Homoeologous copy-specific expression patterns of MADS-box genes for floral formation in allopolyploid wheat

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The consensus model for floral organ formation in higher plants, the so-called ABCDE model, proposes that floral whorl-specific combinations of class A, B, C, D, and E genes specify floral organ identity. Class A, B, C, D and E genes encode MADS-box transcription factors; the single exception being the class A gene *APETALA2*. Bread wheat (*Triticum aestivum*) is a hexaploid species with a genome constitution AABBDD; the hexaploid originated from a cross between tetraploid *T. turgidum* (AABB) and diploid *Aegilops tauschii* (DD). Tetraploid wheat is thought to have originated from a cross between the diploid species *T. urartu* (AA) and *Ae. speltooides* (BB). Consequently, the hexaploid wheat genome contains triplicated homoeologous copies (homoeologs) of each gene derived from the different ancestral diploid species. In this study, we examined the expression patterns of homoeologs of class B, C and D MADS-box genes during floral development. For the class B gene *wheat PISTILLATA2* (*WPI2*), the homoeologs from the A and D genomes were expressed, while expression of the B genome homoeolog was suppressed. For the class C gene *wheat AGAMOUS1* (*WAG1*), the homoeologs on the A and B genomes were expressed, while expression of the D genome homoeolog was suppressed. For the class D gene *wheat SEEDSTICK* (*WSTK*), the B genome homoeolog was preferentially expressed. These differential patterns of homoeolog expression were consistently observed among different hexaploid wheat varieties and synthetic hexaploid wheat lines developed by artificial crosses between tetraploid wheat and *Ae. tauschii*. These results suggest that homoeolog-specific regulation of the floral MADS-box genes occurs in allopolyploid wheat.

Key words: ABCDE model, homoeologous gene, homoeolog, MADS-box gene, polyploidy

INTRODUCTION

The ABCDE model of flower development proposes that floral organ identity is mediated by five classes of homeotic genes, named A, B, C, D and E (Rijkema et al., 2010). According to this model, class A and E protein complexes induce sepal formation as the ground-state floral organs in the first floral whorl; class A, B and E protein complexes specify petals in the second whorl; class B, C and E protein complexes specify stamens in the third whorl; and class C and E protein complexes specify car-

pels in the fourth whorl. The ABCDE homeotic genes in *Arabidopsis* encode MADS-box transcription factors, the exception being the class A gene, *APETALA2* (*AP2*). The *Arabidopsis* genome includes the class A MADS-box gene *AP1* (Mandel et al., 1992), the class B genes *AP3* and *PISTILLATA* (*PI*) (Jack et al., 1992; Goto and Meyerowitz, 1994), the class C gene *AGAMOUS* (*AG*) (Yanofsky et al., 1990), the class D genes *SEEDSTICK* (*STK*), *SHATTERPROOF1* (*SHP1*) and *SHP2* (Favaro et al., 2003; Pinyopich et al., 2003), and the class E genes *SEPALLATA1* (*SEP1*), *SEP2*, *SEP3* and *SEP4*. The class E genes show partially redundant functions in determination of sepal, petal, stamen and carpel identities (Pelaz et al., 2000; Ditta et al., 2004).

The ABCDE model applies to wheat flower develop-

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ment (Murai, 2013). The wheat inflorescence (spike or ear) is composed of spikelets that, in turn, are composed of florets. In each floret, the reproductive organs are enveloped by two leaf-like structures, the lemma and the palea. The lemma is a bract and the palea is a prophyll, the first leaf formed by the axillary meristem (Shitsukawa et al., 2007). An individual wheat flower contains one pistil, three stamens and two lodicules that are modified petals. Thus, the wheat floral organ has a palea, lodicules, stamens and a pistil in whorls 1, 2, 3, and 4, respectively.

Wheat *FRUITFULL2* (*WFUL2*) is a candidate wheat class A gene (Kinjo et al., 2012). *WPI1* (wheat *PISTILLATA1*) and *WPI2* are class B genes (Hama et al., 2004). A phylogenetic analysis using deduced amino acid sequences indicated that *WPI1* and *WPI2* are orthologs of the rice *PI*-type genes *OsMADS4* and *OsMADS2*, respectively (Murai, 2013). *WPI1* and *WPI2* have also been called *TaPI-1* and *TaPI-2/TaAGL26*, respectively (Zhao, T. et al., 2006; Paolacci et al., 2007). The *AG* orthologs of wheat, *WAG1* (wheat *AGAMOUS1*) and *WAG2*, were identified as class C genes (Meguro et al., 2003; Hirabayashi and Murai, 2009). A phylogenetic analysis using the deduced amino acid sequences showed that *WAG1* and *WAG2* are orthologs of the rice *AG*-type genes, *OsMADS58* and *OsMADS3*, respectively (Murai, 2013). *WAG1* and *WAG2* are also known as *TaAG-1* and *TaAG-2/TaAGL39*, respectively (Zhao, T. et al., 2006; Paolacci et al., 2007). Two studies in wheat identified five genes, *TaAGL2*, *TaAGL9*, *TaAGL31*, *TaAG-3A* and *TaAG-3B*, as candidate orthologs of the rice class D gene *OsMADS13* (Zhao, T. et al., 2006; Paolacci et al., 2007). Sequence analyses showed that *TaAG-3A* is identical with *TaAGL9*, and *TaAG-3B* is identical with *TaAGL2*. Furthermore, *TaAGL2* and *TaAGL9* show very high sequence similarity. As *OsMADS13* is orthologous to *Arabidopsis* *STK*, these wheat orthologs have been renamed as *WSTK* (wheat *SEEDSTICK*) (Yamada et al., 2009).

Bread wheat is an allohexaploid with the genomic constitution AABBDD in which each genome originated from a different ancestral species. The A genome is believed to be derived from *Triticum urartu*, the B genome from *Aegilops speltoides* or another species in the Sitopsis section, and the D genome from *Ae. tauschii* (Feldman, 2001). Allopolyploidization leads to the generation of duplicated homoeologous gene-copies (homoeologs) and, consequently, the hexaploid wheat genome contains triplicated homoeologs derived from the three ancestral diploid species. With regard to the class E *SEP*-like genes, two MADS-box genes, *WSEP* (wheat *SEPALLATA*) and *WLHS1* (wheat *LEAFY HULL STERILE 1*) have been identified in wheat (Shitsukawa et al., 2007). Analyses of gene structure, expression patterns and protein functions showed no evolutionary changes in the *WSEP* homoeologs. In contrast, the three *WLHS1* homoeologs

show genetic and epigenetic alterations. The A genome homoeolog (*WLHS1-A*) has a large deletion in the coding region, and does not have a function in floral development. *WLHS1-B* and *WLHS1-D*, located in the B and D genomes, respectively, have the complete MADS-box gene structure; however, *WLHS1-B* is effectively silenced by epigenetic modification. The diversification that has occurred among *WLHS1* homoeologs suggests that homoeologs of other homeotic MADS-box genes may also be differentially regulated in wheat spike formation. The floral homeotic MADS domain proteins have been proposed to interact in the floral tissue in the “floral quartet” model, in which a tetramer of MADS domain proteins functions in specification of floral organ identity (Smaczniak et al., 2012). Complex interactions among the homoeologous copies are probably associated with morphological and physiological diversification of floral development among species with different ploidy levels. In the present study, we examined homoeolog-specific expression patterns of the class B, C and D genes, *WPI2*, *WAG1* and *WSTK*, respectively, in tetraploid and hexaploid wheat. Our results suggest that homoeolog-specific expression patterns of floral MADS-box genes are determined and controlled by an as yet uncharacterized regulatory system that developed during allopolyploidization.

MATERIALS AND METHODS

Plant materials One wild tetraploid species (*T. turgidum* ssp. *dicoccoides*), five cultivated tetraploid species (*T. turgidum* ssp. *durum* and four ssp. *dicoccon*), four hexaploid bread wheat cultivars (*T. aestivum* ssp. *aestivum*), and four hexaploid species (*T. aestivum* ssp. *sphaerococcum*, ssp. *compactum*, ssp. *spelta* and ssp. *macha*) were used in this study (Table 1). These materials were obtained from NBRP-KOMUGI (National Bioresource Project-KOMUGI, Japan; <http://shigen.lab.nig.ac.jp/wheat/komugi>). Four synthetic hexaploid wheat lines were also used. Two were generated previously by crossing *T. turgidum* ssp. *dicoccoides* or ssp. *dicoccon* with *Ae. tauschii*; they were provided by NBRP-KOMUGI. Two synthetic hexaploid lines (F₄ generation) were generated recently from crosses between *T. turgidum* ssp. *durum* cv. Langdon and *Ae. tauschii* (Kajimura et al., 2011). These synthetic hexaploid lines have the normal chromosome set (2n = 42) and grow normally. Wheat plants were grown in the experimental field at Fukui Prefectural University.

Identification of homoeologs for class B, C, and D MADS-box genes The sequences of class B, C, D MADS-box genes were obtained by a database search of DDBJ (DNA Data Bank of Japan) and NBRP-KOMUGI websites. The chromosomal location of each gene was determined by PCR analysis with homoeolog-specific

Table 1. Plant materials

Tetraploid (Wild)				
No.	Species	NBRP No.	Country	Genome
1	<i>T. turgidum</i> ssp. <i>dicoccoides</i> var. <i>kotschyianum</i>	KU-108-2	Syria	AABB
Tetraploid (Cultivated)				
No.	Species	NBRP No.	Country	Genome
1	<i>T. turgidum</i> ssp. <i>durum</i> cv. Langdon	LPGKU2196	unknown	AABB
2	<i>T. turgidum</i> ssp. <i>dicoccon</i> var. <i>farrum</i>	KU-113	unknown	AABB
3	<i>T. turgidum</i> ssp. <i>dicoccon</i> var. <i>atratum</i>	KU-115	unknown	AABB
4	<i>T. turgidum</i> ssp. <i>dicoccon</i> var. <i>liguliforme</i>	KU-111	unknown	AABB
5	<i>T. turgidum</i> ssp. <i>dicoccon</i> var. <i>farrum</i>	KU-123	unknown	AABB
Hexaploid (<i>T. aestivum</i> ssp. <i>aestivum</i> : Bread wheat)				
No.	Variety	NBRP No.	Country	Genome
1	Chinese Spring	KU-184-1	China	AABBDD
2	Nanbukomugi	KT020-617	Japan	AABBDD
3	ND	KU-11809	Greece	AABBDD
4	ND	KU-1005	Spain	AABBDD
Hexaploid (Excepting Bread wheat)				
No.	Species	NBRP No.	Country	Genome
1	<i>T. aestivum</i> ssp. <i>sphaerococcum</i> var. <i>rotundatum</i>	Sphr ¹⁾	unknown	AABBDD
2	<i>T. aestivum</i> ssp. <i>compactum</i> cv. No. 44	Cmp ¹⁾	unknown	AABBDD
3	<i>T. aestivum</i> ssp. <i>spelta</i> var. <i>duhamelianum</i>	Splt ¹⁾	unknown	AABBDD
4	<i>T. aestivum</i> ssp. <i>macha</i> var. <i>subletschchumicum</i>	Mch ¹⁾	unknown	AABBDD
Synthetic hexaploid				
No.	Combination	NBRP No.	Genome	
1	<i>T. turgidum</i> ssp. <i>dicoccoides</i> var. <i>spontaneonigrum</i> KU-109 × <i>Ae. tauschii</i> ssp. <i>strangulata</i> KU-2074	KU-221-12	AABBDD	
2	<i>T. turgidum</i> ssp. <i>dicoccon</i> cv. Vernal × <i>Ae. tauschii</i>	KU-222	AABBDD	
3	<i>T. turgidum</i> ssp. <i>durum</i> cv. Langdon × <i>Ae. tauschii</i> cv. PI476874	6256 ²⁾	AABBDD	
4	<i>T. turgidum</i> ssp. <i>durum</i> cv. Langdon × <i>Ae. tauschii</i> cv. CGN10768	6277 ²⁾	AABBDD	

¹⁾ After Tsunewaki (1980)

²⁾ Kajimura et al. (2011)

ND: not determined

primer sets (described below) (Table 2). These analyses were performed using nulli-tetrasomic lines of bread wheat cultivar ‘Chinese Spring’ in which a pair of A, B, or D genome chromosomes was substituted by a pair of homoeologous chromosomes. The nulli-tetrasomic lines were obtained from NBRP-KOMUGI. We describe homoeologs located on chromosomes A, B and D as ‘gene name-A, gene name-B and gene name-D’, respectively. The accession numbers of homoeologs for class B, C, D MADS-box genes (*WPI2*, *WAG1* and *WSTK*) are shown in Table 3. Based on single nucleotide polymorphisms and/

or nucleotide indels, we designed homoeolog-specific primers for *WPI2*, *WAG1* and *WSTK* (Supplementary Figs. S1, S2 and S3). The sequences of the primer sets used in this study are listed in Table 2. Using the homoeolog-specific PCR primer sets, homoeolog-specific real-time PCR analyses were carried out to investigate differences in expression levels of the MADS-box homoeologs.

Real-time PCR analysis Expression analyses were performed using RNAs extracted from young spikes at the

Table 2. Sequences of primer sets used for real-time PCR analyses

Gene	Primer name	Sequences (5'-3')	Product size (bp)	Annealing temperature (°C)
Actin	actin361-L	TATGCCAGCGGTCTGAACAAC	361	65
	actin361-R	GGAACAGCACCTCAGGGCAC		
WPI2-A	WPI2-AspeL	CATTTTGCCAGTTGTATCCTTCA	240	65
	WPI2-AspeR	CTATTCACCGTCCGGCAAC		
WPI2-B	WPI2-BspeL	CCTTCGTTTGTATCTGTTTGGAC	225	70
	WPI2-BspeR	ACTATTCACCGTCCAGCAATTGT		
WPI2-D	WPI2-DspeL	CTAGGCCACCGACTCCACA	305	70
	WPI2-DspeR	TATTCACCGTCCGGCAATC		
WAG1-A	WAG1-AspeL	TCAACTTCATGCAGCAGCAC	213	70
	WAG1-AspeR	ATAGGATAAAGTCGATTGTGCGC		
WAG1-B	WAG1-BspeL	TCGGGCTCAGGATCGC	358	70
	WAG1-BspeR	TGGAGTTCTGCAAGGTGGTTATT		
WAG1-D	WAG1-DspeL	CGCATCGAGAACACGACC	366	70
	WAG1-DspeR	CAGCCTTCCCTCCAGCTGT		
WSTK-A	WSTK-AspeL	AGGGAGATAGAGCTTCAGAGTGACAA	230	68
	WSTK-AspeR	TGAAGCGAGGCCTGA		
WSTK-B	WSTK-BspeL	GCGGAGGAGGAGCAGCA	166	72
	WSTK-BspeR	CCTGAGCCTGAGCCTGAGTCC		
WSTK-D	WSTK-DspeL	AAAGGGAGATAGAGCTTCAGAGTGACAG	228	72
	WSTK-DspeR	GCTGAAGCGAGGCCTGAGCC		

Table 3. Three homoeologs of *WPI2*, *WAG1* and *WSTK*

Gene	Homoeologs	Accession no.	Synonym/Clone	Reference
<i>WPI2</i>	<i>WPI2-A</i>	CJ683155/CJ576494	whpc11d19 ¹⁾	Mochida et al. 2006
	<i>WPI2-B</i>	AB107992	<i>WPI2</i>	Hama et al. 2004
		AM502881	<i>TaPI-2 (WM15)</i>	Paolacci et al. 2007
		CJ685552/CJ578977	whpc20k17 ¹⁾	Mochida et al. 2006
	<i>WPI2-D</i>	DQ512343	<i>TaAGL26</i>	Zhao et al. 2006
		CJ660742/CJ553194	whhg5o03 ¹⁾	Mochida et al. 2006
<i>WAG1</i>	<i>WAG1-A</i>	CJ686121/CJ579589	whpc4g11 ¹⁾	Mochida et al. 2006
	<i>WAG1-B</i>	AB084577	<i>WAG</i>	Meguro et al. 2003
		CJ682811/CJ576124	whok9l15 ¹⁾	Mochida et al. 2006
	<i>WAG1-D</i>	AM502863	<i>TaAG-1 (WM2)</i>	Paolacci et al. 2007
		CJ677819/CJ570945	whok10e04 ¹⁾	Mochida et al. 2006
	<i>WSTK</i>	<i>WSTK-A</i>	DQ512337	<i>TaAGL2</i>
AM502865			<i>TaAG-3B (WM3B)</i>	Paolacci et al. 2007
CJ676578/CJ569669			whms5h04 ¹⁾	Mochida et al. 2006
<i>WSTK-B</i>		BJ260748/BJ266438	whh26k17 ¹⁾	Mochida et al. 2006
<i>WSTK-D</i>		DQ512364	<i>TaAGL9</i>	Zhao et al. 2006
		DQ512349	<i>TaAGL31</i>	Zhao et al. 2006
		AM502864	<i>TaAG-3A (WM3A)</i>	Paolacci et al. 2007
		CJ629480/CJ520771	whcs19k15 ¹⁾	Mochida et al. 2006

¹⁾ EST clones. The sequence information is shown by 5'-sequence accession no./3'-sequence accession no. The connecting (contig) sequences are considered to be the full-length cDNA sequences and used in this study.

floral organ developmental stage (3–10 mm in length), and from stamens and pistils of heading stage plants grown in the field. Samples were collected from three to five individuals, and each assay was performed with three replications. Total RNAs were extracted using ISOGEN (Nippon-gene, Japan) and cDNAs were subsequently synthesized with oligo-dT primer in accordance with the protocol for the Ready-To-Go T-Primed First-Strand Kit (GE Healthcare Life Sciences). Real-time RT-PCR analyses were performed using a LightCycler 2.0 (Roche Diagnostics GmbH) with homoeolog-specific primer sets for *WPI2*, *WAG1* and *WSTK* (Table 2). The relative quantities of transcripts were normalized against SYBR Green fluorescence of the *Actin* gene. For comparison of expression levels among homoeologs, we examined the amplification efficiency of each primer set using 10-fold dilutions ($\times 1$, $\times 10$, $\times 100$, $\times 1,000$, $\times 10,000$) of the gene-specific plasmids and compared their relative amplification efficiencies (Supplementary Fig. S4). The gene expression level of each homoeolog was standardized based on the amplification efficiency of its primer set and these revised levels of expression were used for comparison of homoeologs. Differences in gene expression levels were analyzed statistically using *t*-tests. Possible nucleotide polymorphisms within the homoeolog-specific primer sites of the varieties were not examined in the present study.

RESULTS

Identification of homoeologs for class B, C, D MADS-box genes in hexaploid wheat The *PI*-type genes *WPI1* (*wheat PISTILLATA1*) and *WPI2* have been identified as class B genes in wheat (Hama et al., 2004). *WPI1* and *WPI2* are also called *TaPI-1* and *TaPI-2/TaAGL26*, respectively (Paolacci et al., 2007; Zhao, T. et al., 2006). Here, sequence and PCR analyses of genomic DNA from nulli-tetrasomics indicated that *TaPI-2* was identical with *WPI2*, and that *WPI2/TaPI-2* and *TaAGL26* were homoeologous and located on chromosomes 3B and 3D, respectively (Fig. 1A, Table 3). In this report, we call these genes *WPI2-B* and *WPI2-D*, respectively. A third homoeolog, *WPI2-A*, was detected in the DDBJ database as an EST clone (whpc11d19; Mochida et al., 2006). The cDNA coding sequences of the homoeologs have eight single nucleotide polymorphisms (SNPs) (Supplementary Fig. S1), and three amino acid alterations (one located in the I-region and two in the K-domain) were identified in the deduced polypeptide sequences of their proteins (Supplementary Fig. S5).

The wheat *AG* orthologs *WAG1* (*wheat AGAMOUS1*) and *WAG2* were identified as class C genes (Meguro et al., 2003; Hirabayashi and Murai, 2009). *WAG1* and *WAG2* are also known as *TaAG-1* and *TaAG-2/TaAGL39*, respectively (Zhao, T. et al., 2006; Paolacci et al., 2007). Sequence and PCR analyses indicated that *WAG1* and

Fig. 1. PCR assay using genomic DNA of nulli-tetrasomics as the template and homoeolog-specific primer sets to determine the chromosomal locations of class B, C and D MADS-box gene homoeologs. (A) The *WPI2-A*, *WPI2-B* and *WPI2-D* homoeologs are located on chromosomes 3A, 3B and 3D, respectively. (B) The *WAG1-A*, *WAG1-B* and *WAG1-D* homoeologs are located on chromosomes 1A, 1B and 1D, respectively. (C) The *WSTK-A*, *WSTK-B* and *WSTK-D* homoeologs are located on chromosomes 5A, 5B and 5D, respectively. Homoeolog-specific primer sets for each MADS-box gene are shown in Table 2. The accession numbers of the homoeologs are shown in Table 3.

TaAG-1 were homoeologs and were located on chromosome 1B and 1D, respectively (Fig. 1B, Table 3); we term these genes *WAG1-B* and *WAG1-D* in this report. In the DDBJ database, we identified *WAG1-A* (whpc4g11) (Mochida et al., 2006), which is located on chromosome 1A. The homoeologous genes had nine SNPs in their cDNA coding sequences (Supplementary Fig. S2), and three amino acid alterations in the C-region were found in the deduced polypeptide sequences (Supplementary Fig. S6). Some of the *AGAMOUS* homologs had an N-region upstream of the MADS-box region. Since it is not certain that *WAG1* has an N-region, we considered the ATG codon immediately preceding the MADS-domain to be the initiation codon.

The genes *TaAGL2*, *TaAGL9*, *TaAGL31*, *TaAG-3A* and *TaAG-3B* have been identified as candidate orthologs of the rice class D gene *OsMADS13* (Zhao, T. et al., 2006; Paolacci et al., 2007). Sequence analyses showed that *TaAG-3A* was identical to *TaAGL9*, and that *TaAG-3B* was identical to *TaAGL2*. Contrary to Zhao, T. et al. (2006), our sequence analysis indicated that *TaAGL31* was identical to *TaAGL9*. In the wheat EST database in DDBJ, we identified another *WSTK* homolog, clone whh26k17 (Mochida et al., 2006). Our PCR assay indi-

cated that *TaAGL2*, whh26k17 and *TaAGL9* corresponded to *WSTK-A*, *WSTK-B* and *WSTK-D*, respectively (Fig. 1C, Table 3). The homoeologs have 16 SNPs and six base insertion/deletions in the cDNA coding sequences (Supplementary Fig. S3), and ten amino acid alterations and two amino acid insertion/deletions in the deduced polypeptide sequences of their proteins (Supplementary Fig. S7). There was a single amino acid alteration in the K-box domains and nine in the C-regions of the homoeologs, suggesting functional divergence among the three *WSTK* homoeologs.

Expression pattern of class B gene *WPI2* in hexaploid and tetraploid wheat The levels of expression of *WPI2* homoeologs were compared in hexaploid wheat varieties (Fig. 2). The A genome and D genome homoeologs, *WPI2-A* and *WPI2-D*, were expressed in young spikes and stamens (Fig. 2, A and B). In contrast,

Fig. 2. Expression patterns of three *WPI2* homoeologs examined by real-time PCR in hexaploid wheat using homoeolog-specific primers for *WPI2-A*, *WPI2-B* and *WPI2-D*. *Actin* was used as the endogenous control. Total RNAs were isolated from (A) young spikes (3 to 10 mm in length) and (B) stamens of hexaploid species: (1) *T. aestivum* ssp. *aestivum* cv. Chinese Spring; (2) *T. aestivum* ssp. *aestivum* cv. Nanbukomugi; (3) *T. aestivum* ssp. *aestivum* (KU-11809); (4) *T. aestivum* ssp. *aestivum* (KU-1005); (5) *T. aestivum* ssp. *sphaerococcum*; (6) *T. aestivum* ssp. *compactum*; (7) *T. aestivum* ssp. *spelta*; (8) *T. aestivum* ssp. *macha*. Data represent means $\pm$ standard errors from three replicate experiments. “ns” indicates no significant difference; asterisk indicates a significant difference in a *t*-test (*, $P < 0.05$; **, $P < 0.01$).

the B genome homoeolog *WPI2-B* was significantly down-regulated in all varieties, suggesting that suppression of *WPI2-B* expression was a common feature of hexaploid wheat. The suppression of *WPI2-B* was also observed in the stamens of tetraploid wheat varieties (Fig. 3B). Interestingly, in young spikes, *WPI2-B* was not down-regulated, and showed higher expression than *WPI2-A* (Fig. 3A). These observations indicated that the up-regulation of *WPI2-A* and down-regulation of *WPI2-B* in young spikes occurred during the allohexaploidization process in progeny of crosses of tetraploid wheat and the diploid D genome donor *Ae. tauschii*.

Expression pattern of class C gene *WAG1* in hexaploid and tetraploid wheat The levels of expression of *WAG1* homoeologs were compared in hexaploid wheat varieties (Fig. 4). In young spikes from hexaploid wheat lines, *WAG1-A* was expressed but *WAG1-B* and *WAG1-D* were significantly down-regulated (Fig. 4A). In stamens and pistils, *WAG1-B* was up-regulated compared

Fig. 3. Expression patterns of three *WPI2* homoeologs examined by real-time PCR in tetraploid wheat using homoeolog-specific primers for *WPI2-A* and *WPI2-B*. *Actin* was used as the endogenous control. Total RNAs were isolated from (A) young spikes (3 to 10 mm in length) and (B) stamens of tetraploid species: (1) *T. turgidum* ssp. *dicoccoides*; (2) *T. turgidum* ssp. *durum*; (3) *T. turgidum* ssp. *dicoccon* (KU-113); (4) *T. turgidum* ssp. *dicoccon* (KU-115); (5) *T. turgidum* ssp. *dicoccon* (KU-111); (6) *T. turgidum* ssp. *dicoccon* (KU-123). Data represent means $\pm$ standard errors from three replicate experiments. “ns” indicates no significant difference. Asterisk indicates a significant difference in a *t*-test (*, $P < 0.05$; **, $P < 0.01$).

Fig. 4. Expression patterns of three *WAG1* homoeologs examined by real-time PCR in hexaploid wheat using homoeolog-specific primers for *WAG1-A*, *WAG1-B* and *WAG1-D*. *Actin* was used as the endogenous control. Total RNAs were isolated from (A) young spikes (3 to 10 mm in length), (B) stamens and (C) pistils of hexaploid species: (1) *T. aestivum* ssp. *aestivum* cv. Chinese Spring; (2) *T. aestivum* ssp. *aestivum* cv. Nanbukomugi; (3) *T. aestivum* ssp. *aestivum* (KU-11809); (4) *T. aestivum* ssp. *aestivum* (KU-1005); (5) *T. aestivum* ssp. *sphaerococcum*; (6) *T. aestivum* ssp. *compactum*; (7) *T. aestivum* ssp. *spelta*; (8) *T. aestivum* ssp. *macha*. Data represent means $\pm$ standard errors from three replicate experiments. "ns" indicates no significant difference. Asterisk indicates a significant difference in a *t*-test (*, $P < 0.05$; **, $P < 0.01$).

with *WAG1-D* except in stamens of the bread wheat cv. Nanbukomugi (Fig. 4, B and C). At present, it is not clear why the expression level of *WAG1-B* in stamens of Nanbukomugi was so low compared to other varieties. *WAG1-D* was silenced or down-regulated in young spikes and reproductive floral organs (stamens and pistils) in all hexaploid wheat varieties, suggesting that suppression of the D genome homoeolog of *WAG1* is a common feature of

Fig. 5. Expression patterns of three *WAG1* homoeologs examined by real-time PCR in tetraploid wheat using homoeolog-specific primers for *WAG1-A* and *WAG1-B*. *Actin* was used as the endogenous control. Total RNAs were isolated from (A) young spikes (3 to 10 mm in length) (B) stamens and (C) pistils of tetraploid species: (1) *T. turgidum* ssp. *dicoccoides*; (2) *T. turgidum* ssp. *durum*; (3) *T. turgidum* ssp. *dicoccon* (KU-113); (4) *T. turgidum* ssp. *dicoccon* (KU-115); (5) *T. turgidum* ssp. *dicoccon* (KU-111); (6) *T. turgidum* ssp. *dicoccon* (KU-123). Data represent means $\pm$ standard errors from three replicate experiments. "ns" indicates no significant difference. Asterisk indicates a significant difference in a *t*-test (*, $P < 0.05$; **, $P < 0.01$).

hexaploid wheat. Both *WAG1-A* and *WAG1-B* were expressed in young spikes, stamens and pistils of wild and cultivated tetraploid wheat lines (Fig. 5). This suggests that the down-regulation of *WAG1-B* in young spikes occurred during the allohexaploidization process between tetraploid wheat and *Ae. tauschii*.

Expression pattern of class D gene *WSTK* in hexaploid and tetraploid wheat The levels of expression of *WSTK* homoeologs were determined in hexaploid wheat varieties (Fig. 6). In young spikes and pistils from all hexaploid wheat lines, *WSTK-B* was highly expressed but *WSTK-D* was significantly down-regulated and *WSTK-A* was silenced. In tetraploid wheat varieties, silencing of *WSTK-A* was observed in young spikes and very low expression was found in pistils (Fig. 7), suggesting that the suppression of *WSTK-A* occurred during allo-tetraploidization following the crossing of A and B genome donor species.

Expression pattern in synthetic hexaploid wheat lines To investigate the cause of the homoeolog-specific expression patterns, we examined *WPI2*, *WAG1* and *WSTK* expression in four synthetic hexaploid wheat lines

(Table 1). The synthetic hexaploid wheat lines were generated by crossing the tetraploid wheat *T. turgidum* (AABB) and diploid wheat *Ae. tauschii* (DD). Two types of synthetic hexaploid lines were used: first, previously generated synthetic hexaploids between *T. turgidum* ssp. *dicoccoides* or ssp. *dicoccon* and *Ae. tauschii* strains (old synthetic hexaploids) obtained from NBRP-KOMUGI; and second, newly generated synthetic hexaploids between *T. turgidum* ssp. *durum* cv. Langdon and *Ae. tauschii* strains (new synthetic hexaploids) generated by ourselves (Kajimura et al., 2011).

For the class B MADS-box gene *WPI2*, we found that homoeolog-specific expression patterns were similar among the four synthetic hexaploid wheat lines: *WPI2-A* and *WPI2-D* were expressed, while *WPI2-B* was down-regulated in both young spikes and stamens except in the stamens of one of the old synthetic lines (#1) (Fig. 8). This expression pattern is similar to that of natural hexaploid wheat varieties (Fig. 2). These results indicated that the homoeolog-specific expression patterns of

Fig. 6. Expression patterns of three *WSTK* homoeologs examined by real-time PCR in hexaploid wheat using homoeolog-specific primers for *WSTK-A*, *WSTK-B* and *WSTK-D*. *Actin* was used as the endogenous control. Total RNAs were isolated from (A) young spikes (3 to 10 mm in length) and (B) pistils of hexaploid species: (1) *T. aestivum* ssp. *aestivum* cv. Chinese Spring; (2) *T. aestivum* ssp. *aestivum* cv. Nanbukomugi; (3) *T. aestivum* ssp. *aestivum* (KU-11809); (4) *T. aestivum* ssp. *aestivum* (KU-1005); (5) *T. aestivum* ssp. *sphaerococcum*; (6) *T. aestivum* ssp. *compactum*; (7) *T. aestivum* ssp. *spelta*; (8) *T. aestivum* ssp. *macha*. Data represent means $\pm$ standard errors from three replicate experiments. “ns” indicates no significant difference. Asterisk indicates a significant difference in a *t*-test (*, $P < 0.05$; **, $P < 0.01$).

Fig. 7. Expression patterns of three *WSTK* homoeologs examined by real-time PCR in tetraploid wheat using homoeolog-specific primers for *WSTK-A* and *WSTK-B*. *Actin* was used as the endogenous control. Total RNAs were isolated from (A) young spikes (3 to 10 mm in length) and (B) pistils of tetraploid species: (1) *T. turgidum* ssp. *dicoccoides*; (2) *T. turgidum* ssp. *dicoccon* (KU-113); (3) *T. turgidum* ssp. *dicoccon* (KU-115); (4) *T. turgidum* ssp. *dicoccon* (KU-111); (5) *T. turgidum* ssp. *dicoccon* (KU-123). Data represent means $\pm$ standard errors from three replicate experiments. “ns” indicates no significant difference. Asterisk indicates a significant difference in a *t*-test (*, $P < 0.05$; **, $P < 0.01$).

Fig. 8. Expression patterns of three *WPI2* homoeologs examined by real-time PCR in synthetic hexaploid wheat using homoeolog-specific primers for *WPI2-A*, *WPI2-B* and *WPI2-D*. *Actin* was used as the endogenous control. Total RNAs were isolated from (A) young spikes (3 to 10 mm in length) and (B) stamens of synthetic hexaploid species: (1) *T. turgidum* ssp. *dicoccoides* var. *spontaneonigrum* KU-109 $\times$ *Ae. tauschii* ssp. *strangulata* KU-2074; (2) *T. turgidum* ssp. *dicoccon* cv. Vernal $\times$ *Ae. tauschii*; (3) *T. turgidum* ssp. *durum* cv. Langdon $\times$ *Ae. tauschii* PI476874; (4) *T. turgidum* ssp. *durum* cv. Langdon $\times$ *Ae. tauschii* CGN10768. Data represent means $\pm$ standard errors from three replicate experiments. “ns” indicates no significant difference. Asterisk indicates a significant difference in a *t*-test (*, $P < 0.05$; **, $P < 0.01$).

WPI2 were a consistent and reproducible feature of hexaploid lines.

For the class C MADS-box gene *WAG1*, we found high expression of *WAG1-A*, very low expression of *WAG1-B* and silencing of *WAG1-D* in young spikes (Fig. 9A). *WAG1-A* and *WAG1-B* were expressed in stamens and pistils, but *WAG1-D* showed silencing or a very low expression level (Fig. 9, B and C). These homoeolog-specific expression patterns in different organs were similar to those of natural hexaploid wheat varieties (Fig. 4) and indicate that expression patterns of *WAG1* homoeologs were a consistent and reproducible feature of hexaploid lines.

For the class D MADS-box gene *WSTK1*, only *WSTK-B* was preferentially expressed in both young spikes and pistils (Fig. 10). This expression pattern was similar to that in natural hexaploid wheat lines (Fig. 6) and indicates that expression patterns of *WSTK* homoeologs were a consistent and reproducible feature of hexaploid lines.

Fig. 9. Expression patterns of three *WAG1* homoeologs examined by real-time PCR in synthetic hexaploid wheat using homoeolog-specific primers for *WAG1-A*, *WAG1-B* and *WAG1-D*. *Actin* was used as the endogenous control. Total RNAs were isolated from (A) young spikes (3 to 10 mm in length), (B) stamens and (C) pistils of synthetic hexaploid lines: (1) *T. turgidum* ssp. *dicoccoides* var. *spontaneonigrum* KU-109 $\times$ *Ae. tauschii* ssp. *strangulata* KU-2074; (2) *T. turgidum* ssp. *dicoccon* cv. Vernal $\times$ *Ae. tauschii*; (3) *T. turgidum* ssp. *durum* cv. Langdon $\times$ *Ae. tauschii* PI476874; (4) *T. turgidum* ssp. *durum* cv. Langdon $\times$ *Ae. tauschii* CGN10768. Data represent means $\pm$ standard errors from three replicate experiments. “ns” indicates no significant difference. Asterisk indicates a significant difference in a *t*-test (*, $P < 0.05$; **, $P < 0.01$).

DISCUSSION

Allopolyploidization, the doubling of chromosome number in species hybrids, generates polyploids with genome sets derived from related species. For example, hexaploid bread wheat has the AABBDD genome composed of genomic contributions from three different ancestral species. Allopolyploidization is an important process

Fig. 10. Expression patterns of three *WSTK* homoeologs examined by real-time PCR in synthetic hexaploid wheat using homoeolog-specific primers for *WSTK-A*, *WSTK-B* and *WSTK-D*. *Actin* was used as the endogenous control. Total RNAs were isolated from (A) young spikes (3 to 10 mm in length) and (B) pistils of synthetic hexaploid species: (1) *T. turgidum* ssp. *dicoccoides* var. *spontaneonigrum* KU-109 $\times$ *Ae. tauschii* ssp. *strangulata* KU-2074; (2) *T. turgidum* ssp. *dicoccon* cv. Vernal $\times$ *Ae. tauschii*; (3) *T. turgidum* ssp. *durum* cv. Langdon $\times$ *Ae. tauschii* PI476874; (4) *T. turgidum* ssp. *durum* cv. Langdon $\times$ *Ae. tauschii* CGN10768. Data represent means $\pm$ standard errors from three replicate experiments. "ns" indicates no significant difference. Asterisk indicates a significant difference in a *t*-test (*, $P < 0.05$; **, $P < 0.01$).

both for angiosperm evolution and for functional innovations, such as the development of inflorescence structure (Shitsukawa et al., 2009). The polyploid genome displays dynamic changes in gene expression patterns caused by so-called "genomic shock" (McClintock, 1984). Feldman and Levy (2005) suggested that this phenomenon might induce rapid genomic changes (revolutionary change), through either genetic or epigenetic alterations, over a comparatively short period during allopolyploidization. These changes could include: (1) non-random elimination of coding and non-coding DNA; (2) epigenetic changes of coding and non-coding DNA; and (3) activation of genes and retroelements induced by alteration of adjacent DNA sequences. Recently, studies on the molecular pathways affected by polyploidization have greatly progressed. In particular, the genetic diversification and epigenetic modifications of transcription factors have been shown to influence many steps in the intricate and

coordinately regulated biological pathways of allopolyploid species (Chen, 2007).

The expression profiles of homoeologous genes have previously been investigated in hexaploid wheat using the frequencies of ESTs (Mochida et al., 2003). This study indicated that expression patterns could be classified into two major groups: genes that are almost equally expressed from all three genomes; and genes that are expressed differentially in the three genomes, and which vary between tissues. Subsequently, the differential expression of homoeologs was measured using cDNA-SSCPs and microarray analysis (Pumphrey et al., 2009), while more recently, genome-wide gene expression changes during allopolyploidization were investigated using Affymetrix GeneChip Wheat Genome Arrays (Changue et al., 2010; Akhunova et al., 2010; Qi et al., 2012) and an Agilent 38K oligo-DNA microarray (Jung et al., 2014). These studies indicate that up to 20% of genes show non-additive expression with homoeolog-specific up- or down-regulation. Leach et al. (2014) performed RNA-seq analysis using next generation sequencing with nullisomic-tetrasomic lines of chromosome groups 1 and 5 in hexaploid wheat to identify homoeolog-specific expression patterns. They assessed 2354 genes from homoeologous chromosomes and showed that at least 45% are expressed from all three homoeologs, but that most of the genes have strikingly biased expression patterns among the homoeologs. The remaining 55% of genes are expressed from one or two homoeologs only. These observations indicate that differentiation of expression among homoeologs is common in the hexaploid wheat genome.

In order to understand the meaning of homoeolog-specific expression differences, we have started to study functional differentiation in genes involved in floral organ formation. Ultimately, our goal is to elucidate the role of homoeolog-specific expression of MADS-box genes in this developmental pathway. This will also involve determining the function of homoeolog-specific protein-protein interactions. As a first step in this investigation, we examined homoeolog-specific expression patterns of class B, C and D MADS-box genes in floral development in allopolyploid wheat.

Homoeolog-specific expression patterns varies among MADS-box genes Our quantitative real-time PCR analysis showed that in young spikes of hexaploid wheat, the class B *WPI2* genes of the A and D genomes (*WPI2-A* and *WPI2-D*) were preferentially expressed (Fig. 2A), the class C *WAG1* gene in the A genome (*WAG1-A*) was preferentially expressed (Fig. 4A), and the class D *WSTK* gene of the B genome (*WSTK-B*) was preferentially expressed (Fig. 6A). Our previous study revealed that class E MADS-box gene *WSEP* homoeologs did not show any change in expression patterns and protein functions

(Shitsukawa et al., 2007). However, only the D genome copy of *WLHS1*, another class E gene, functions in hexaploid wheat. These results indicate that the homoeologs of each MADS-box gene for floral organ development show a specific differential expression pattern.

The concept of 'genomic expression dominance' has been developed in allotetraploid cotton; in this species, homoeolog expression is biased in favor of the D genome over the A genome (Rapp et al., 2009; Flagel and Wendel, 2010). Genomic expression dominance has also been reported in synthetic hexaploid wheat which shows a biased contribution from the AB genome of the tetraploid parent (Akhunova et al., 2010). The AB genome homoeologs involved in protein biosynthesis and photosynthesis were up-regulated, suggesting that they may contribute to restoration of growth vigor in allopolyploid wheat. The present study is the first to demonstrate that there is no common genomic expression dominance mechanism among floral MADS-box genes in hexaploid wheat, but rather that the pattern of differential expression of homoeologs varies among the genes (Figs. 2, 4, and 6). Future studies will be need to elucidate the common features of the gene systems involved in developmental pathways such as flower development in allopolyploid plants.

Homoeolog-specific expression patterns are consistently, reproducibly and rapidly established during allopolyploidization The homoeolog-specific expression patterns of class B, C, D MADS-box genes were consistent in all hexaploid wheat lines studied, including synthetic hexaploid wheat lines (Figs. 2, 4, 6, 8, 9 and 10), suggesting that these patterns of floral MADS-box gene expression are not stochastically determined but are controlled by an as yet unidentified mechanism that develops during allopolyploidization. Bottley and Koebner (2008) examined the silencing of 15 EST homoeologs in 16 modern northern European winter wheat varieties by SSCP analysis. The homoeolog-specific silencing patterns differed among varieties, and only two varieties shared the same pattern. More recently, Qi et al. (2012) compared homoeolog-specific expression patterns among two newly synthesized hexaploid wheat lines based on data from Affymetrix GeneChip Wheat Genome Arrays. This analysis showed that only 27 of 3539 genes had the same homoeolog-specific expression pattern in two synthetic hexaploid lines. Taken together with the present study, a unique feature of floral MADS-box genes has been revealed, namely, that the homoeolog-specific expression patterns are determined in a consistent manner among different polyploid varieties.

In young spikes of tetraploid wheat, *WPI2-B* was more highly expressed than *WPI2-A* (Fig. 3A). However, the expression of *WPI2-B* was significantly down-regulated compared to that of *WPI2-A*, which was up-regulated in

hexaploid wheat (Fig. 2A). Newly synthesized hexaploid wheat lines showed the same pattern of down-regulation of *WPI2-B* as natural hexaploid wheat varieties (Fig. 8A). These results suggest that homoeolog-specific down-regulation in young spikes occurred early in allohexaploidization of tetraploid wheat and *Ae. tauschii* hybrids. We also found that *WAG1-B* was significantly down-regulated in young spikes of hexaploid wheat varieties (Fig. 4A), while *WAG1-B* was expressed in the young spikes of tetraploid varieties (Fig. 5A). Synthetic hexaploid lines showed down-regulation of *WAG1-B* similar to natural hexaploid wheat varieties (Fig. 9A). These results suggest that the down-regulation of *WAG1-B* in young spikes occurred early in allohexaploidization between tetraploid wheat and *Ae. tauschii*. In summary, as newly generated synthetic hexaploid wheat lines showed similar homoeolog-specific expression patterns for floral MADS-box genes as natural hexaploid wheat varieties, then this control mechanism must develop relatively rapidly and reproducibly at an early stage in the generation of allopolyploids.

One of the causes of non-additive expression associated with homoeolog-specific up- or down-regulation is the trans-regulation of regulatory factor(s) and target genes located on different genomes in allopolyploid species (Soltis et al., 2014). In diploid species, only cis-regulation occurs between regulatory factor(s) and target genes. Complicated trans-regulation together with cis-regulation could be involved in homoeolog expression in allopolyploids, leading to the homoeolog-specific non-additive expression patterns.

It is also known that epigenetic alterations accompany allopolyploidization and can, occasionally, lead to gene silencing. Such epigenetic alterations have been reported in polyploids of *Arabidopsis* (Lee and Chen, 2001; Wang et al., 2004), *Triticum* (Shitsukawa et al., 2007; Zhao, N. et al., 2011), and *Brassica* (Lukens et al., 2006). Therefore, it will be important to determine in future studies whether homoeolog-specific expression patterns in floral MADS-box genes are regulated by an epigenetic mechanism. A recent RNA-seq analysis in synthetic hexaploid wheat suggested that a mechanism involving small RNAs may mediate dynamic homoeolog regulation (Li et al., 2014). Identification of micro RNAs and small interfering RNAs that target floral MADS-box genes would be a further step in elucidating the molecular mechanism of homoeolog-specific expression.

Organ-specific patterning of homoeolog-specific expression We found that *WAG1-B* was significantly down-regulated in young spikes, but was expressed in stamens and pistils of hexaploid wheat varieties (Fig. 4). Furthermore, in tetraploid wheat varieties, *WPI2-A* was more weakly expressed than *WPI2-B* in young spikes, while *WPI2-A* was more highly expressed than *WPI2-B* in

stamens (Fig. 3). An SSCP analysis identified organ-specific partitioning of the expression levels of homoeologs of *Alcohol dehydrogenase A (AdhA)* in cotton flowers (Adams et al., 2003). In this case, both *AdhA* homoeologs were expressed in vegetative tissues, but one copy was predominantly expressed in petals and stamens while the other copy was more highly expressed in carpels. Another SSCP analysis found organ-specific silencing of homoeologs in hexaploid wheat (Bottley et al., 2006). Such functional differentiation is likely to have occurred at an early stage in allopolyploidization (Adams et al., 2004). The differences in homoeolog-specific expression levels in different tissues and organs suggest that the MADS-box gene homoeologs are functionally divergent in floral development after allopolyploidization.

CONCLUSION

The present study identified homoeolog-specific expression patterns in floral MADS-box genes in allopolyploid wheat and showed: 1) the homoeolog-specific expression pattern was different in each MADS-box gene; 2) the homoeolog-specific expression patterns were consistently, reproducibly and rapidly established during allopolyploidization; and 3) organ-specific expression changes occurred among homoeologs in some MADS-box genes. The floral homeotic MADS domain proteins interact in floral tissues as proposed in the “floral quartet” model; in this model, a tetramer of MADS-domain proteins functions in the specification of floral organ identity (Smaczniak et al., 2012). The amino acid sequences of WPI2, WAG1 and WSTK differ among homoeologs, suggesting that the complex protein-protein interactions of homoeologs of MADS-box genes are probably associated with morphological and physiological diversification in floral development in wheat plants with different ploidy levels.

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