Introduction

Polyploidy has been a primary mechanism of plant evolution, and related gene expression, primarily by allopolyploids, has been the subject of a great deal of recent research. Expression is affected not only by ploidy type but also by the hybridization of different genomes in allopolyploidy. However, it is difficult to distinguish between those two factors (Chen and Ni 2006). A comparison of expression profiles among the synthetic Brassica napus (CCAA), a diploid hybrid (CA), and its homozygous diploid progenitors B. rapa (AA) and B. oleracea (CC) has indicated that hybridization has a greater effect than genome-doubling (Albertin et al. 2006). Similar results have been reported from work with maize (Zea mays; Auger et al. 2005) and Senecio (Hegarty et al. 2006). Galitski et al. (1999) and Guo et al. (1996) have suggested that autopolyploids could be used for accurately estimating the influence of ploidy on gene expression. This is particularly interesting if one agrees that all changes are due strictly to genome duplication rather than to hybridization. In some studies with autopolyploids, alterations in expression have also been observed between ploidy types, albeit to a much smaller degree (Church and Spaulding 2009). Stupar et al. (2007) found with microarray analyses of potato (Solanum tuberosum) that, for 9000 loci, only 10% show a change in their level of expression among haploid, diploid, and tetraploid plants. Autopolyploidy in cabbage (Brassica oleracea) does not significantly alter the proteomes of green tissues (Albertin et al. 2005).

The size of plant organs depends upon the regulation of cell division and cell expansion. Signals from the ARGOS protein are transferred to the gene for the AINTEGUMENTA (ANT) encoding transcription factor, which in turn controls cellular division in the aboveground organs (Wang et al. 2009) by regulating the expression of cyclin CYCD3;1 (Hu et al. 2003, Mizukami and Fischer 2000). Furthermore, an increase in the transcription of ARGOS can lead to enhanced expression by protein factors that modulate cell expansion, e.g., by AtGRF1 and AtEXP10 (Wang et al. 2009). Most studies of ARGOS have to-date focused on transgenic plants, such as tobacco (Nicotiana tabacum) that expresses ARGOS

Research Paper

Analyses of phenotype and ARGOS and ASY1 expression in a ploidy Chinese cabbage series derived from one haploid

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The aim of this research was to improve our understanding of how ploidy level influences phenotype and gene expression in Chinese cabbage (Brassica rapa L. ssp. pekinensis). Haploid plants (2n = 10) was induced by 0.2% colchicine to produce diploid (2n = 20) and tetraploid plants (2n = 40). The aneuploid (2n = 24) was also obtained by hybridization between diploid plants as the female and tetraploid plants. The ploidy levels of all plants were identified through chromosome counts and flow cytometry. Leaves and petals became larger as the ploidy level increased from haploid to diploid, and from aneuploid to tetraploid. Similarly, expression of ARGOS was regulated by genome size, increasing in parallel with the level of ploidy. Among the four ploidy types, expression was stronger in the floral buds than in the leaves. Expression by ASY1 also differed according to ploidy level, being highest in diploid plants, followed in order by tetraploids. Expression was similar between haploids and aneuploids at two stages—prior to and after meiosis—but was higher in the haploids during meiosis. When buds were compared within the same ploidy type at different stages, ASY1 expression was obviously higher during meiosis than either before or after. Our study demonstrated the generation and phenotype of a ploidy Chinese cabbage series derived from one haploid. Expression of genes ARGOS and ASY1 were modulated by genome size in this ploidy series, and the regulated patterns of the two genes was different.

Key Words: Chinese cabbage, ploidy series, organ size, ARGOS, ASY1.
from *Arabidopsis thaliana* (Kuluev *et al.* 2013, 2014a), as well as *Arabidopsis* transgenics that express this gene from Chinese cabbage (*Brassica rapa* L. ssp. *pekinesis*; Wang *et al.* 2010).

The *ASY1* (*ASYNAPTIC 1*) gene is essential for homologous chromosome synapsis. Because *asy1* mutants of *Arabidopsis* are characterized by the development of two univalents by most homologous chromosomes, compared with only one bivalent in the wild type (Armstrong *et al.* 2002). We have previously shown that one to two bivalents or one trivalent (as well as univalents) occur in haploid Chinese cabbages, whereas diploid Chinese cabbages have 10 bivalents, and aneuploid Chinese cabbages can have two to four univalents, one to two trivalents, or even one quadrivalent (as well as bivalents), likewise, in tetraploid Chinese cabbages, one quadrivalent, two bivalents, or a trivalent plus a univalent, are formed from four homologous chromosomes (Liu *et al.* 2013). However, the relationship between the expression of *ASY1* and patterns of homologous chromosome synapsis has not been previously studied.

In the present research, we generated a ploidy Chinese cabbage series of plants from one genotype of a haploid that had different-sized leaf and flower organs. And detected whether and/or how *ARGOS* expression changed and to evaluate the relationship between organ size and genome dosage. Based on the diverse patterns of homologous chromosome synapsis observed in that ploidy series, we also explored the expression of *ASY1* among haploid, diploid, aneuploid, and tetraploid plants.

**Materials and Methods**

**Production of a ploidy Chinese cabbage series**

The haploid Chinese cabbage ‘13-1’ was obtained from an isolated microspore culture. Cloned haploid seedlings were then treated with 0.2% colchicine for 48 h to develop diploid plants, which were then exposed to 0.2% colchicine for 48 h to produce tetraploids. From a diploid cabbage and a tetraploid reciprocal cross, ovaries were removed at 8 to 10 d post-pollination and cultured on an improved White culture medium. Afterward, the ovules were stripped out and cultured in an MS medium for 20 to 25 d to obtain vigorous plants. The levels of ploidy were determined via flow cytometry and the counting of chromosomes.

**Morphological characterizations and plant growing conditions**

Rooted plantlets from the haploid, diploid, aneuploid, and tetraploid vegetative lines were transferred to a greenhouse (Agricultural University of Hebei, Baoding, China) in January of 2013 for standard management under a regimen of natural illumination and ambient temperatures. Organ sizes were evaluated when flowering began. The three largest leaves and flowers were measured from three plants per ploidy type.

**Pollen viability**

The percentage of pollen grains that were stained with 0.5% triphenyl tetrazolium chloride, normal grains were densely stained, while aborted grains were lightly stained or completely colorless.

**Quantitative real-time PCR (qRT-PCR)**

Total RNA was extracted from leaves and floral buds with Trizol reagent. First-strand cDNA was prepared with a ReverTra Ace qPCR RT Master Mix (TOYOBO, Shanghai, China). The primers CTCGTCGAGAAAAGGT and CCAACGGAAGTATCAACAG were used for qRT-PCR of *ARGOS* whereas ATTCGGATTCTGATAGCC and CTCC TCATTTGATTGG were used for *ASY1*. Our reference gene was *β-actin*. Calculations were performed according to the 2^–ΔΔCt_ method (Livak and Schmittgen 2001), with the level of expression in the diploid set as 1.00. Amplifications were performed in a total volume of 20 μL that contained 0.5 μL of appropriate primers and reverse primers, 2 μL of cDNA template, 10 μL of KOD SYBR qPCR Mix.
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Results

Generation of a ploidy Chinese cabbage series

We cloned 23 lines from haploid 13-1 (Figs. 1a, 2a, 3a) and treated them with 0.2% colchicine for 48 h. This resulted in the production of four diploid Chinese cabbages plants (Fig. 1b), based on screening with flow cytometry (Fig. 2b) and the counting of chromosomes (Fig. 3b). The other 19 plants were chimeras. When 15 cloned plants from the diploid were treated with 0.2% colchicine for 48 h, only one tetraploid was produced (Fig. 1c), as confirmed by flow cytometry (Fig. 2c) and chromosome counts (Fig. 3c). After the diploid as female was crossed with this tetraploid, 78 ovaries were removed at 8 to 10 d post-pollination and cultured in the improved White medium. However, none of the ovules survived.

(TOYOB0, Shanghai, China), and 7 μL of water. The PCR conditions included initial denaturation for 2 min at 98°C; followed by 40 cycles of denaturation for 30 s at 98°C, hybridization for 10 s at 58°C, and elongation for 10 s at 68°C.

Fig. 2. DNA concentrations for Chinese cabbage plants with different levels of ploidy: (a) haploid, (b) diploid, (c) tetraploid, and (d) aneuploid.

Fig. 3. Mitotic chromosomes at metaphase for Chinese cabbage plants with different levels of ploidy: (a) haploid, (b) diploid, (c) tetraploid, and (d) aneuploid.

Fig. 4. Leaves from Chinese cabbage plants with different levels of ploidy: (a) tetraploid, (b) aneuploid, (c) diploid, and (d) haploid.

Fig. 5. Flowers from Chinese cabbage plants with different levels of ploidy: (a) haploid, (b) diploid, (c) tetraploid, and (d) aneuploid.
Comparative morphological analysis of a ploidy Chinese cabbage series

As the ploidy level rose, both leaves (Fig. 4) and floral buds (Fig. 5) increased in size (Table 1) during the flowering period. For example, leaves from aneuploid and tetraploid plants were 5.44% and 23.38% longer, respectively, than those from diploid plants while haploid leaves were 14.33% shorter. The degree of difference in leaf widths among ploidy types was not as dramatic. Petal length for aneuploid and tetraploid plants was increased by 56.41 and 76.92%, respectively, when compared with diploids, while haploid petals were 26.92% shorter. Finally, petal widths were 7.14 (aneuploid) and 29.43% (tetraploid) larger than for the diploid, while those of the haploid were 50.00% smaller. Overall, the contrast was greater among ploidy types for petal size than for leaf dimensions. We also measured the size of floral buds and guard cells and found that both increased along with ploidy status (Table 1, Figs. 6, 7). Haploid plants tended to have narrow anthers and no pollen. The number of pollen grains per anther and pollen viability were highest for diploids, followed by aneuploids (Table 1). The ordering of flowering time, from earliest to latest, was haploid, tetraploid, and diploid. Respective flower and leaf colors were similar regardless of ploidy type.

Quantitative analysis of ARGOS expression in leaves and floral buds

When compared with the diploid type, ARGOS expression in the leaves during the flowering period was increased in the haploid, aneuploid, and tetraploid types by 0.45-fold, 1.58-fold, and 2.14-fold, respectively (Fig. 8). These changes in expression paralleled the change in genome dosage as well as the trend in the dimensions of leaf organs. In floral buds, ARGOS expression was 0.23-fold, 1.81-fold, and 2.45-fold higher in the haploid, aneuploid, and tetraploid types, respectively, than in the diploid (Fig. 9). The magnitude of this difference among ploidy types was greater than that observed in the leaves because changes in petal sizes were more dramatic.

Quantitative analysis of ASY1 expression in leaves and floral buds

Regardless of developmental stage, ASY1 expression was not detected in the leaves of haploid, diploid, aneuploid, or tetraploid Chinese cabbage plants. However, using floral buds taken from first-order lateral branches, we were able to monitor expression before, during, and after meiosis in samples from all four ploidy types (Table 2). Bud sizes at meiosis were determined by microscopic observation; the

Table 1. Characteristics of leaf, flower, and pollen from Chinese cabbages with different levels of ploidy

|                      | Haploid       | Diploid       | Aneuploid     | Tetraploid    |
|----------------------|---------------|---------------|---------------|---------------|
| Leaf width (cm)      | 8.100 ± 1.510 | 9.630 ± 1.560 | 9.680 ± 1.350 | 9.730 ± 1.440 |
| Leaf length (cm)     | 10.700 ± 1.480| 12.490 ± 1.280| 13.170 ± 1.270| 15.410 ± 1.550|
| Floral bud length (cm)| 0.435 ± 0.023 | 0.589 ± 0.028 | 0.592 ± 0.062 | 0.688 ± 0.023 |
| Floral bud width (cm)| 0.176 ± 0.011 | 0.261 ± 0.011 | 0.245 ± 0.017 | 0.371 ± 0.024 |
| Petal length (cm)    | 0.565 ± 0.058 | 0.778 ± 0.038 | 1.222 ± 0.053 | 1.377 ± 0.047 |
| Petal width (cm)     | 0.352 ± 0.016 | 0.700 ± 0.013 | 0.751 ± 0.011 | 0.906 ± 0.024 |
| Number of pollen grains per anther | 2.30 × 10^5 | 2.12 × 10^5 | 1.94 × 10^5 |
| pollen viability (%) | 94.01          | 82.20         | 72.87         |

Fig. 6. Floral buds from Chinese cabbage plants with different levels of ploidy: (a) haploid, (b) diploid, (c) aneuploid, and (d) tetraploid.

Fig. 7. Stomatal sizes from Chinese cabbage plants with different levels of ploidy: (a) haploid, (b) diploid, (c) aneuploid, and (d) tetraploid.
smallest and largest buds from a single inflorescence were examined before and after meiosis, respectively. Prior to meiosis (Fig. 10a), expression was similar between the haploid and aneuploid, but was 0.28-fold and 0.31-fold higher, respectively, when compared with the diploid. Expression in the tetraploid was 0.57-fold higher than in the diploid and was also greater than in either the haploid or aneuploid. During meiosis (Fig. 10b), ASY1 expression was 0.63-fold, 0.45-fold, and 0.90-fold higher in the haploid, aneuploid, and tetraploid, respectively, than in the diploid. Transcript levels were lowest in aneuploid buds. After meiosis (Fig. 10c), expression was similar between the haploid and aneuploid, but was 0.30-fold and 0.29-fold higher, respectively, than in the diploid. Transcript levels in the tetraploid were 0.56-fold greater than in the diploid and were also higher than in either the haploid or aneuploid. Among this ploidy series, these changes in expression paralleled the change in the rate of normal homologous chromosome
synapsis. When buds were compared within the same ploidy type during different meiotic stages (Fig. 11), similar levels of transcripts were detected both before and after meiosis. However, expression was obviously much lower than that measured during meiosis.

**Discussion**

The diversity of homologous chromosome synapsis in a tetraploid plant form leads to a variety of gametes. This explains why we were able to generate the $2n = 24$ aneuploid after crossing the diploid as female with the tetraploid. Using loquat fruit, Wang (2008) has obtained 16 aneuploid plants from 30 seeds after performing hybridizations between the autotetraploid as female and the diploid.

Organ sizes were in proportion to the extent to which $\textit{ARGOS}$ was expressed in our haploid, diploid, aneuploid, and tetraploid Chinese cabbage. This has also proven true for transgenic plants of tobacco and $\textit{Arabidopsis}$ plants that over-express $\textit{ARGOS}$ or show reduced expression of that gene and produce larger or smaller aerial organs, respectively, than their wild-type counterparts (Kuluev et al. 2014b, Wang et al. 2009). In transgenic tobacco expressing $\textit{ARGOS}$ from $\textit{Arabidopsis}$, leaves are approximately 25% larger while their flower sizes are increased by only 5% (Kuluev et al. 2011, 2014b). However, we noted here that, as ploidy increased, the magnitude by which petal size was enlarged was more significant than that of leaf dimensions. Moreover, the rise in $\textit{ARGOS}$ expression was more significant for floral buds than for leaves. This discrepancy may have resulted not only because of organ position on the plant and dosage of $\textit{ARGOS}$ gene but also because of the genome dosage effect associated with haploid, diploid, aneuploid, and tetraploid types.

Armstrong et al. (2002) reported that Asy1 was not detected in the vegetative tissues of the stem and leaf in $\textit{B. oleracea}$ by western blots analysis. Our research showed that $\textit{ASY1}$ was particularly expressed in bud but not in leaf, and its expression was the highest during meiosis in buds. In Chinese cabbage ploidy series, $\textit{ASY1}$ expression was modulated by genome size, being highest in diploid, next to tetraploid, followed by haploid and aneuploid. These changes in expression were similarly with the change in the rate of normal homologous chromosome synapsis among ploidy series. In our previously study (Liu et al. 2013), in Chinese cabbage the homologous chromosome synapsis were all normal in diploid, most of homologous chromosome synapsis were normal in tetraploid, the rate of abnormal homologous chromosome synapsis were increased in haploid and aneuploid.

Genes for which expression is modulated by genome dosage have been identified in yeast (Galitski et al. 1999), potato (Stupar et al. 2007), and maize (Riddle et al. 2010). Yao et al. (2011) have also monitored transcript levels for eight genes in diploid (2X), tetraploid (4X), and hexaploid (6X) maize, and have described five separate patterns of expression based on ploidy level, i.e., $2X < 4X < 6X$, $4X < 2X < 6X$, $2X = 4X < 6X$, $2X > 6X > 4X$, or no significant differences. However, this variation between any two ploidy types is not uniform for all genes. In addition, as ploidy increased in our Chinese cabbage, $\textit{ARGOS}$ expression was enhanced, but contrasts in transcript abundance were not as dramatic in the leaves as in the floral buds. Likewise, the ordering of expression for $\textit{ASY1}$ followed diploid > tetraploid > haploid and aneuploid. Compared with expression in the diploids, expression in any of the other three ploidy types varied.

![Fig. 11.](image-url) Relative expression by $\textit{ASY1}$ before (1), during (2), and after (3) meiosis in Chinese cabbage plants within the same ploidy type: (a) haploid, (b) diploid, (c) aneuploid, and (d) tetraploid.
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According to the stage of bud development before, during, and after meiosis. All of these results provided evidence that expression could be up-regulated, down-regulated, or not significantly altered by a change in ploidy. Moreover, the degree of expression was not consistent for all genes monitored here. And transcript levels measured for any particular developmental stage or tissue type might not have been the same when comparing two different ploidy types.

In the present research, expression of used genes were modulated by genome size in Chinese cabbage ploidy series, showing different regulated patterns. We will further explore and study the modulated mechanism by which ploidy influences gene expression. The fluctuations in expression among ploidy types might be explained by dosage and dosage compensation effects (Guo and Birchler 1994, Guo et al. 1996). In addition, Galitski et al. (1999) have suggested that changes in cell size among four ploidy types of yeast (haploid, diploid, triploid, and tetraploid) can have an impact on the import and nuclear concentration of regulatory proteins, thereby leading to alterations in transcription. Moreover, DNA methylation (Lee and Chen 2001, Wang et al. 2004) and microRNA (Kashkush et al. 2003, Yamada et al. 2003) have a clear effect on the regulatory mechanism. The role that ploidy has in controlling expression has also been associated with the level and patterns of DNA methylation (Beaulieu et al. 2009, Wang et al. 2004) and the number and expression of microRNA (Hu et al. 2011, Kashkush et al. 2003). Among our ploidy Chinese cabbage series derived from one haploid, leaves and floral organ became larger as ploidy increased, and expression regulated patterns of the related genes were different, which would lay the foundation to comprehensive understand modulated patterns of gene expression by ploidy level at whole genomics. It will clarify reasons/mechanism of gene expression affected only by ploidy but not by the hybridization of different genomes, better knowing the function and meaning of polyploidy in plant evolution.

Acknowledgments

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All authors declare that they have no conflict of interest.

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