The Axial Element Protein DESYNAPTIC2 Mediates Meiotic Double-Strand Break Formation and Synaptonemal Complex Assembly in Maize

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During meiosis, homologous chromosomes pair and recombine via repair of programmed DNA double-strand breaks (DSBs). DSBs are formed in the context of chromatin loops, which are anchored to the proteinaceous axial element (AE). The AE later serves as a framework to assemble the synaptonemal complex (SC) that provides a transient but tight connection between homologous chromosomes. Here, we showed that DESYNAPTIC2 (DSY2), a coiled-coil protein, mediates DSB formation and is directly involved in SC assembly in maize (Zea mays). The dys2 mutant exhibits homologous pairing defects, leading to sterility. Analyses revealed that DSB formation and the number of RADIATION SENSITIVE51 foci are largely reduced, and synopsis is completely abolished in dys2 meiocytes. Super-resolution structured illumination microscopy showed that DSY2 is located on the AE and forms a distinct alternating pattern with the HORMA-domain protein ASYNAPTIC1 (ASY1). In the dys2 mutant, localization of ASY1 is affected, and loading of the central element ZIPPER1 (ZYP1) is disrupted. Yeast two-hybrid and bimolecular fluorescence complementation experiments further demonstrated that ZYP1 interacts with DSY2 but does not interact with ASY1. Therefore, DSY2, an AE protein, not only mediates DSB formation but also bridges the AE and central element of SC during meiosis.

INTRODUCTION

Meiosis is the type of cell division that is required for the generation of haploid gametes during sexual reproduction. The most profound characteristic of meiosis is the correct pairing of homologous chromosomes during prophase I; in most organisms, this is achieved by DNA double-strand break (DSB)-dependent recombination (Szostak et al., 1983; Sun et al., 1989; Cao et al., 1990; Barzel and Kupiec, 2008). The paired chromosomes are eventually held together by crossovers (COs) and later segregate to complete the reductional division at anaphase I (Hunter, 2007). The second meiotic division then separates the sister chromatids to give rise to haploid daughter cells.

Meiotic recombination is initiated by the DSB formation catalyzed by SPORULATION11 (SPO11), a conserved topoisomerase-like protein (Keeney et al., 1997; Grellon et al., 2001). DSBs are then resected by exonucleases to generate 3' single-stranded DNA (ssDNA) overhangs, which further associate with DNA recombinase, DISRUPTED MEIOTIC CDNA1 (DMC1), and RAD51, and their accessory factors (Bishop, 1994; Da Ines et al., 2012; Kurzbauer et al., 2012). These ssDNA nucleoprotein complexes, also called presynaptic filaments, mediate the homology search process and form joint DNA molecule intermediates by invading homologous sequences (Shinohara et al., 1992; Baumann et al., 1996; Pradillo et al., 2014). After strand invasion, recombination intermediates are repaired either by the DSB repairing pathway via the formation of double Holliday junctions to generate CO products or by the synthesis-dependent strand-annealing pathway that leads to non-CO products (Allers and Lichten, 2001; San Filippo et al., 2008). Interestingly, despite the fact that hundreds of DSBs are usually created, only a small subset of DSB sites give rise to COs; at least one CO between each homologous pair is required for accurate segregation (Martini et al., 2006; Chen et al., 2008; Serrentino and Borde, 2012).

Meiotic DSBs are not uniformly distributed; they tend to occur at preferred locations, which are called hot spots. Although the detailed controls are unclear, the overall chromatin structure and epigenetic marks appear to influence hotspot activity (Youds and Boulton, 2011). For example, in Arabidopsis thaliana, hot spots are associated with active chromatin modification, including H2A.Z, histone H3 Lys4 trimethylation, low nucleosome density, and low DNA methylation (Choi et al., 2013). Moreover, genetic and molecular evidence suggested a close relationship between...
chromosome axes and DSBs (Kleckner, 2006; de Massy, 2013). At the onset of meiosis, the axial element (AE) is required to organize sister chromatids into arrays of multiple DNA loops, with bases that are anchored on AEs (Borde and de Massy, 2013). Studies in many organisms, such as budding yeast, Caenorhabditis elegans, mouse, and plants have shown that mutations of AE components affect DSB formation (Mao-Draayer et al., 1996; Blat et al., 2002; Goodyer et al., 2008; Ferdous et al., 2012; Luo et al., 2014). In addition, several Spo11-accessory proteins, for example, yeast Recombination114 (Rec114p), Meiotic Recombination2014). In addition, several Spo11-accessory proteins, for example, et al., 2002; Goodyer et al., 2008; Ferdous et al., 2012; Luo et al., components affect DSB formation (Mao-Draayer et al., 1996; Blat eukaryotic organisms (Hunter, 2007; Baudat et al., 2013; Luo et al., 2014). Interestingly, ASY3 and PAIR3 are required for normal localization of ASY1 and PAIR2, respectively. Ferdous et al. (2012) further demonstrated that Arabidopsis ASY3 interacts with ASY1 and suggested that Arabidopsis ASY3 is the functional analog for yeast Red1 (Ferdous et al., 2012).

Maize, one of the most important crops, is an excellent model organism for studying meiosis because of its superb morphology of large meiogic chromosomes, synchronized meiotic progression, and a large collection of meiotic mutants (Cande et al., 2009). Among maize mutants, only absence of first division1 (afd1), a cohesion protein, has been identified as a component of AE/LE (Golubovskaya et al., 2006). Previously, studies of the maize desynaptic2 (dsy2) mutant showed that Dsy2 is required for homologous recombination and synopsis (Franklin et al., 2003; Golubovskaya et al., 2011). In this study, we cloned maize Dsy2 and showed that it encodes an AE protein containing coiled-coil domains and is likely an ortholog of Arabidopsis ASY3 and rice PAIR3. The results presented here suggest that DSY2 is required not only to establish normal levels of DSB but also for SC assembly. We further demonstrate that DSY2 interacts with ASY1 and, more importantly, with the central element ZYP1. Our study provides insight into the role of chromosome axis-associated proteins and reveals the molecular mechanism of SC assembly at the molecular level in plants.

RESULTS

The Maize dsy2-1 Mutant Showed Defective Homologous Recombination with Various Numbers of Univalents

The dsy2-1 mutant exhibited normal vegetative growth, but showed male and female sterility. We analyzed the meiotic progression using acetocarmine staining of male meiocytes (Figure 1A). In the dsy2-1 mutant, meiosis appeared to progress normally at leptotene and early zygotene; however, normal pachytenes with fully synapsed chromosomes was not observed. Consistent with the results from a previous study (Franklin et al., 2003), the most obvious defects were found at diakinesis with variable numbers of univalents. The average number of bivalents was 2.42 (n = 69) in the mutant, compared with 9.9 (n = 92) in the wild type (Figure 1B), suggesting that Dsy2 is important for homologous chromosome recombination. These unpaired univalents in the mutant segregate randomly at metaphase I, leading to incomplete chromosome...
sets in the daughter cells. Polyads at the tetrad stage were often observed in the mutant (Supplemental Figure 1). As a consequence, the mutant pollen was not viable, as shown by Alexander staining (Figure 1C). Female meiosis was also affected as univalents were observed previously (Franklin et al., 2003).

**Dsy2 Encodes a Coiled-Coil Protein**

The original *dsy2* maize mutant was isolated from a mutagenesis screen using the mutagen *N*-nitroso-*N*-methyl urea and was shown to harbor a monogenic recessive mutation (Golubovskaya, 1989). The mutation was mapped to chromosome 5 and cosegregated with simple sequence repeat markers *bnlg1208* and *dupssr10* (Franklin et al., 2003). In this study, *dsy2* was further mapped to a position linked with the single-nucleotide polymorphism (SNP) marker 18689W2 by bulk segregation analysis using the Sequenom-based SNP assay (Liu et al., 2010) (Figure 2A; Supplemental Figure 2). The putative maize gene AC210848.3_FG004 around this region shares similarities with rice *PAIR3* and Arabidopsis *ASY3*, both of which show meiotic defects when mutated. DNA sequence analysis in the *dsy2* mutant uncovered a nucleotide substitution from G to A at the splicing donor site of the 12th intron (Figure 2B). We confirmed by RT-PCR that the point mutation results in the intron being mis-spliced

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**Figure 1.** The *dsy2-1* Mutant Exhibits Various Numbers of Univalents, Leading to Sterility.

(A) Male meiocytes were stained with aceticarmine. The *dsy2-1* mutant shows abnormal pachytene chromosomes. In contrast to 10 bivalents at wild-type diakinesis, mutant plants exhibit univalents and some open bivalents (arrows) at diakinesis. At metaphase I, univalents are distributed unevenly as 8 and 12 chromosomes observed in the representative *dsy2-1* cell. Dotted lines mark the metaphase plate. Bar = 20 μm.

(B) *dsy2-1* mutant shows a significant reduction in the number of bivalents. Open squares indicate values from individual cells.

(C) Aborted pollens with no cytoplasm were observed in the *dsy2-1* mutant by Alexander’s staining. Bar = 50 μm.

**Figure 2.** *Dsy2* Encodes a Coiled-Coil Protein.

(A) Previously, the *Dsy2* gene was mapped to chromosome 5 and found to cosegregate with the simple sequence repeat markers *bnlg1208* and *dupssr10*. In this study, *dsy2* was further mapped to a position closely linked with the SNP marker 18689W2.

(B) Map of the 6.8-kb *Dsy2* locus showing the exon (box) and intron (line) organization. The point mutation of the *dsy2-1* allele (arrow) and Mu transposon insertion site in the *dsy2-2* allele (triangle) are indicated. A partial sequence in the lower panel shows a G-to-A substitution (arrowhead) in the splicing donor site of the 12th intron in the *dsy2-1* mutant. An in-frame stop codon (underlined tga) is predicted in mutant transcripts.

(C) RT-PCR analysis demonstrates an intron retention that results from the mis-splicing in the *dsy2-1* mutation.
(Figure 2C); thus, we designated the original mutant as the dsy2-1 allele. From the trait utility system for maize (TUSC) transposon mutagenesis program at Pioneer Hi-Bred (Bensen et al., 1995), we obtained an additional mutant allele, named dsy2-2, with a Mu insertion in the first exon, at 103 bp upstream of the ATG. The new allele was sterile and displayed similar phenotypes to the dsy2-1 during meiosis (Supplemental Figure 3); therefore, we conclude that we had cloned Dsy2.

A 2814-bp full-length cDNA was isolated from the A344 inbred line (GenBank accession number KR029723). It encodes a 758-amino acid protein that shares 83 and 31% similarity with rice PAIR3 and Arabidopsis ASY3, respectively. The maize DSY2 contains several α-helices at the C terminus, and this region was predicted to form two coiled-coil domains (amino acids 649 to 679 and 718 to 740) by COILS (Lupas et al., 1991) (Supplemental Data Set 1). Several related genes were found in the GenBank/EMBL databases, all of which contain coiled-coil motifs at the C terminus, with a higher level of conservation. Phylogenetic analysis indicated that maize DSY2 and rice PAIR3 form a branch with other monocot genes, suggesting that Dsy2 is a putative ortholog of rice PAIR3 and the closest maize homolog of Arabidopsis ASY3 (Supplemental Figure 4 and Supplemental Data Set 1).

RT-qPCR showed that Dsy2 is highly expressed in wild-type anthers (Figure 3A). The dsy2-1 RNA level did not display a significant difference with that in the wild-type anthers; however, a severe reduction of transcript was detected in dsy2-2. An immunoblot analysis using an antibody against a region of DSY2 (amino acids 237 to 503) showed that DSY2 is present in the meiotic anthers of the wild type, with the highest abundance at prophase I, whereas DSY2 was not detectable in either mutant alleles (Figure 3B). This result explains the identical meiotic phenotypes observed for both alleles. Although dsy2-1 expresses a normal level of mRNA, abnormal dsy2-1 transcripts are predicted to produce a truncated DSY2 protein lacking the last coiled-coil domain, implying that the C terminus may be important for DSY2 stability. Alternatively, the intron-retention transcripts of dsy2-1, which is caused by a point mutant at a splice junction, may fail to be exported from the nucleus to the cytoplasm for protein translation, a phenomenon that was recently found in Arabidopsis (Göhring et al., 2014).

Figure 3. Dsy2 Is Located on the Axial/Lateral Elements of SC.

(A) RT-qPCR shows that Dsy2 mRNA is highly expressed in wild-type and dsy2-1 anthers, but not in dsy2-2.

(B) Immunoblot analysis indicates that DSY2 is specifically produced in meiotic anthers, whereas it was not detected in either of the mutant alleles.

(C) Immunofluorescence results show that DSY2 (green or grayscale) appears as numerous foci at late interphase and becomes a linear structure along chromosomes at leptotene. By pachytene, DSY2 is located on the coiled lateral elements of SC. Bar = 5 µm. Magnified images in the right panel show closed-up views from blocked regions of leptotene and pachytene cells. Bar = 2 µm.
DSY2 Is an Axial/Lateral Element Component of the Synaptonemal Complex

Previous studies have demonstrated that rice PAIR3 and Arabidopsis ASY3 are located on the AE/LE of the SC (Wang et al., 2011; Ferdous et al., 2012). However, the spacing of the AE in SC is below the resolution limit of conventional light microscopy, which makes it difficult to resolve the structure of the tripartite SC. To investigate the distribution of DSY2 in detail, we used a super-resolution structured illumination microscope (SIM). The high resolving power of this microscope provides detailed protein localization (Gustafsson et al., 2008; Wang et al., 2009). In the wild type, DSY2 first appears as numerous foci during late interphase and begins to form discontinuous stretches along the chromosome axes at leptotene (Figure 3C). Later, during zygotene and pachytene, the DSY2 signals become more continuous with variable intensity along the developing SC. When synopsis is complete, DSY2 is located on the coiling LEs of the SC as two parallel tracks. The coiling of the SC was similar to that previously observed using SIM when maize AEs were stained with REC8/AFD1 antibody (Wang et al., 2009). At late pachytene, when the SC disassembles, DSY2 staining is diminished (Supplemental Figure 5). Super-resolution imaging of DSY2 showed that it is a protein component of the AE/LE of the SC.

dsy2 Is Required for Normal Levels of DSBs

Meiotic recombination is initiated by DSB formation, the location and frequency of which are influenced by many factors (Keeney et al., 2014). To determine whether DSY2 is important for DSB formation, TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assays were conducted. As predicted, in the wild type, TUNEL signals appeared during early zygotene and disappeared at the late pachytene stage, when DSBs are repaired. When treated with DNase I, pachytene cells exhibit strong staining as a positive control. In a comparison of the wild type and dsy2-1 at zygotene, TUNEL signals were reduced in the dsy2 mutant, suggesting that DSB formation is affected in the absence of DSY2 (Figure 4A). Although TUNEL signals do not appear as punctate foci on meiotic chromosomes in maize, which is consistent with a previous report (Pawlowski et al., 2004), it is evident that the overall signals in the dsy2 mutant are weaker. To further determine

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Figure 4. DSB Formation and RAD51 Foci Were Reduced in the dsy2-1 Mutant.

(A) The TUNEL assay shows that DSB level (green) in dsy2-1 zygotene meiocytes was reduced compared with wild-type meiocytes at the zygotene stage. DSB was not detected during late pachytene in the wild type, as DSBs are repaired. DNase I-treated pachytene meiocytes served as a positive control. Bar = 5 μm.

(B) The number of RAD51 foci increases from ~250 to ~500 during zygotene in the A344 wild-type and dsy2-1 heterozygous meiocytes, while reduced RAD51 foci were observed in the dsy2-1 homozygous mutant. Open squares indicate values from individual cells. Average numbers are listed above each box plot. Representative images are shown in Supplemental Figure 6.

(C) The RAD51 focus morphology was examined by super-resolution microscopy at mid-zygotene in the wild type and dsy2-1 mutant. Projection images of partial nuclei are presented. RAD51 (green) often forms elongated filaments with one end attached or close to chromosome axes (magenta; labeled with the AFD1 antibody, as shown in blocked regions). A similar conformation can be seen in dsy2-1 meiocytes, although fewer RAD51 foci are observed. Bar = 5 μm. Magnified images of the blocked regions with merged channels (upper middle), green channel separated out (lower middle), and surface-rendered images (right) are shown in the right panels. Bar = 2 μm. See corresponding Supplemental Movies 1 and 2 for three-dimensional visualizations.
DSB levels in the dsy2 mutant, we examined the distribution of RAD51, a recombinase protein that binds DSB sites to facilitate homology-directed repair of DSBs (Sung, 1994; Franklin et al., 2003). Immunostaining results were first imaged by deconvolution microscopy, and quantitative analyses showed that in wild-type and heterozygous dsy2 plants, RAD51 appeared at the beginning of zygotene and rose to ~500 foci at mid-zygotene, when homologous chromosomes are actively pairing (Figure 4B; Supplemental Figure 6). Chromosomes in meiocytes from the dsy2 mutant were fairly normal in appearance before pachytene and became shorter and thicker over time, which helps us to determine meiotic stages in the mutant (Supplemental Figure 6). In contrast to the wild type, only ~30% of the normal number of RAD51 foci was observed in the dsy2 meiocytes, with an average of 87 foci at early zygotene and 144 foci at mid-zygotene being detected. The reduction in the number of RAD51 foci is likely due to the lower numbers of DSBs at the earlier stages of meiosis. To examine this phenotype in more detail, we used super-resolution SIM to compare the RAD51 morphology relative to the AEs labeled by cohesion protein AFD1 antibody in the wild-type and dsy2 mutant. The projection images (Figure 4C) and series of optical sections (Supplemental Figure 7) showed that RAD51 forms elongated filaments during zygotene, and one end of these filaments is often attached or close to the chromosome axes in the wild type. From surface-rendered 3D models analyzed by Imaris software, some RAD51 signals appeared to be embedded in the chromosome axes (Figure 4C; Supplemental Movies 1 and 2). The elongated RAD51 signals protruding from the axes likely represent ssDNAs engaging in homologous searches. Consistent with deconvolution images, fewer RAD51 foci were observed in the dsy2 mutant by SIM (Supplemental Figures 6 and 7). Importantly, similar conformations of elongated RAD51 signals relative to the chromosome axes were observed in the mutant, suggesting that these residual signals are not RAD51 aggregates. The RAD51 signals observed by SIM were slightly longer in the dsy2 mutant, which is consistent with previous research (Supplemental Figure 8) (Franklin et al., 2003). From our results, we conclude that loss of DSY2 results in a significant reduction in DSB formation and that RAD51 loading does not appear to be affected.

Homologous Pairing Is Defective in the dsy2 Mutant

In most organisms, pairing of homologous chromosomes during zygotene requires DSBs, and a small subset of DSBs gives rise to COs at the late pachytene stage. To assess homologous pairing in the dsy2 mutant, we performed fluorescence in situ hybridization (FISH) on 3D-preserved meiocytes at the zygotene stage. This method allows visualization of the pairing process of a single locus without disrupting the spatial configuration of chromosomes. Meiotic stages were carefully determined based on chromosome thickness, condensation, and compaction (Bass et al., 1997; Cande et al., 2009), and only zygotene meiocytes were subjected to the analysis (Supplemental Figure 6). The dynamic pairing status is determined by spatial distances of 5S rDNA signals in 3D stacks of a cohort of meiocytes during zygotene (Figure 5A). Analyses showed that pairing is efficient in the wild type, as many 5S signals are approaching each other or even paired during zygotene, whereas signals in the mutant were more randomly distributed. Only 4 of 42 cells during zygotene in dsy2 contained close signals with a distance shorter than 1 μm (Figure 5B), suggesting a defect in homologous pairing during zygotene. On the other hand, the observation of these close 5S signals in the mutant suggested that a certain level of homologous alignment may be maintained. Normal pachytene with fully synapsed chromosomes was never observed in the mutant, which makes it more difficult to define pachytene according to the canonical definition; thus, we next analyzed CO formation at diakinesis. We estimated CO numbers using the following method: rod-shaped bivalents have one CO, whereas ring-shape bivalents have at least two COs (Jones and Franklin, 2006). This analysis revealed that

![Figure 5](image-url)

**Figure 5.** Homologous Pairing Is Defective in the dsy2 Mutant.

(A) Homologous pairing is monitored by 5s rDNA signals (green) on 3D-preserved meiocytes at the zygotene stage in the wild type and dsy2-1 mutant. Arrows indicate 5s rDNA signals. Bar = 5 μm.

(B) Quantitative analysis of spatial distances of 5s rDNA signals showed that homologous pairing is defective in the dsy2 mutant. Open circles indicate values from individual cells.

(C) Metaphase I cells probed with Cent-C repeat (green), 180-bp knob repeat (red), and 5s rDNA sequence (yellow) showed all bivalents with symmetric FISH signals in the wild type. In the dsy2 mutant, remaining bivalents (arrows) were aligned at the metaphase plate with paired FISH signals. Bar = 10 μm.
meiocytes at diakinesis in dsy2 contain 0 to 7 COs, with an overall mean of 3.2, compared with 19.9 in the wild type (Supplemental Figure 3C). To further determine whether COs are formed between homologous chromosomes, we performed FISH using a probe mixture (Figure 5C). All examined bivalents have a paired pattern of FISH signals, indicating that they are homologous chromosomes. We did not observe nonhomologous associations in the dsy2 mutant. These results suggest that the CO number was reduced, which corresponds to the defective pairing observed at earlier stages in the mutant. Nevertheless, COs are only formed between homologous chromosomes, despite impaired pairing in the dsy2 mutant.

**DSY2 Is Essential for ZYP1 Installation and Normal ASY1 Distribution**

To investigate the role of DSY2 in SC formation, immunolocalization of DSY2 together with other SC proteins, including AFD1, ASY1, and ZYP1, was examined. AFD1, a meiotic α-kleisin cohesion protein, is thought to hold sister chromatids together and to establish mono-oriented kinetochores during metaphase I (Golubovskaya et al., 2006). ASY1, a HORMA protein, is another AE component in plants and shares amino acid sequence similarities to yeast Hop1p (Armstrong et al., 2002; Golubovskaya et al., 2011). ZYP1, a central element protein, acts as a transverse filament that polymerizes between AEs for SC assembly (Golubovskaya et al., 2011). These three components are important for synapsis and CO formation (Page and Hawley, 2004; de Boer and Heyting, 2006). Using super-resolution SIM on wild-type nuclei, we showed that AFD1 signals appear as discontinuous dots and patches that are distributed along with DSY2 staining on the AE/LE without an obvious pattern (Figure 6A). AFD1 staining was not significantly altered in the mutant, although the mutant AEs exhibited slightly crooked paths. Immunostaining of the dsy2 mutant meiocytes did not reveal any DSY2 signal, which confirms the specificity of the anti-DSY2 antibody and supports the immunoblot results. Interestingly, DSY2 and the other AE protein, ASY1, showed a distinct alternating pattern of brighter and dimmer signals (Figure 6B). Often, when one component gave a prominent signal, the other component was faint. When z-stacks of images were visualized by surface-rendering modeling, ASY1 and DSY2 appeared as a series of patches that connect to each other along the axes (Supplemental Movie 3). In the synapsed regions of wild-type chromosomes, ASY1 signals are diminished and DSY2 remains as two parallel lines on the LEs, so that the alternate pattern of the two proteins is no longer observed (arrow in Figure 6D). These results may reflect a specified organization of the two components in the unsynapsed AEs, and their organization may change rapidly upon synapsis. Relative to the more uniform staining of ASY1 in the wild type, loss of DSY2 results in discontinuous ASY1 signals with lower intensity and longer gaps along the AEs (Figure 6B; Supplemental Table 1), suggesting that DSY2 is required for ASY1 localization or stability.

Immunolocalization analysis using an antibody against the N terminus of ZYP1 and anti-DSY2 demonstrated that the central element protein ZYP1 is located between two DSY2-labeled LEs, all along the chromosomes, during pachytene (Figure 6C). Supplemental Movie 4 demonstrates that the DSY2 and ZYP1 signals form the tripartite SC structure, seen as a left-handed coiling ribbon. By contrast, ZYP1 signals in the mutant at pachytene are present as only a few short stretches that are not aligned with the AEs (seen in Figure 6C by ASY1 staining for the mutant), which suggests that ZYP1 installation, which is normally required to assemble mature SC, is abolished in the dsy2 mutant (Figure 6C). In summary, this result indicates that the installation of the central element ZYP1 requires the AE protein DSY2.

**DSY2 Interacts with ZYP1 and ASY1**

The observation that normal localization of ASY1 in the AE and loading of ZYP1 were dependent on DSY2 suggested a possible physical interaction between DSY2, ASY1, and ZYP1. To obtain evidence for protein-protein interactions, we conducted yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) assays with plasmids containing the Dsy2, Asy1, and Zyp1 sequences. In the quantitative Y2H experiments, the coding regions for each gene were cloned as in-frame fusions with the LexA DNA binding domain and the GAL4 activation domain, respectively. The interaction of the two proteins as quantified by β-galactosidase activity is dependent on the expression of the reporter lacZ (Yeast protocols handbook; Clontech). We found that DSY2 not only interacts with itself, but also interacts with ASY1 and ZYP1 (Table 1). Further validation using BiFC with the split YFP fragments fused with their corresponding genes confirmed these findings (Figure 7). The interaction between ZYP1 and DSY2, as well as between ASY1 and DSY2, allow the reconstitution of YFP to generate fluorescent signals in rice protoplast cells. By contrast, Y2H and BiFC assays did not detect interaction between ASY1 and ZYP1.

**DISCUSSION**

In this study, we identified maize DSY2 and showed that it is an AE protein containing coiled-coil domains. Our results demonstrated that DSY2 is essential for normal levels of DSBs and SC formation in maize. More importantly, protein-protein interaction analyses showed that DSY2 physically interacts with the transverse filament ZYP1. This finding accounts for the synaptic phenotype observed in the dsy2 mutant and provides novel insight into the molecular mechanism of SC formation in plants. The phylogenetic and phenotypic analyses suggest that maize Dsy2 is an ortholog of Arabidopsis Asy3 and rice PaI/R3, whose mutants cause similar meiotic phenotypes with the dsy2 mutant (Wang et al., 2011; Ferdous et al., 2012). Moreover, Ferdous et al. (2012) suggested that Arabidopsis Asy3 is the functional analog of yeast Red1p (Ferdous et al., 2012). We further propose that maize DSY2 also shares functional similarities with Red1p, based on their roles in recombination and synapsis and on the relationship between DSY2 and the HORMA-domain proteins, yeast Hop1p and maize ASY1 (see below).

**DSY2 Supports the Chromosome Axis Integrity in Maize**

Super-resolution imaging of DSY2 in the wild-type meiocytes demonstrated that it is located on the AEs. Interestingly, dual immunolocalization with DSY2 and ASY1 antibodies revealed that
these two proteins have an alternating localization, which is a distinct organization that has not previously been shown experimentally for axial element proteins. By surface-rendering analyses, we further showed that patches of the DSY2 and ASY1 proteins are organized alternately, but also exhibit some overlapping regions. In budding yeast, Red1p and Hop1p were found to colocalize on the AE of chromosome spreads by conventional light microscopy (Smith and Roeder, 1997); however, their organization by super-resolution imaging has not yet been reported. Nonetheless, the patterns of DSY2 and ASY1 in maize are reminiscent of Arabidopsis ASY1, i.e., they are present as alternating hyperabundant and lower-abundant regions on meiotic chromosome axes (Ferdous et al., 2012). The functional implication of this distribution pattern remains unclear. It may be required for AE integrity, since Arabidopsis asy1 and asy3 mutants, as well as the maize dsy2 mutant, seem to have compromised AE structures (Figure 6) (Pradillo et al., 2007; Ferdous et al., 2012). In addition, an earlier study in budding yeast suggested that the stoichiometric ratio of Hop1p to Red1p is crucial for AE function (Bailis et al., 2000), implying that the organization of the two AE proteins along chromosome axes is important.

Loss of DSY2 resulted in impaired localization of ASY1, such that there was lower intensity of the ASY1 signals, with much longer gaps between the axis-associated foci. Consistent with our

Figure 6. Synaptonemal Complex Formation in the dsy2 Mutant.

(A) Immunostaining of DSY2 (green) and AFD1 (red/magenta) in wild-type and dsy2-1 mutant meiocytes at zygotene. (B) Immunostaining of DSY2 (green) and ASY1 (red/magenta) at the leptotene stage showed an alternate pattern of two axial proteins in the wild type, whereas the ASY1 signals are reduced in the mutant. (C) The central element protein ZYP1 (red/magenta) is localized between DSY2-labeled lateral elements (green) in the wild type at pachytene. In the mutant, ZYP1 was only found as short stretches and not seen as the normal tripartite SC. Magnified images of the blocked regions are shown in the right panels with channels separated out (top, middle) and merged (bottom). (D) A projection image of a partial wild-type nucleus at zygotene showed a significant reduction of ASY1 signals in synaptic regions (arrow). Bars = 5 μm in whole-nucleus images and 2 μm in the magnified images.
observation, axis association of ASY1/PAIR2 is dependent on ASY3/PAIR3 in Arabidopsis and rice, respectively (Wang et al., 2011; Ferdous et al., 2012). Similarly, in yeast, localization of Hop1p requires Red1p (Smith and Roeder, 1997). Our results suggest that a similar relationship exists between DSY2 and ASY1 in maize. However, it is worth noting that although ASY1 localization is defective in the absence of DSY2, some ASY1 is loaded independently of DSY2. This raises the possibility that DSY2/ASY3 is required to stabilize ASY1 rather than for its initial loading, and this could explain the observation that Arabidopsis ASY1 is epistatic to ASY3 with regard to CO formation (Ferdous et al., 2012).

### The Role of DSY2 in Meiotic Recombination

It has been suggested in many species that DSB formation is indispensable for meiotic recombination (Barzel and Kupiec, 2008). In maize, the level of DSBs can be detected by TUNEL assay and the number of RAD51 foci observed in meiocytes during early prophase I (Pawlowski et al., 2004; Cande et al., 2009). Our results show a consistent reduction of DSBs during dsy2 zygotene of ~70%. Similarly, in Arabidopsis, the loss of ASY3 also interrupts DSB formation, despite the fact that the reduction was found to be around 33% (Ferdous et al., 2012). Interestingly, we observed on average 87 and 144 RAD51 foci in dsy2 meiocytes at early and mid-zygotene, respectively. Compared with the 302 and 471 RAD51 foci detected in the +/dsy2 heterozygous plants at early and mid-zygotene, respectively, there is a similar decline in DSBs at both stages in the dsy2 (71 and 69% reduction at early and mid-zygotene, respectively), suggesting that DSY2 has a similar influence on early and late DSB formation. On the other hand, the continuous increase in the number of RAD51 foci implies that the stage dependent—progressive increase in DSBs is independent of DSY2. It has been proposed that the DSB machinery can be tethered to chromosome axes prior to break formation; thus, disrupting axial proteins could affect the ability or efficiency of DSB formation. Indeed, studies in yeast have showed that both Red1 and Hop1 are required for this process (Kim et al., 2010; Panizza et al., 2011). However, this raises the question of whether the decrease in DSBs in the dsy2 mutant is due to the lack of DSY2 or is indirectly due to improper ASY1 localization. Since the maize asy1 mutant is not yet available, the observation that the Arabidopsis asy1 mutant has a normal level of DSBs supports the former scenario. If this is also the case in the maize asy1 mutant, the DSB reduction observed here is likely to be a direct effect of the absence of DSY2. Nevertheless, the link between AE and DSB remains elusive in plants; further studies are required to understand this process.

After DSBs are formed, homologous chromosomes are increasingly juxtaposed at a distance of 400 nm, a process often called presynaptic alignment. This initial pairing soon leads to SC nucleation such that, in organisms with long chromosomes such as maize, alignments between homologous chromosomes and SC nucleation are often initiated from chromosome distal regions at the leptotene-zygotene transition (Burnham et al., 1972; Harper et al., 2004). The dsy2 mutant exhibited a 70% reduction in the number of DSBs, which probably affects presynaptic alignment of homologous chromosomes during the initial pairing stage. Our FISH results indicate that pairing of the 5S rDNA locus during zygotene is much less efficient in the dsy2 mutant. Thus, the residual DSBs may not be sufficient to establish connections between all homologous chromosomes. However, meiotic pairing is a dynamic process; we cannot exclude the possibility that chromosome movements disrupt some presynaptic alignments in the mutant, especially in the absence of SC formation.

In wild-type maize meiocytes, the RAD51 foci displayed a filament-like morphology in the super-resolution microscopy images. In the rotating movie of the surface-rendering model, we show that the RAD51 elongated foci are attached to or close to the chromosome axes, with the other end of the RAD51 focus projecting away from the chromosome axes. This configuration likely represents the presynaptic filaments that mediate strand invasion into homologous duplex DNAs. Our observations are consistent with the current model of DSB-dependent recombination, where DSBs are formed on DNA associated with chromosome axes, resected to

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### Table 1. Y2H Analyses for Determining the Interaction between Synaptonemal Complex Proteins

<table>
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<tr>
<th>LexA-X</th>
<th>Gal4-Y</th>
<th>X-Y Interactiona</th>
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<tbody>
<tr>
<td>DSY2</td>
<td>DSY2</td>
<td>69.97 ± 6.71</td>
</tr>
<tr>
<td>DSY2</td>
<td>–</td>
<td>0.16 ± 0.06</td>
</tr>
<tr>
<td>ASY1</td>
<td>DSY2</td>
<td>10.16 ± 0.64</td>
</tr>
<tr>
<td>ASY1</td>
<td>–</td>
<td>0.23 ± 0.27</td>
</tr>
<tr>
<td>ZYP1</td>
<td>DSY2</td>
<td>93.31 ± 4.17</td>
</tr>
<tr>
<td>ZYP1</td>
<td>ASY1</td>
<td>0.10 ± 0.08</td>
</tr>
<tr>
<td>ASY1</td>
<td>ZYP1</td>
<td>0.24 ± 0.41</td>
</tr>
<tr>
<td>ZYP1</td>
<td>–</td>
<td>0.07 ± 0.03</td>
</tr>
</tbody>
</table>

aThe X-Y interaction was determined by ß-galactosidase activity and its calculation is described in Methods.
form ssDNA tails, and then coated with recombinase proteins for homology search. RAD51 foci in the dsy2 mutant have a similar shape to those in the wild type, suggesting that RAD51 loading was unaffected in the dsy2 mutant. Unfortunately, Arabidopsis or commercial DMC1 antibodies did not recognize the maize DMC1 in meiocytes; thus, whether the loss of DSY2 interrupts DMC1 localization remains unknown.

The CO frequency in the dsy2 mutant was 17% of the wild-type level; however, DSB formation was around 30% of the wild-type level. This difference in the reductions of DSB and CO formations was also seen in the Arabidopsis asy3 mutant. This difference might be explained by the observation that interhomolog recombination is severely compromised in the Arabidopsis asy1 mutant. It is possible that defective ASY1 localization caused by the loss of DSY2 in maize may impair interhomolog recombination, so that more DSBs are repaired by sister chromatids. Alternatively or in addition, the synopsis failure observed in the dsy2 mutant may alter the ratio of CO to non-CO formation. Moreover, our FISH results showed no sign of nonhomologous chromosome association at dsy2 metaphase I, suggesting that homolog recognition is normal and that COs form only between homologous chromosomes. Finally, DNA fragmentation was not present at any stage in dsy2 meiocytes, indicating that all DNA breaks in the mutant were likely repaired.

**DSY2 Acts as a Structural Protein to Connect the Axial Element to the Central Element for SC Assembly**

The 3D-SIM enabled the visualization of structures smaller than 100 nm, providing a suitable method for dissecting the SC structure (Wang et al., 2009). Our observation of the maize SC is similar to that in a recent study in barley; however, we did not observe the separated ZYP1 tracks seen in barley (Phillips et al., 2012). Instead, our ZYP1 signals formed a line between the AE. This might be due to different regions of antibody recognition being used; we used an antibody against the N terminus of maize ZYP1, whereas Phillips et al. (2012) used an Arabidopsis antibody against the C terminus. Despite different ZYP1 regions being detected by different antibodies, these results reflect that the orientation of the transverse filament is conserved across kingdoms, suggesting that the major transverse filaments form a zipper-like structure with their N termini oriented at the center of the SC and their C termini oriented toward the chromosome axes (de Boer and Heyting, 2006).

DSY2 is required for synopsis, similar to Arabidopsis ASY3 and rice PAIR3. In the maize dsy2 mutant, ZYP1 signals appear as a few short stretches that are not sandwiched between AEs, indicating that ZYP1 fails to assemble. The observations of pairing of 25% of the 5S rDNA signals (Franklin et al., 2003) and an average of 3.2 COs in this study, suggest that some of the homologous chromosomes are identified and prealigned; however, synopsis (i.e., ZYP1 assembly) failed to start in the dsy2 mutant. Our finding that DSY2 interacts with the transverse filament ZYP1 explains this synaptic phenotype. DSY2 also interacts with another AE protein, ASY1. This work thus reveals the mechanism for the assembly of the SC in plants at the molecular level. Our findings show that DSY2 serves as a structural protein that connects the AE and the transverse filament.

**METHODS**

**Plant Material**

The dsy2-1 mutant was previously identified in a screen for meiotic mutants by Inna Golubovskaya (Golubovskaya and Mashnenkov, 1976; Golubovskaya, 1989). The original mutant line was a gift from Inna Golubovskaya and W. Zacheus Cande (UC Berkeley). The dsy2-2 mutant allele was obtained from the TUSC population by reverse genetics. Plants for cytology were grown in Academia Sinica, Taipei, and the mapping population was grown in Potzu Branch Station, Tainan District Agricultural Research and Extension Station, Chiayi, Taiwan.

**Genetic Mapping and Genotyping**

The dsy2-1 mutant in the inbred line A344 background was outcrossed to Mo17. The F1 individuals were self-crossed to obtain F2 individuals. Leaf tissues were collected from individuals within the F2 family. DNA was isolated and two bulk DNA samples were prepared from dsy2 mutants and non-mutant plants (Supplemental Figure 2). The dsy2 gene was mapped by Sequenom-based SNP-typing assays provided by Genomic Technologies Facility, Iowa State University (Liu et al., 2010). After mutations were identified, homozygous dsy2-1 plants were genotyped using the gene-specific primers Dsy2-F1 and Dsy2-R1. The dsy2-2 mutant (BT94-169-B-10) from the Maize TUSC line was genotyped using primers DO156291, DO156292, MuTIR, and Dsy2-F2 (Supplemental Table 2).

**cDNA Cloning, RT-PCR, and RT-qPCR Analysis**

Total RNA was isolated from immature tassels using TRIzol (Invitrogen) following the manufacturer’s protocol. cDNA synthesis was performed using the First-Strand cDNA Synthesis Kit (Roche) and oligo(dT) primers. The full-length cDNA was amplified using the GeneRacer kit (Invitrogen). The retention of the 12th intron in the *dsy2*-1 allele was assayed by PCR using primers Dsy2-516 and Dsy2-517. RT-qPCR reactions were performed using FastStart Universal SYBR Green Master (Roche). Cyanase was used as the internal normalization control. PCR was performed on the ABI 7500 real-time PCR system in a 64-well reaction plate. Primers used for RT-qPCR are listed in Supplemental Table 2. The RT-qPCR reaction was performed in triplicate for each RNA sample. Specificity of the amplifications and Ct values were analyzed using ABI prism dissociation curve analysis software.

**Database Searches and Sequence Analysis**

BLAST searches were conducted at the NCBI. Amino acid sequences were then aligned using ClustalW multiple alignment (BioEdit version 7.2.1). Phylogenetic analysis was performed by the maximum likelihood method with the bootstrap test (1000 replicates) (MEGA, version 6.05).

**Protein Extraction and Immunoblot Analysis**

Anthers were dissected from immature tassels and snap frozen in liquid nitrogen. The tissue was then ground and suspended in buffer containing 10% trichloroacetic acid in acetone and 20 mM DTT. The precipitates were suspended twice with 1 mM PMSF in acetone containing 20 mM DTT to remove pigments and lipids. The protein precipitates were dissolved in the buffer containing 8 M urea, 2 mM thiourrea, 1% Triton X-100, and 1% DTT. Protein concentration was determined using the Pierce 660-nm protein assay reagent. Thirty micrograms of protein was loaded on a 4% to 12% precast gel (Invitrogen), run for 3.5 h at 100 V, and then transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was blocked with 5% milk in Tris-buffered saline-Tween (TBST) solution (containing 1× TBS and 0.1% Tween 20) overnight at 4°C. Subsequently,
the PVDF membrane was incubated with anti-DSY2 antibody (1:3000 dilution in 5% milk/TBST) and then with the horsedijish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:5000 dilution in TBST). After a TBST wash, signals were detected using the UVP Bio-spectrum 600 imaging system.

Chromosome Behavior Analysis and FISH Analysis

Immature tassels were fixed using 3 parts 95% ethanol:1 part glacial acetic acid. After 16 h in the fixative, tassels were stored in 70% ethanol at −20°C. Meiotic chromosomes were stained using 2% carmine in 45% acetic acid using the squash method, according to the protocol of Golubovskaya (1994). The slides were imaged under a Zeiss Axiosmager Z1 microscope. FISH analysis was performed as described previously (Wang et al., 2006) (1994). The slides were imaged under a Zeiss AxioImager Z1 microscope. The optical density of the reaction was measured by spectrophotometry at 420 nm, and the β-galactosidase activity was calculated using the following equation: units = 1/[1000 × OD_{420}]/[T(min) × Y × OD_{600}], where T is time in minutes and V is volume in milliliters of culture used. Values were the averages of three duplicate assays, along with the sd.

Antibody Production

To generate anti-DSY2 antibody, a partial cDNA corresponding to amino acids 237 to 503 was cloned into the pET28a vector in trans-lational fusion with 6× His tag. The protein was expressed in BL21 and purified by Ni Sepharose 6 Fast Flow (GE Healthcare). Purified antigen was sent to LTK BioLaboratories (Taiwan) to generate a rabbit polyclonal antibody.

Immunostaining and Image Processing

Immature tassels were carefully removed from 4- to 6-week-old plants and kept in moist paper towel until dissection or fixation. Anthers were dissected using fine forceps and immunostaining was performed as described by Wang (2013). Anti-DSY2 and anti-ASY1 antibodies were used at 1:400 dilution, and anti-RAD51, anti-AFD1, and anti-ZYP1 antibodies were used at 1:200 dilution. The polyclonal antibodies against ASY1, ZYP1, and AFD1 were gifts from W. Zacheus Cande (UC Berkeley), and the anti-RAD51 antibody was a gift from Wojciech Pawlowski (Cornell University). Conjugated secondary antibodies were obtained from Molecular Probes. All secondary antibodies were used at a dilution of 1:200. For ASY1 and DSY2 dual immunostaining, primary antibodies were directly labeled with fluorophores using Alexa Fluor 488 and Alexa Fluor 568 by APEX antibody labeling kits (Invitrogen). Slides were imaged using either a Zeiss LSM 780 or a Deltavision core microscope equipped with a Plan Apochromat 63× oil immersion objective lens.

Y2H Analysis

The coding regions of Dsy2, Asy1, and Zyp1 were cloned into Y2H bait and prey vector (pGADT7 and pBHA). Plasmids pGADT7 and pBHA were gifts from Ting-Fang Wang. The yeast reporter strain L40 [MATa trp1 leu2 his3 lys2::lexA-HIS3 ura3::lexA- lacZ] was used. The protein interaction was quantitated using the β-galactosidase assay as described by Cheng et al. (2006). The coding regions of Dsy2, Asy1, and Zyp1 were cloned into the Gateway prey vector (pGADT7 and pBHA). The constructs were then sent to the Plant BioTechnology Core Facility, ABRC, Academia Sinica for rice protoplast transformation and imaging.

Accession Numbers

Maize Dsy2 sequence data from this article can be found in the GenBank data libraries under accession number KR029723. The accession numbers for the GenBank/EMBL protein sequences shown in the alignments are as follows: OsPAIR3 (FJ449712), Hordeum vulgare (AK375886), Sorghum bicolor (CM000763), Brachypodium distachyon (XP_003570685), Glycine max (XP_006574518), Arabidopsis thaliana, ASY1 (AK228456), Arabidopsis lyrata (GL348716), Brassica rapa (XP_009142576), Solanum lycopersicum (XP_004229554), Cucumis sativus (XP_011656946), and Populus trichocarpa (XP_002320932).

Supplemental Data

Supplemental Figure 1. Meiotic progression in wild-type and dsy2-2 mutant meiocytes by acetoxyamine staining.

Supplemental Figure 2. Mapping scheme of dsy2 gene.

Supplemental Figure 3. The dsy2-2 mutant allele exhibits similar phenotypes to dsy2-1 mutant.

Supplemental Figure 4. The sequence relatedness among 12 DSY2 homologs in an unrooted maximum-likelihood phylogenetic tree.

Supplemental Figure 5. DSY2 protein starts degenerating when SC disassembles at late pachytene.

Supplemental Figure 6. RAD51 foci at early and middle zygotene of wild type and dsy2-1 mutant by deconvolution microscopy.

Supplemental Figure 7. RAD51 foci of wild type and dsy2-1 mutant by super-resolution microscopy.

Supplemental Figure 8. Quantification of RAD51 length distribution in the wild type and dsy2-1 mutant.

Supplemental Table 1. Fluorescence intensity of ASY1 in wild-type and dsy2-1 mutant meiocytes.

Supplemental Table 2. Primers used in this study.

Supplemental Data Set 1. Amino acid alignment of maize DSY2 and related homologs.

Supplemental Movie 1. 3D surface render of RAD51 and AFD1 in the wild type.

Supplemental Movie 2. 3D surface render of RAD51 and AFD1 in the dsy2-1 mutant.
Supplemental Movie 3. 3D surface render of DSY2 and ASY1 in the wild type.

Supplemental Movie 4. 3D surface render of DSY2 and ZYP1 in the wild type.

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AUTHOR CONTRIBUTIONS

D.H.L. and C.-J.R.W. designed the research, analyzed the data, and wrote the article. D.H.L., Y.-H.K., J.-C.K., C.-Y.L., and Y.-S.J. performed the research. R.M. contributed experimental materials.

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# The Axial Element Protein DESYNAPTIC2 Mediates Meiotic Double-Strand Break Formation and Synaptonemal Complex Assembly in Maize

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