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# Homologous chromosome pairing is completed in crossover defective *atzip4* mutant

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ABSTRACT

In transposon-tagged lines of *Arabidopsis*, we found a mutant that was defective in meiotic chromosome segregation. This mutant, named *atzip4-4*, was due to a novel mutant allele of *AtZIP4*, which has sequence similarity to yeast *ZIP4/SPO22*, which codes a ZMM protein that is a proposed unit of the synapsis initiation complex. The chiasma distribution in *atzip4-4* differed from that in the wild-type, involved in a deficiency of interfering crossovers in the mutant genome. On the other hand, FISH staining of loci on two independent chromosomes in mutant meiocytes indicated that homologous chromosome pairing to synapse progresses normally until the pachytene stage, yet homologous chromosomes often separated abruptly at diplotene and diakinesis. These results suggest that *AtZIP4* plays an important role in normal crossover formation and meiotic chromosome segregation, but not in homolog search. The relationship of AtZIP4 and other related proteins in meiotic events is discussed and compared with that in yeast.
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A model of the meiotic recombination pathways of prophase I of meiosis in yeast and plants has been created along the cytological progression [1–3]. First, double-strand breaks (DSBs) are induced after meiotic DNA replication, then are processed to form single strands. Next, strand invasion occurs at the corresponding sites of the homologous chromosome as a repair template. Finally, the repair process results in any of three different products: interfering crossovers (COI), non-interfering crossovers (COII), and noncrossovers, the latter of which are not genetically detected [3].

In yeast, COI formation is accomplished by the recruitment to a subset of DSB sites of a set of meiosis-specific proteins, the ZMM proteins—Zip1, Zip2, Zip3 (also known as Cst9), Zip4 (Spo22), Msh4, Msh5, and Mer3 (Hfm1) [3–5]. A detailed study of a set of *Saccharomyces cerevisiae zmm* mutants demonstrated that the corresponding ZMM proteins were necessary for the correct progression from DSBs to stable single-end invasion intermediates in the meiotic recombination pathway [3–5].

Two *Arabidopsis* ZMM proteins, AtMSH4 and AtMER3 (RCK), were reported to be involved in meiotic crossovers [6–8]. The chiasma frequency of *atmsh4* mutants was greatly reduced to 15% of the wild-type [6]. That of *atmer3* mutants was also considerably lower than in the wild-type [7,8]. In both cases, the defect lay in COI formation in the meiotic recombination pathway [6–8].

In budding yeast, Zip1 is a component of the synaptonemal complex (SC) central region [9]. Zip1 consists of a coiled-coil domain flanked by globular domains, and two Zip1 dimers lying head-to-head span the width of the SC [10]. Recent molecular data from budding yeast studies showed that the formation of a mature SC depends on a protein complex called the synapsis initiation complex (SIC) [11]. Several known components of the SIC are all ZMM proteins [5,12,13]. It appears that binding of Zip3 onto chromatin recruits both Zip2 and Zip4, which in turn induce Zip1 polymerization [5,13]. As Zip proteins are poorly conserved among species, a recent in silico study has sought to overcome the lack of primary sequence homology for identification of functionally homologous proteins [14]. Specifically, ZYP1 in *Arabidopsis* is considered to correspond to Zip1 in yeast [15].

In this report, we describe a semi-sterile mutant, *atzip4-4*, isolated in transposon-tagged lines of *Arabidopsis* [16]. *atzip4-4* has a transposon insertion in the gene-coding region of another Zip gene, *AtZIP4*, which has a weak but significant sequence similarity with yeast *ZIP4/SPO22*. The *atzip4-4* mutants showed typically desynaptic phenotypes: pairing and synapsis looked normal, but afterward, homologous chromosomes separated abruptly. Particularly we are convinced that the homologous chromosome pairing is completed in this mutant by fluorescence *in situ* hybridization (FISH) analysis. In addition, crossover frequency was reduced in the *atzip4-4* mutants, representing partly residual recombination activity by COII. We also show that tagged AtZIP4 protein was

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accumulated as foci on chromosomes in early meiotic prophase. We discuss the different roles of AtZIP4 in *Arabidopsis* and Zip4 in yeast in the orchestration of meiotic progression, including chromosome pairing, synapsis, and crossover formation.

## Materials and methods

*Plant materials, GUS staining and meiotic crossover assay*. The *atzip4-4* mutant was isolated from a *Ds* transposon-tagged mutant population of the Nossen ecotype [16]. The *atzip4-2* allele (salk_068052) was obtained from the Arabidopsis Biological Resource Center (Ohio State University, OH, USA). To determine the genotype of *atzip4-4*, we used the following primers: 11-5412_5′ (5′-GTTTCAGCAACTTCTCTT TGCT-3′), 11-5412_3′ (5′-AGCAAACGTCTTTACCACAAGT-3′), and Ds5-3 (5′-TAC CTCGGGTTCGAAATCGAT-3′). GUS staining was performed according to a standard protocol [17]. The percentage of meiotic crossovers was calculated according to the previous work [18,19].

*Nucleic acid analysis*. Genomic DNA of *Arabidopsis* plants was prepared by using an automatic DNA isolation system (Kurabo, Osaka, Japan). Total RNA from *Arabidopsis* plants was prepared for RT-PCR by using an RNeasy Plant Mini Kit (Qiagen, CA, USA). RT-PCR was performed with a One Step RT-PCR kit (Takara Bio, Shiga, Japan) and the primers 11-5412_RT-PCR_5′ (5′-TTGTTGCTCTCTTTGCTAGTGA-3′) and 11-5412_3′. A cDNA for *At5g48390* was amplified with the primers 90–18 (5′-CCCG CCAACAATCGAAATGAG-3′) and 90–11 (5′-GACAGTTAATATGAATGAAACTAA-3′) from the total RNA of a Columbia wild-type plant, and sequenced (Accession No. AB354634). The ORF of the *At5g48390* cDNA was cloned into the pENTR/D/TOPO vector (Invitrogen, CA, USA), and integrated into the YFP-fusion protein vector pH35GY [20]. The plasmid was then electroporated into *Agrobacterium* GV3101 to generate the transgenic plants by floral dipping of Columbia wild-type plants.

*DAPI staining, FISH and immunolocalization*. Meiotic chromosome spreads were prepared from pollen mother cells (PMCs) as described [18,21]. The number of chiasmata on individual bivalents was estimated as described previously [22]. The probes for FISH were prepared from BAC clones (F25B8, F15G16) used as templates, and synthesized through either a Nick Translation Kit (GE Healthcare, Buckinghamshire, UK) and an ARES Alexa Fluor 488 DNA Labeling kit (Invitrogen), or Cy3-dCTP (GE Healthcare) and Ready-To-Go DNA Labeling Beads (GE Healthcare). Immunolocalization was carried out as described previously [23] with minor modifications. The PMCs were incubated at 4 °C overnight in anti-GFP (rabbit) antibody (Invitrogen) as anti-YFP antibody diluted 400-fold with PBS/1% BSA/0.05% Triton X-100.

## Results

### *Isolation of atzip4-4 mutant*

Previously we systematically observed phenotypes of about 4000 transposon-tagged *Arabidopsis* lines [16]. Some lines had clearly decreased seed yields because of sterile or semi-sterile phenotypes including *spo11-2* [16,19]. We focus on line 11-5412-1 here. According to the flanking sequence database we developed previously [24], 11-5412-1 has the *Ds* element in the fourth exon of a predicted open reading frame (ORF) of the *At5g48390* gene (Fig. 1A).

Line salk_068052, which has a T-DNA insertion in the second exon of *At5g48390*, showed the same phenotypes as our *Ds* mutant, suggesting that mutation of *At5g48390* results in the semi-sterile phenotype. salk_068052 was recently reported to contain *atzip4-2*, one of three mutant alleles of *At5g48390* [25]. We therefore designated the *Ds* insertional allele *atzip4-4*. RT-PCR analysis showed that the allelic homozygous lines of neither *atzip4-2* nor *atzip4-4* contained detectable amounts of transcripts, indicating that these mutants are transcriptional knockouts (Fig. 1B).

The *atzip4-4* allele has a *Ds* insertional element containing the *GUS* reporter gene as a gene-trap system [26]. To check the tissue-specific expression of *AtZIP4* under the control of the authentic promoter, we GUS-stained an *atzip4-4* heterozygous mutant. GUS signals were detected in floral organs, especially anthers and ovaries (Fig. 1C). This gene expression pattern was consistent with the semi-sterile phenotype of the *atzip4-4* mutants.

### *The atzip4-4 mutant is defective in crossover formation*

A couple of *Arabidopsis* mutants of ZMM-like gene were defective in crossover formation during meiotic recombination [6–8]. To examine whether AtZIP4 is involved in meiotic crossover, we crossed heterozygous *atzip4-4* (ecotype Nossen) to the wild-type (ecotype Columbia), and generated homozygous mutants (*atzip4-4/atzip4-4*) with a hybrid background (Nossen/Columbia) for two molecular markers on each of chromosomes I and V. We estimated the frequency of meiotic crossover by determining the genotypes of these markers in $F_3$ progeny of self-fertilized $F_2$ plants. Co-segregating wild-type plants (+/+) with a hybrid background (Nossen/Columbia) for the same markers were used as positive controls. 24.7% of wild-type (+/+) chromosomes crossed over between NGA280 and ATPASE on chromosome I, but only

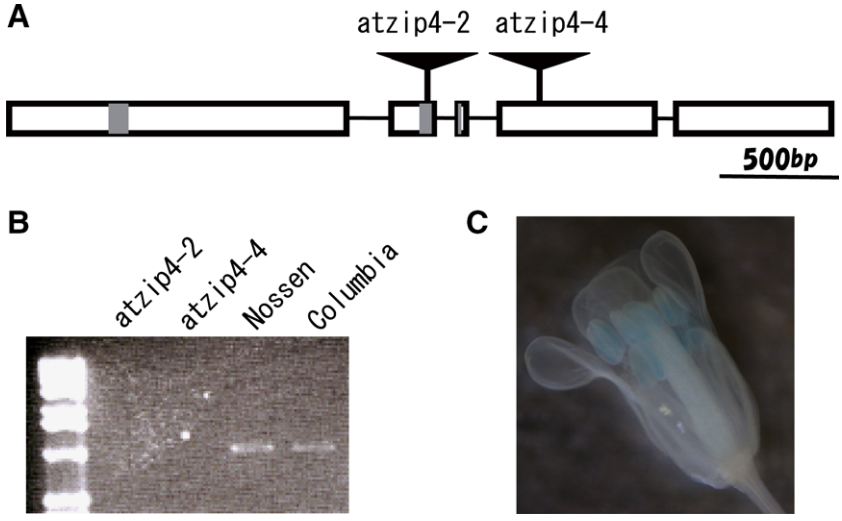


**Fig. 1.** Identification of *AtZIP4* gene and *atzip4* mutant alleles. (A) *AtZIP4* gene structure and insertional mutation sites of two *atzip4* alleles. Square boxes represent exons, and black bars represent introns. The sites of transposon insertion in *atzip4-4* (Ds11-5412-1) in the fourth exon and of T-DNA insertion in *atzip4-2* (salk_068052) in the second exon are indicated. Gray bars represent the regions of tetratricopeptide repeat domains. (B) RT-PCR analysis of *AtZIP4*. Total RNAs were prepared from flowers of *atzip4-4* (Nossen ecotype), *atzip4-2* (Columbia ecotype), Nossen wild-type, and Columbia wild-type (left to right). No transcription was observed in either mutant allele. (C) GUS staining of *atzip4-4* flowers using the gene-trap system in the transposable element. Tissue-specific expression of the *GUS* reporter gene was detected in heterozygous *atzip4-4* plants. GUS activity was observed in anthers and ovaries of mutant flowers.

6.4% of mutant (*atzip4-4/atzip4-4*) chromosomes did so, indicating a severe defect in the crossover process. Similarly, the crossover frequency between NGA106 and NGA76 on chromosome V was 20.0% in the wild-type (+/+), but only 3.5% in mutants (*atzip4-4/atzip4-4*). These results strongly suggest that crossover formation during meiosis in *Arabidopsis* requires AtZIP4.

Chiasmata are generally considered to be cytological representations of crossover events. The number of chiasmata per cell can be counted by examining DAPI-stained chromosomes in PMCs. The *atzip4-4* mutant was not entirely devoid of recombinations, forming an average of 2.55 chiasmata per cell (58 cells). The distributions of chiasmata per cell in *atzip4-4* and wild-type (Nossen) plants are shown in Fig. 2A and B. Despite the low mean chiasma frequency of *atzip4-4* mutants, the number of chiasmata per cell was quite variable, ranging from 0 to 9, although the distribution was markedly skewed at the mode to the two-chiasmata class. This distribution did not differ significantly from a Poisson distribution (Fig. 2B), indicating that the distribution of these residual chiasmata among cells was random. In contrast, the wild-type had more chiasmata (10.33 per cell), and their distribution among cells deviated significantly from a Poisson distribution (Fig. 2A). Wild-type meiocytes had a large excess of chiasmata around the mean, and no cases of fewer than 8 or more than 15 chiasmata per cell were observed (39 cells). We similarly analyzed the distributions of chiasmata per chromosome. Whereas the distribution of chiasmata per chromosome in the wild-type deviated strongly from a Poisson distribution (Fig. 2C), that in *atzip4-4* mostly fitted the predicted distribution (Fig. 2D). These results are additional evidence that the residual chiasmata that are formed in *atzip4-4* are randomly distributed.

### *Homolog pairing was completed in the atzip4-4 mutant*

In spite of the crossover deficiency, the early meiotic progression of *atzip4* mutants looked indistinguishable from that of the wild-type [25]. To examine the progression more closely, we investigated synapsis and chromosome organization by FISH analysis with BAC probes of prophase nuclei. We reasoned that if synapsis were locally incomplete in *atzip4-4* cells, this might be seen as a greater proportion of double spots as opposed to single FISH signals. For this analysis we selected two *Arabidopsis* BAC probes (F25B8 and F15G16), which hybridize near the telomere region of the short arm of chromosome I and on the short arm of chromosome III, respectively. Fig. 3A and B shows the results with the F25B8 sequence as the probe. Signals first appeared at different loci at early prophase I (Fig. 3A1 and B1), and came together at mid prophase I (Fig. 4A2 and B2) in both wild-type and mutant cells. But later, whereas signals from the wild-type stayed together until metaphase I (Fig. 3A3 and A4), signals from the mutant started separating as early as diplotene (Fig. 3B3 and B4). Signal separation was observed in the wild-type too, but the distances were much smaller than in the mutant. We measured the distance between the two signals by probe, comparing it with the whole expansion of meiotic chromosomes at each stage. In wild-type meiocytes, two signals could be detected in premeiotic cells, but they started getting closer in leptotene cells, lay mostly together in zygotene and pachytene cells, then began to separate gradually in diplotene and diakinetic cells (Fig. 3C). In mutant meiocytes, separate signals could be detected as in the wild-types from premeiotic to pachytene cells (Fig. 3D). In pachytene cells, FISH signals were observed as a single dot for each probe, indicating that pairing and synapsis could be completed even if *AtZIP4* was disrupted (Fig. 3B2). However, they separated abruptly after the diplotene stage (Fig. 3B3 and B4), not gradually as in wild-type cells (Fig. 3A3 and A4).

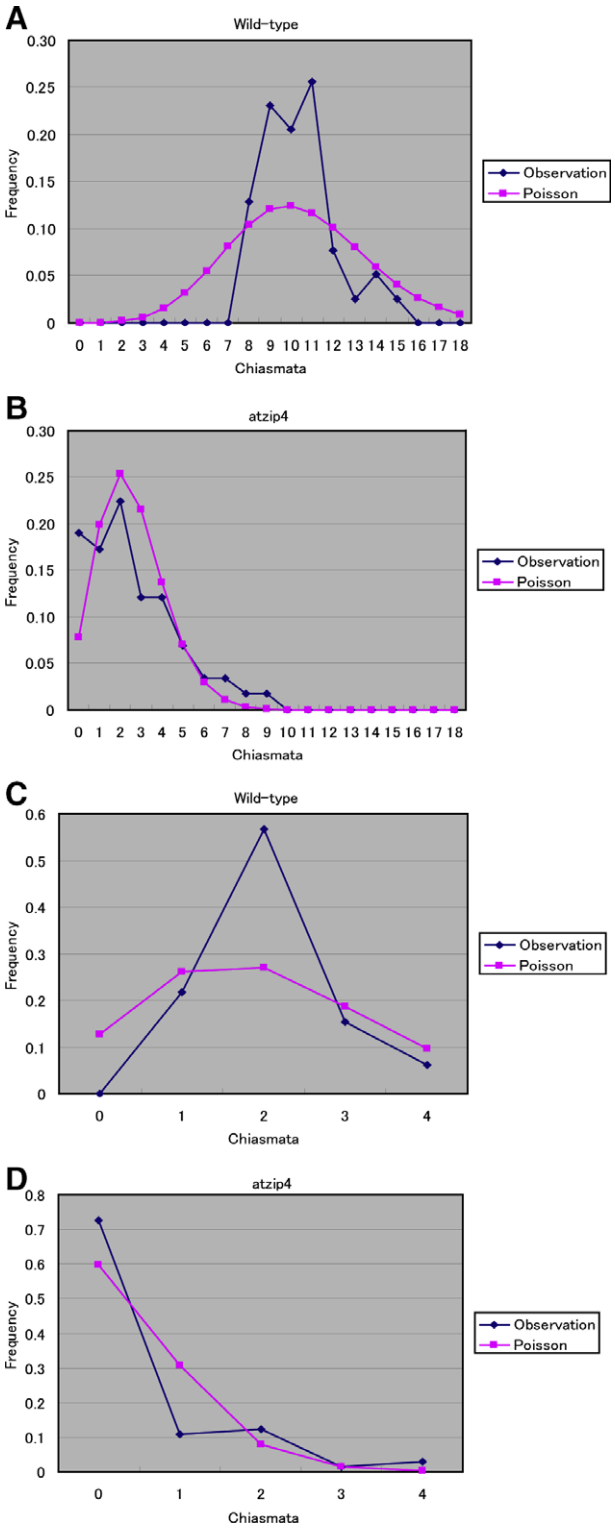


**Fig. 2.** Chiasmata distribution in wild-type and *atzip4-4* cells. Numbers of chiasmata per meiocyte (A,B) and per chromosome (C,D) were counted in wild-type cells (A,C) and *atzip4-4* cells (B,D). The observed distributions of the chiasmata deviate from predicted distributions in the wild-type cells, but not from the predicted Poisson distribution in mutant cells.

### *AtZIP4 proteins are localized as foci on meiotic chromosomes*

To investigate AtZIP4 protein localization in PMCs, we tried first to detect the protein signals directly by using a peptide antibody

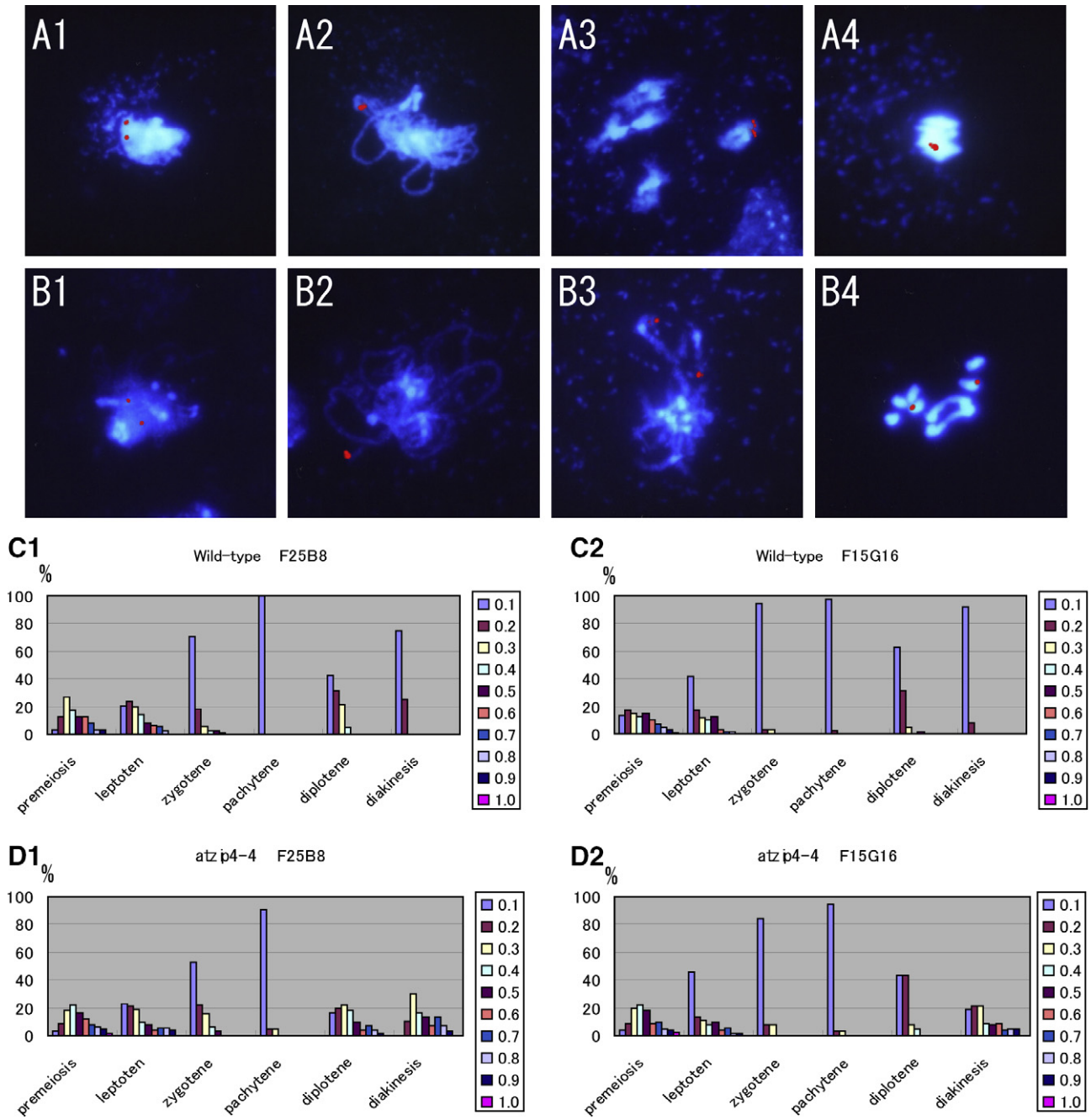


**Fig. 3.** FISH analysis of homologous chromosomes at various stages of meiotic prophase I. (A,B) FISH signals of DNA probes hybridized to pachytene-stage nuclei of wild-type cells (A) and *atzip4-4* cells (B). (A1,B1) Early prophase I. (A2,B2) Mid prophase I. (A3, B3) Late prophase I. (A4) Normal metaphase I. (B4) Defective metaphase I. Red signals show the location of BAC F25B8 loci on chromosome I. (C,D) Distribution of cells at each stage in wild-type cells (C) and *atzip4-4* cells (D). The relative distance between signals of homologous chromosomes was calculated as the actual measurement divided by the whole expansion of meiotic chromosomes. Loci were examined by FISH using probes specific to BAC clone F25B8 (C1,D1) or F15G16 (C2,D2). The distribution is shown as a percentage of the total number of cells examined at each stage.

against AtZIP4. However, we could not find any signals, which suggested that there was little active expression of this protein. Indeed, examination of the public AtGenExpress microarray database [27] showed that *At5g48390* (corresponding to *AtZIP4*) was expressed at a low level throughout development, although it was moderately upregulated in floral tissue. In yeast research, Tsubouchi et al. [5] investigated the localization of Zip4 by anti-tag antibody against tagged Zip4 proteins instead of by direct detection. So we generated transgenic plants that produced AtZIP4 fused with a tagging protein under the control of the 35S promoter. Fluorescence immunolocalization on spread preparations of anthers from immature flower buds was performed with a commercial antibody against YFP-tagged proteins. The results indicated that AtZIP4 was restricted to meiocytes at prophase I, and numerous fluorescent foci appeared temporarily in leptotene nuclei in a restricted period (Fig. 4), whereas very few foci could be detected at other meiotic stages. This result suggests that AtZIP4 has an important role very specifically in meiotic early prophase I.

## Discussion

We isolated a novel mutant allele of *AtZIP4* producing sterile phenotype in transposon-tagged lines of *Arabidopsis* (Fig. 1). We found that the mutant had meiotic defects, typically a desynaptic

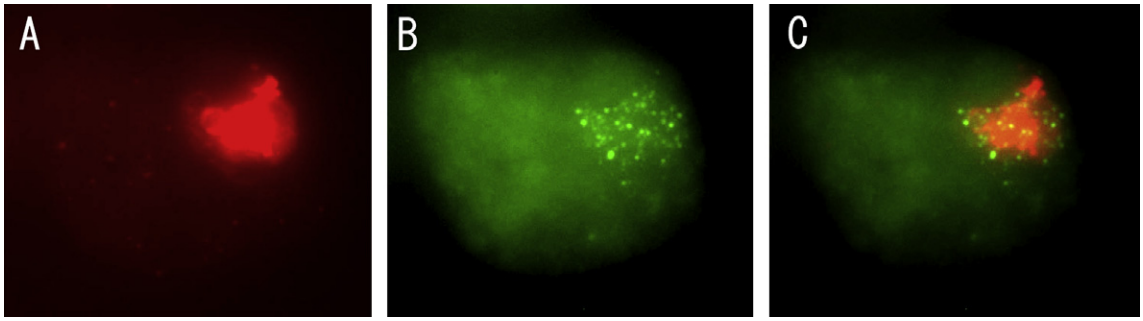


**Fig. 4.** Immunolocalization of AtZIP4 protein. A meiotic cell carrying tagged AtZIP4 was stained with DAPI (A) and anti-tag antibodies (B). Images are merged in (C). Numerous signals appeared in the leptotene stage of meiotic cells.

phenotype (Fig. 3). In particular, we demonstrated that successful pairing of homologous chromosomes at the pachytene stage was followed abruptly by unpairing in *atzip4-4* mutant chromosomes (Fig. 3). We also showed a reduced crossover frequency in the mutant (Fig. 2), almost the same level as in the *atmsh4* mutant, which was reported to be defective in COI [6]. These results suggest that AtZIP4 protein is necessary for normal crossover formation and chromosome segregation, especially COI, in meiotic prophase I in *Arabidopsis*. Indeed, *AtZIP4* was specifically expressed in young flowers, especially anthers and ovaries, and labeled proteins were localized as foci during a restricted period in leptotene nuclei (Figs. 1 and 4).

In *S. cerevisiae*, Zip4/Spo22 was characterized as a meiosis-specific protein essential for chromosome synapsis [5]. In the absence of Zip4, the synaptonemal complex (SC) protein Zip1 failed to polymerize along chromosomes [5]. Zip4 forms a functional unit with Zip2, which was characterized as a component of the synapsis initiation complex (SIC) [5]. Crossing-over was decreased in the *zip4* mutant as in other *zmm* mutants, but *zip4* displayed a novel phenotype: negative crossover interference, meaning that crossovers tended to cluster, dependent on Zip1 [5]. Our results reveal a couple of differences related to the functional roles of yeast ZIP4 and *Arabidopsis* AtZIP4. First, in yeast, the *zip4* mutant phenotype could be explained by the defect in SC formation [5]; but in *Arabidopsis* the *atzip4* mutants looked normal to synapsis before metaphase I, then homologous chromosomes separated after synapsis (Fig. 3). Second, in yeast, the *zip4* mutant displayed negative crossover interference [5]; but in *Arabidopsis* the *atzip4* mutants did not, although crossover formation was greatly reduced (Fig. 2). Third, in yeast, Zip4 formed a functional unit with Zip2 [5]; but in *Arabidopsis* no *zip2*-like factor is known yet. So far, the *Zip3* gene is also missing in *Arabidopsis*. Whether AtZIP4 has any functional partner is yet to be resolved.

The functions of Zip1 in yeast and ZYP1 in *Arabidopsis* in SC formation and crossover formation differ. In budding yeast, Zip1 has been described as having two important roles in meiosis: the formation of the SC as a component of the SIC, and the formation of COI as a component of the ZMM proteins [5,11]. In *zip4* mutants, few or no linear stretches of Zip1 were observed, indicating that Zip4 or a Zip4 complex, such as Zip2/Zip4, is needed for exact localization of Zip1 on meiotic chromosomes [5]. The DSBs, or recombination processes after the DSBs, are necessary for Zip4 protein localization, because no Zip4 signal was detected on chromosomes in *spo11* mutants [5]. This evidence suggests that Zip1 works in both SC formation and COI formation, at least under Zip4-related control.

On the other hand, ZYP1 in *Arabidopsis* has been described as having a role in the formation of the SC, but not as much as the function of ZMM proteins in yeast, because crossover formation was only slightly reduced when ZYP1a and ZYP1b, which are two *Arabidopsis* homologs of yeast ZIP1, were inactivated by RNA interference [15]. In addition, multivalents between non-homologous chromosomes were observed when *ZYP1* genes were inactivated [15], suggesting that ZYP1 proteins are specialized to function in SC formation with the inhibition of non-homologous chromosome association, rather than in the COI pathway. ZYP1 polymerization to bring about homologous chromosome synapsis did not occur if recombination was blocked at an early step [15], suggesting that some recombination proteins are necessary for ZYP1 polymerization. In addition, there are some other descriptions related to AtMSH4 analysis: AtRAD51, which is a main member of the recombination proteins, co-localized with AtMSH4, and the synapsis of homologous chromosomes in the *atmsh4* mutant might be incomplete [6]. This evidence suggests that some relationships between recombination, the COI pathway, and SC formation are independent of ZYP1 proteins.

We now wonder whether AtZIP4 forms a complex and, if so, with what; whether it has any relation to DSBs or early recombination intermediates; and whether it directly targets AtMSH4. The ternary structures in the relationship would be interesting. We dissected the complete homolog search phase, but the mechanism that maintains the pairing for precise chromosome segregation is still inconclusive. These issues need be investigated to explain the network of factors involved in SC formation and the CO pathway in meiotic progression.

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