covered with a coverslip. Subsequently, the slides were placed on a hotplate at 60°C for 10 minutes. After determining the correct stages under a light microscope, the rest of the anthers were fixated in different ways depending on the assay.

7. Phenotypic analysis in Arabidopsis

For the complementation experiments in Arabidopsis, all the plants were analyzed under the confocal microscope to detect the presence of GFP signal in male meiocytes. The phenotypic analysis was performed in the positive plants by studying the pollen viability (using the Peterson staining method; Appendix **Table 5**), silique length, and seed abortion. Three replicates per construct were assessed and 10 siliques per plant were checked, and the seeds were counted distinguishing between green viable seeds and white shrunken aborted seeds.

8. Chromosome spreads

For the chromosome spreads in maize, the whole tassel was stored in ethanol and acetic acid (3:1) solution for 1 day. This process was repeated until the green color was gone. Afterwards, the tassel was put in ethanol 70% and stored at -4°C until use. The maize anthers were put into a plate with ethanol 70% and dissected under a binocular. In the case of Arabidopsis, the flower buds were

fixated, separated, and organized according to their size. Spreading was performed as described before by Wijnker et al., in 2012. To sum up, both maize anthers and Arabidopsis flower buds were digested using an enzyme solution dissolved in 0,01 M citrate buffer and incubated for 1h (maize) and 3h (Arabidopsis) respectively at 37°C. For spreading, 1 anther/flower bud was placed on a slide with 15-30 µl of 45% acetic acid, on a hotplate at 46°C. The slides were rinsed with ice-cold ethanol/acetic acid (3:1). To stain the DNA, a drop of mounting medium Vectashield with DAPI (Vector Laboratories) was added, and the slide was covered with a coverslip. For the buffer solutions, see Appendix **Table 5**.

9. Immunolocalization assay

Tassels were taken at the right stage and anthers were dissected using tweezers. The fixation, as well as the immunostaining procedure, were carried out as described by Wang (2013). In summary, anthers were fixed with 4% paraformaldehyde in 1X Buffer A for 45 minutes. Anthers were squeezed with a cannula under a binocular and placed the solution on a slide. Acrylamide pads (15% Acrylamide/Bisacrylamide in 1x buffer A) were made to cover the meiocyte solution and left drying for 40 minutes. Later, the pads were incubated for 1 h in a membrane permeability solution. They were subsequently dipped into the blocking solution for 2h. The first antibody was added and incubated in a humid chamber overnight at room temperature. Pads were washed three times (30 minutes each) with washing buffer. A secondary antibody was added and incubated for 1h. After washing three times with the previous solution and another three times with 1x PBS 10 minutes each, an anti-fade DAPI solution (Vector Laboratories) was incorporated to stain the chromosomes. The pads were washed with 1x PBS three times for 20 minutes each and added a drop of DABCO (Sigma D2522). After 10 minutes, the pad was covered by a coverslip. Anti-DSY2 antibody was used at 1:200 dilution. Goat anti-rabbit Alexa Fluor 488 secondary antibody (Invitrogen) was used at 1:100 dilution. The pictures were taken using a Zeiss LSM 780 confocal microscope and ZEN 2.3 SP1 software (Carl Zeiss AG, Oberkochen, Germany). Buffers and solutions can be seen in Appendix **Table 5**. The antibodies can be seen in Appendix **Table 8**.

10. Confocal microscopy analysis and sample preparation

To take pictures of the reporter lines, an LSM880 Zeiss confocal microscope was used. For the analysis of the maize *PRO$_{ZmSDS1}$:ZmSDS1-RFP* transgenic plants, anthers on the right stages were taken and put onto a slide in a drop of water. For the Arabidopsis complementation experiments, small flower buds were selected and dissected; afterwards, several anthers were deposited on the slide with a drop of water. All the slides containing water were visualized with a 40X water objective. For the immunolocalization and chromosome spread experiments, a 63X oil objective was used. The samples containing GFP or Alexa Fluor dye 488 were excited at λ = 488 nm and detected between 498 and 560 nm. On the other hand, RFP, Alexa Fluor dye 594, and Alexa Fluor dye 568 were excited at λ = 561 and detected between 578 and 603. For the auto-fluorescence, a range of 680 to 750 nm was applied.

11. Pollen viability assay

The pollen grains were analyzed using the Peterson staining method (Peterson et al., 2010; Appendix **Table 5**). The maize anthers were dissected under a binocular and with the help of a cannula, squeezed onto a slide with 18 µl of Peterson staining solution. In the case of Arabidopsis, mature flower buds were opened and a total of 10 anthers were dipped into 30 µl of solution for 20 seconds. All the slides were covered with a coverslip and left staining overnight. Afterwards, the slides were heated on a hotplate at 80 °C for 1 h, and later the pollen grains were analyzed, quantified, and imaged using a light microscope. Three anthers from three biological replicates were used for quantification.

12. Accession numbers

The maize protein SDS1 and SDS2 sequence data from this article can be found in the GenBank data libraries under accession numbers AFW89131 and DAA44170 respectively. The accession numbers for the protein alignment and the phylogenetic tree are the following: *Arabidopsis thaliana* (Q1PFW3), *Oryza sativa* (AK065907), *Sorghum bicolor* (XP_021318147) and *Brassica napus* (XP_013696127).

13. Quantification of RAD51 foci

The quantification of RAD51 foci was processed as a three-part problem in a stepwise elimination, based on the estimation of intensity above the global mean intensity of the image. The scanning time was optimized by applying no overlapping region on the predefined window. The z-stacks were split in the RAD51 and DAPI channels. In the RAD51 channel, the location of peak intensities was estimated. Several boundary conditions were set to locate the foci, such as the nucleus size and the segmented chromosomes from the DAPI channel. A maximum projection was performed on the z-stacks of the DAPI channel and generated a histogram-based segmentation. In a final step, the channels were merged to do the final count. More details of the boundary conditions and the flow chart can be found in **Supplemental Figure 6** and **Supplemental Table 1**.

14. Image processing

All the images and z-stack series taken by confocal microscopy in this research were saved as .czi image format and were converted to TIFF images or AVI video formats using the program ImageJ (Schindelin et al., 2012). Brightness and contrast were changed and scale bars were applied also using this program.

15. Statistical analysis

Normality tests were performed to see if the data followed a normal Gaussian distribution using the Kolmogorov-Smirnov or the Shapiro-Wilk tests. To assess the variance, the F-test was applied. To determine a statistical difference between the two groups, the two-tailed student´s t-test was used. In the case of having more groups, the analysis of variance one-way ANOVA test followed by Tukey´s test was calculated. Whenever the data was not normally distributed, alternative non-parametric tests were applied, independent samples Mann-Whitney U test and Kruskal-Wallis depending on the number of data sets. The level of significance determined from the p-values between two or more samples are represented by asterisks: $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

Appendix

Table 3. Primers used in this study.

Primer name	Sequence 5´- 3´	Purpose
btgCrisprSDS2-ex1.1up	TGTTGTGCGATGAGCTGACCCCGT	Cloning CRISPR/Cas9
btgCrispSDS2-ex1.1dw	AAACACGGGGTCAGCTCATCGCAC	
bsaCrisprSDS2-ex1.3up	GTGTGCACGTTTTGGGGTGCACGC	
bsaCrisprSDS2ex1.3dw	AAACGCGTGCACCCCAAAACGTGC	
ZmSDS1-F	ATGCCTCCCACCATGCTCGCGCC	Cloning gZmSDS1 and cZmSDS1 reporters in Arabidopsis
ZmSDS1-R	TCACGAGACGTACTTGATCAGCC	
ZmSDS1-attb1-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTAAATGCCTCCCACCATGCTCGCGCC	
ZmSDS1-attb2-R	GGGGACCACTTTGTACAAGAAAGCTGGGTTCGAGACGTACTTGATCAGCC	
AtSDS-I1-SLiCE-F	CCAAGTCCTCAAACCGCCTCCCCTAAAACGAAAATGTATACTTAAG	Cloning $_{AtSDS\text{-}intron1}$:ZmSDS1 reporter
AtSDS-I1-SLiCE-R	CCACTCTCGATCTCCTGACGGTAAGTGGTATAATGATTTCATATC	
ZmSDS1-I1-del-F	GGGAGGCGGTTTGAGGACTTGG	
ZmSDS1-I1-del-R	CGTCAGGAGATCGAGAGTGGAAC	
AtSDS-I2-SLiCE-F	CATGGTGAACTGGATCATCCAGGTGAATTTTAACTTTCTG	Cloning $_{AtSDS\text{-}intron2}$:ZmSDS1
AtSDS-I2-SLiCE-R	GAGCTTCATCAGACGTGAATGCTGCTTGTAATCAGGGAGCAGA	
ZmSDS1-I2-del-F	CATTCACGTCTGATGAAGCTC	
ZmSDS1-I2-del-R	CTGGATGATCCAGTTCACCATG	
AtSDS-I1-del-F	TTGCTAAGGTTTGATGATGAGG	Cloning $AtSDS^{\Delta I1}$
AtSDS-I1-del-R	TTCAGAGTGAATTTCTTCCTCG	
AtSDS-I2-del-F	CAATGTTCTGACATGGGGCTTCAG	Cloning $AtSDS1^{\Delta I2}$
AtSDS-I2-del-R	CTTTACAATCCATTGAACCATGATGG	
SDSp-attb4-F	GGGGACAACTTTGTATAGAAAAGTTGGTGTAACATGAACAACTGTTCGGTGCT	Cloning AtSDS promoter
SDSp-attb1-R	GGGGACTGCTTTTTTGTACAAACTTGGTTTTTCTCCGTACGAAAGCTTGAAA	
AtSDS-F	ATGAAGGAGATCGCGATGAGGA	Cloning gAtSDS and cAtSDS reporter
AtSDS-R	CTGCCCAAGCAACCAGTCCA	
AtSDS-attb1-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTAAATGAAGGAGATCGCGATGAGGAA	

AtSDS-attb2-R	GGGGACCACTTTGTACAAGAAAGCTGGGTTCTGCCCA AGCAACCAGTCCA	
AtSDS - LP	CTGCTCCCTGATTACAAGCAG	Genotyping AtSDS
AtSDS - RP	CTTAACGCATTCAGGCAACTC	
atsds - BP	TAGCATCTGAATTTCATAACAATCTCGATACAC	
gSDS-F1	CGCTTGACTGTCTCGCTTTCTAC	Cloning gZmSDS1 reporter
gSDS-R1	TATGATTCCAGCCAGACATACCA	
gSDS-F2	GCTGGTATGTCTGGCTGGAATCA	
gSDS-R2	AGATTCCCCGGATAGAGACTGCT	
pENTR2B-SDS-F	AAAGCAGTCTCTATCCGGGGAATCTGCGGCCGCACTC GAGATATCTAG	
pENTR2B-SDS-R	TTGTAGAAAGCGAGACAGTCAAGCGGGATCCAGTCGA CTGAATTGGTTC	
SDS-SmaI-F	GGGTGATACCCAGAGCTCCCAGGTG	
SDS-SmaI-R	GGGCGAGACGTACTTGATCAGCCACTC	
SDS_Fw_seq1	TAGCAGATGATGGCAATTCG	Sequencing ZmSDS1 reporter
SDS_Fw_seq2	AATTGGCGAAACTCACTTGC	
SDS_Fw_seq3	ATACGGTGGGACTTGTGAGC	
SDS_Fw_seq4	GCCTCATCGCAAAAACCTAC	
SDS_Fw_seq5	GAGGTCGAGGTCTCCGAATC	
SDS_Fw_seq6	CTCCATACCCGACAGCTACC	
SDS_Fw_seq7	GGCAGCCAACAGAGTTAGGA	
SDS_Fw_seq8	TTCGCATCTGAACCCCTATC	
SDS_Fw_seq9	CAATTGGTAATGTTCTCCATTGT	
SDS_Fw_seq10	GGGTTTCCTGATTGTGCTGT	
SDS_Fw_seq11	CCTGGCCTTCATCTCACTTC	
SDS_Fw_seq12	GCAAATTTTTAGCCAACCAA	
SDS_Fw_seq13	CCTGTCATGTTGCCTGCTAA	
ZmSDS2-LP	CCTCGATCAGACTGCGACGGCTG	Genotyping ZmSDS2 and sequencing
ZmSDS2-RP	CAAGCATTCCCACAAAACATTACC	
ZmSDS2-SEQ	ATCGAACCCCTCCCAACAAATTG	
ZmSDS16 Ex1 F	ACCGAAGATGACAACGACGAC	Sequencing ZmSDS2
ZmSDS16 Ex2 F	ATGGTGGACTGGATCATTGAG	
ZmSDS16 int2 F	TTTCTCTGAATTTCCCTTGCA	
ZmSDS16 Ex3 F	TGCTGGCCATTGCCTGCATCA	
ZmSDS16 Ex4 F	CTTGCGTTTAGAAGTGTCCAT	
ZmSDS16 Ex5 F	AACACCTCACCATTTCCTATG	
ZmSDS16 Ex6 F	TCATGCCATTTGGTGATGGAG	
ZmSDS16 Ex7 F	GATCTACCAGAATGCCTAATG	

Table 4. Plant material used in this research

A188 (*Zea mays*)	Maize Genetics Cooperation Stock Center
B73 (*Zea mays*)	Maize Genetics Cooperation Stock Center
A188 x B73	This thesis
sds1sds2-1 (A188) - (*Zea mays*)	This thesis
sds1sds2-2 (A188) - (*Zea mays*)	This thesis
sds (*Arabidopsis thaliana*)	SAIL_129_F09

Table 5. PCR master mix used in this research.

PCR reaction mix	**Volume**	**Purpose**
PrimeSTAR Max Premix (2X)	25 µl	Cloning
Primer F	1.5 µl	constructs
Primer R	1.5 µl	
DNA template	< 200 ng	
Sterile distilled water	Up to 50 µl	
5X Phusion GC buffer	10 µl	Cloning
Phusion DNA Polymerase	0.5 µl	constructs
dNTPs (10 mM)	1 µl	
DMSO	1.5 µl	
Primer F	1.5 µl	
Primer R	1.5 µl	
DNA template	< 200 ng	
Sterile distilled water	Up to 50 µl	
DreamTaq Green PCR mastermix	7.5 µl	Genotyping
Primer F	0.5 µl	Arabidopsis/C
Primer R	0.5 µl	olony PCR
Sterile distilled water	Up to 14 µl	
2X Terra PCR Direct Buffer	25µl	Genotyping
Primer F	2.5 µl	maize
Primer R	2.5 µl	
DNA template	1µl	
Sterile Water	Up to 25µl	

Table 6. PCR conditions used in this research

PCR Master Mix	**PCR conditions**	**Temperature**	**Time**	
PrimeSTAR	Initial denaturation	98 °C	30 s	
	Denaturation	98 °C	10 s	x 35
	Annealing	55 °C	5s	
	Elongation	72 °C	10 s / Kb	
	Final elongation	72 °C	2 min	
	Hold	16 °C	∞	

Phusion Polymerase	Initial denaturation	95 °C	30 s	
	Denaturation	98 °C	10 s	x 35
	Annealing	*	30s	
	Elongation	72 °C	30 s / Kb	
	Final elongation	72 °C	10 min	
	Hold	16 °C	∞	
DreamTaq Green	Initial denaturation	95 °C	5 min	
	Denaturation	95 °C	30 s	x 30
	Annealing	55 °C	3s	
	Elongation	72 °C	1 min	
	Final elongation	72 °C	5 min	
	Hold	16 °C	∞	
Terra Polymerase	Initial denaturation	98 °C	2 min	
	Denaturation	98 °C	10 s	x 35
	Annealing	60 °C	15s	
	Elongation	68 °C	1 min	
	Final elongation	68 °C	5 min	
	Hold	16 °C	∞	

* The annealing temperature was calculated according to: https://tmcalculator.neb.com/#!/main

Table 7. Buffers, solutions, and media

LB medium	1% Tryptone 0.5 % Yeast Extract 0.5 % NaCl 0.8% Agar ddH_2O up to volume
YEB medium	1% Peptone 0.5 % Yeast Extract 0.5 % NaCl 0.8 % Agar ddH_2O up to volume, pH 6.8
SOC medium	0.5% Yeast extract 2% Tryptone 10 mM NaCl 2.5 mM KCl 10 mM MgCl 10 mM $MgSO_4$ 20 mM Glucose ddH_2O up to volume
Infection-Medium (1X)	For 1L 100 mL N_6-Macro-Salts 1 mL N_6-Micro-Salts

	1 mL N6-Vitanime 2 mL NaFe-EDTA 0.7 g Proline 1.5 mL 2,4-D (1mg/mL) 68.4 g Sucrose 36 g Glucose pH 5.2 acetosyringone (100µM) is added prior to use
Co-cultivation medium (2X)	For 1L 8.6 MS basal powder 2 ml MS Vitaminstock (1000X; 100 ml with 200 mg glycine, 50 mg Thiamin HCL, 50 mg Pyridoxin, 5 mg Nicotinic acid) 60 g Sucrose 4 ml myo-Inositol (50 mg/ml) 200 mg cas-Aminoacids 1.4 g Proline 1 ml Dicamba (30 mM dissolved in 50% EtOH) pH 5.8 + Silvernitrate (50 mM) + L-Cysteine (15mg7ml) + Acetosyringone (100 mM)
Resting and Selection medium (2X)	For 2L 17.2 MS basal powder 4 ml MS Vitaminstock (see above) 2 ml Dicamba (30 mM) 120 g Sucrose 2 g MES 400 mg myo-Inositol (50 mg/ml) 400 mg cas-Aminoacids 2.8 g Proline pH 5.8 + Silvernitrate (50 mM) + Carbenicillin (250 mg/ml) + Basta (20 mg/ml) 37,5 µl for Selection I; 75 µl for Selection II; 125 for Selection III
Regeneration medium I (2X)	For 2L 17.2 MS basal powder 4 ml MS Vitaminstock (see above) 240 g Sucrose 8 ml myo-Inositol (50 mg/ml) pH 5.8

	+ Cefotaxim (250 mg/ml) + Basta (20 mg/ml)
Regeneration medium II	For 500 mL 250 ml 2XMS 250 ml Phytagel (1.5 g/250 ml) 1 ml myo-Inositol (50 mg/ml) + Basta
Murashige and Skoog (1/2 MS)	0.2% MS basal powder 1% sucrose 1% agar pH 5.8
Acetocarmine staining	0.5 g Carmine in 45% Acetic acid
Infiltration medium (BiFCs)	For 100 mL 42,5 mL NaH_2PO_4 7.5 mL Na_2HPO_4 2.5 mL $MgCl_2$ 47.5 mL ddH_2O
Magic buffer	For 1 L 50 mL 1M TrisHCl, pH 7.5 60 mL 5M NaCl 100 g Sucrose
Enzyme mix (Chromosome spreads)	1% Cellulase 1% Pectolyase 1% Cytohelicase Dissolve in 0.01 M Citrate buffer ph 4.5
0.01 M Citrate buffer	For 100 mL, pH 4.5 4.45 mL of 0.1 M Sodium Citrate 5.55 mL of 0.1 M Citric Acid
1X SDS-page sample buffer (Co-IP)	62.5 mM Tris-Cl, pH 6.8 2% (w/v) SDS 10% (w/v) glycerol 1% β-mercaptooethanol 0.005% bromophenol blue
Ni-NTA binding buffer (Co-IP)	NaH_2PO_4 50 mM NaCl 100 mM 10% (w/v) glycerol Imidazole 25 mM pH 8.0
Ni-NTA washing buffer (Co-IP)	NaH_2PO_4 50 mM NaCl 250 mM 10% (w/v) glycerol Imidazole 25 mM pH 8.0
Transfer blot buffer (Co-IP)	For 1L Tris-Cl 29.1g Glycine 14.15 g SDS 2.85 g

	20% Methanol
10X TGS buffer	For 5 L, pH 8.3 25 mM Tris 192 mM Glycine 0.1% (w/v) SDS
Coomassie blue staining solution	0.1% Coomassie Brilliant Blue R-250 50% Methanol 10% Glacial acetic acid
Coomassie blue destaining solution	40% Methanol 10% Glacial acetic acid
10X Buffer A salts (Immunolocalization)	150 mM PIPES 800 mM KCl 200 mM NaCl 20 mM EDTA 5 mM EGTA Distilled H_2O up to volume, pH 6.8
2X Buffer A (Immunolocalization)	For 50 mL 2 mL of 10X Buffer A salts 10 µl Spermine stock 25 µl Spermidine stock 20 µl DTT stock 3.2 mL sorbitol
10X PBS (Immunolocalization)	For 1 L, pH 7.4 80g NaCl 2g KCl 14.4g Na_2HPO_4 2.4g KH_2PO_4
Permeabilization buffer (Immunolocalization)	1X PBS 1% Triton X-100 1 mM EDTA
Blocking buffer (Immunolocalization)	1X PBS 5% BSA 1 mM EDTA 0.1% Tween 20
Washing buffer (Immunolocalization)	1X PBS 0.1 % Tween 20 1 mM EDTA
DABCO antifade solution (Immunolocalization)	2.5 % (w/v) DABCO 50 mM Tris pH 8.0 90% Glycerol
Carnoy´s solution	10 mL Acetic acid 30 mL Ethanol
TBST buffer	For 1L, pH 7.4 2.41 g Tris 8.7 g NaCl 1 mL Tween 20
10X SLiCE buffer	For 1mL

	500 µl 1M Tris-HCL pH 7.5 50 µl 2M $MgCl_2$ 100 µl 100 mM ATP 10 µl 1 M DTT ddH_2O
TE buffer	Tris-HCl pH 7.5 10 mM EDTA 1 mM ddH_2O up to volume
Plant transformation media (*Arabidopsis*)	5% Sucrose 0.05% Silwet-77 ddH_2O up to volume
Peterson staining	10% Methanol 0.001% Malachite green 25% Glycerol 0.005% Acid fuchsin 0.0005% Orange G 4% Acetic acid ddH_2O up to volume
1X TAE buffer	Tris-Acetate 40 mM EDTA 2 mM

Table 8. Commercial kits

Gateway BP Clonase II Enzyme mix	Thermo Fisher Scientific (CAT #11789020)
Gateway LR Clonase II Enzyme mix	Thermo Fisher Scientific (CAT #11791020)
NucleoSpin® Gel and PCR Clean-up	Macherey-Nagel (CAT #740609.250)
Presto™ Mini Plasmid kit	Geneaid (CAT #PDH300)
VECTASHIELD® with DAPI	Vector Laboratories (CAT #H-1200)
PrimeSTAR® Max DNA Polymerase	TAKARA BIO INC® (CAT #R045A)
Phusion® High-Fidelity DNA Polymerase	New England Biolabs (CAT #M0530S)
DreamTaq Green PCR Master Mix (2X)	Thermo Scientific™ (CAT #K1081)
Terra™ PCR Direct Red Dye Premix	TAKARA BIO INC® (CAT #639286)
Ligation mix	TAKARA BIO INC® (CAT #6023)
Clarity™ Western ECL Substrate	Bio RAD (CAT #1705061)
RNeasy Plant Mini kit (50)	Qiagen (CAT #74904)
RNase-Free DNase Set (50)	Qiagen (CAT #79254)
DNeasy Plant Pro kit (250)	Qiagen (CAT #769206)
Transcriptor First Strand cDNA Synthesis kit	Roche (CAT #04379012001)
Light Cycler 480 SYBR Green I Master	Roche (CAT #04707516001)
Plant protease inhibitor	Sigma (CAT #P9599)
Mini-PROTEAN®TGX Stain-Free 4-15%	Bio RAD (CAT #4568086)
Roti®-PVDF membrane	Carl Roth (CAT #T860.1)

Table 9. Bacteria strains used in this research

E. coli TOP10	Thermo Fisher Scientific (CAT #C404010)
E. coli SoluBL21(DE3)	AMS Biotechnology (CAT #C700200)
A. tumefaciens GV3101 PMP90	DNA Cloning Service
A. tumefaciens LBA4404	Thermo Fisher Scientific (CAT #18313015)

Table 10. Antibodies used in this research

Anti-DSY2 (maize)	Rachel Wang
Anti-RAD51 (maize)	Wojtek Pawlowski
Anti-HEI10 (maize)	Wojtek Pawlowski / (Wang et al., 2012)
Anti-MLH3 (maize)	Wojtek Pawlowski
Anti-ASY1 (maize)	Changbin Chen / (Zhang et al., 2021)
Anti-ZYP1 (maize)	Changbin Chen / (Zhang et al., 2021)
Goat anti-Guinea pig polyclonal secondary antibody Alexa Fluor™ 594	Thermo Fisher Scientific (CAT #A-11076)
Goat anti-rabbit polyclonal secondary antibody Alexa Fluor™ 488	Thermo Fisher Scientific (CAT #A32731)
RFP-Booster Alexa Fluor® 568	Chromotek (CAT #rb2AF568-10)
Anti-Strep-Tag monoclonal antibody	Thermo Fisher Scientific (CAT #MA5-17283)
Anti-MBP tag	GenScript (CAT #A00190)
Mouse monoclonal anti-His	Qiagen (CAT #34660)

Table 11. Constructs and vectors used in this research

Constructs for expression in *Arabidopsis* (Col-0)	
pUC-DONR-P4P1r/SDSpro (*Arabidopsis* SDS promoter)	Dr. Shinichiro Komaki
R4PGWB504/SDSpro::gAtSDS-GFP	This thesis
R4PGWB504/SDSpro::cAtSDS-GFP	This thesis
R4PGWB504/SDSpro::gAtSDS$^{\Delta I1}$-GFP	This thesis
R4PGWB504/SDSpro::gAtSDS$^{\Delta I2}$-GFP	This thesis
R4PGWB504/SDSpro::gZmSDS1-GFP	This thesis
R4PGWB504/SDSpro::cZmSDS1-GFP	This thesis
R4PGWB504/SDSpro::gZmSDS1^{AtSDSi1}-GFP	This thesis
R4PGWB504/SDSpro::gZmSDS1^{AtSDSi2}-GFP	This thesis
R4PGWB504/SDSpro::gZmSDS1$^{\Delta I2}$-GFP	This thesis
Constructs for expression in *Zea mays* (A188)	

p7oM-LH-GW/SDS1pro::gZmSDS1-mRFP1	Dr. Martina Balboni
pENTRY4 sgRNA4 (CRISPR/Cas9)	This thesis
pENTRY4 sgRNA5 (Mini) (CRISPR/Cas9)	This thesis
Constructs for expression in *Nicotiana tabacum*	
pBiFC-2in1-CC/ZmSDS1-ZmCDKA;1	This thesis
pBiFC-2in1-CC/ZmSDS1-ZmCDKA;3	This thesis
pBiFC-2in1-NC/SWI1-PDS5	Dr. Chao Yang; (Yang et al., 2019)
Constructs for expression in *E. coli*	
pCDFDuet-GST-Cak1/AtCDKA;1	Dr. Chao Yang
pCDFDuet-GST-Cak1/ZmCDKA;1	This thesis
pCDFDuet-GST-Cak1/ZmCDKA;3	This thesis
pHMGWA/ZmSDS1	This thesis

Publications and presentations

Publications

Sanz, O., Balboni, M., Brettschneider, R., Schnittger, A. Functional characterization of the meiotic cyclins SOLO DANCERS 1 and SOLO DANCERS 2 in maize. (Paper in preparation).

Bustillo-Avendaño, E., Ibáñez, S., **Sanz, O.**, et al. Regulation of Hormonal Control, Cell Reprogramming, and Patterning during De Novo Root Organogenesis. Plant Physiol. 2018. 176(2):1709-1727. DOI: 10.1104/pp.17.00980.

Oral presentation

- 3rd Edition European Maize Meeting, Montpellier, France. May 15-17, 2019.

Poster presentations

- 4th Edition European Maize Meeting, Regensburg, Germany. September 15-17, 2021.

- 12th International PhD School in “Plant Development”, Zellingen-Retzbach, Germany. October 2-4, 2019.

- 60th Annual Maize Genetics Conference, Saint-Malo, France. March 22-25, 2018.

- 2014: BIOVEGEN meeting, “Science, Technology, and Innovation in plant production”. Madrid (Spain).

Eidesstattliche Versicherung

Declaration on oath

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

I hereby declare, on oath, that I have written the present dissertation by my own and have not used other than the acknowledged resources and aids.

Hamburg, den 21/09/2022 **Unterschrift** 

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