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Heterochronic development of the floret meristem determines grain number per spikelet in diploid, tetraploid and hexaploid wheats

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• *Background and Aims* The inflorescence of grass species such as wheat, rice and maize consists of a unique reproductive structure called the spikelet, which is comprised of one, a few, or several florets (individual flowers). When reproductive growth is initiated, the inflorescence meristem differentiates a spikelet meristem as a lateral branch; the spikelet meristem then produces a floret meristem as a lateral branch. Interestingly, in wheat, the number of fertile florets per spikelet is associated with ploidy level: one or two florets in diploid, two or three in tetraploid, and more than three in hexaploid wheats. The objective of this study was to identify the mechanisms that regulate the architecture of the inflorescence in wheat and its relationship to ploidy level.
• *Methods* The floral anatomy of diploid (*Triticum monococcum*), tetraploid (*T. turgidum* ssp. *durum*) and hexaploid (*T. aestivum*) wheat species were investigated by light and scanning electron microscopy to describe floret development and to clarify the timing of the initiation of the floret primordia. *In situ* hybridization analysis using *Wknox1*, a wheat *knotted1* orthologue, was performed to determine the patterning of meristem formation in the inflorescence.
• *Key Results* The recessive natural mutation of tetraploid (*T. turgidum* ssp. *turgidum*) wheat, *branching head* (*bh*), which produces branched inflorescences, was used to demonstrate the utility of *Wknox1* as a molecular marker for meristematic tissue. Then an analysis of *Wknox1* expression was performed in diploid, tetraploid and hexaploid wheats and heterochronic development of the floret meristems was found among these wheat species.
• *Conclusions* It is shown that the difference in the number of floret primordia in diploid, tetraploid and hexaploid wheats is caused by the heterochronic initiation of floret meristem development from the spikelet meristem.

Key words: *Triticum*, wheat, inflorescence, spikelet, floret, meristem, heterochrony, heterochronic development, *knotted1*, polyploidy.

INTRODUCTION

Organogenesis in the plant shoot is controlled by a group of stem cells (called the meristem) at the shoot apex. In grass species such as rice (*Oryza sativa*), wheat (*Triticum aestivum*) and maize (*Zea mays*), when the growth stage switches from the vegetative phase to the reproductive phase, the shoot apical meristem stops differentiating leaves and gradually elongates and changes into an inflorescence meristem (IM). The architecture of the inflorescence is strongly influenced by the timing of meristem initiation and the hierarchical structures of different types of meristem. The spikelet is a reproductive unit unique to the grass inflorescence (Schmidt and Ambrose, 1998; Bommert *et al.*, 2005). The spikelet is comprised of florets and is encompassed by two small bract leaves (called glumes in wheat) (Murai *et al.*, 2002). During inflorescence development, the inflorescence meristem produces spikelet meristem (SM), and the SM produces the floret meristem (FM). Finally, the FM produces the floral organs.

Cereal crops are the major source of carbohydrates for humans, and the yield of grain from these crops is largely dependent on inflorescence architecture (Ashikari *et al.*, 2005). Studies on the genetic regulation of inflorescence formation have provided valuable information for crop breeding. Molecular genetic studies have identified a number of genes involved in controlling inflorescence branching in maize and rice (Bommert *et al.*, 2005). The inflorescence of maize and rice is more complicated than that of wheat because of the presence of additional axillary branch meristems: the tassel branch and spikelet pair meristem in maize, and the panicle branch meristem in rice (Schmidt and Ambrose, 1998; Shitsukawa *et al.*, 2006). In wheat, branching of the inflorescence is regulated by two types of meristem, SM and FM (Murai *et al.*, 2002; Shitsukawa *et al.*, 2006). A comparative analysis of the genetic networks required for axillary meristem formation in wheat, maize and rice should lead to a more complete understanding of inflorescence architecture in these grass species.

The genes that regulate inflorescence formation are largely conserved in rice and maize. Thus, for example, *BARREN STALK* (*BA1*) in maize is the orthologue of *LAX PANICLE* (*LAX*) in rice (Komatsu *et al.*, 2001, 2003; Gallavotti *et al.*, 2004). *BA1* and *LAX* encode a helix–loop–helix transcription factor that regulates axillary meristem initiation. The gene that functions to initiate axillary meristem and lateral organ

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development in the maize inflorescence was identified in the *barren inflorescence2* (*bif2*) mutant (McSteen and Hake, 2001; McSteen *et al.*, 2007). BIF2, and its rice orthologue, OsBIF2 (OsPID), is a PINOID serine/threonine kinase that is expressed in all axillary meristems and lateral primordia during axillary meristem initiation (McSteen *et al.*, 2007; Morita and Kyozuka, 2007). A recent study showed that the BIF2 protein is associated with auxin transport, and functions in axillary meristem initiation by interaction with *BA1* in the nucleus (Skirpan *et al.*, 2008).

SM identity is regulated by the action of the orthologous genes, *BRANCHED SILKLESS 1* (*BD1*) in maize and *FRIZZY PANICLE* (*FZP1*) in rice (Chuck *et al.*, 2002; Komatsu *et al.*, 2003). *BD1* and *FZP1* belong to the *APETALA2* (*AP2*) transcription factor family, and knockout mutants of these genes exhibit defects in the FM and branch structures at the floret position. Other orthologous *AP2*-like genes, I*NDETERMINATE SPIKELET 1* (*IDS1*) and *SISTER OF INDETERMINATE SPIKELET 1* (*SID1*) of maize and *SUPERNUMERARY BRACT* (*SNB*) of rice, play a major role in the determination of the SM (Chuck *et al.*, 1998, 2008; Lee *et al.*, 2007). In *ids1* and *sid1* mutants, the spikelet loses determinacy and multiple florets are formed by one spikelet. In rice, *SNB* regulates the transition from SM to FM, and its loss-of-function mutant produces extra bract-like structures at the base of the spikelets and shows abnormal development of the floral organs. *AP2*-like genes are also associated with inflorescence formation in wheat. The major domestication *Q* gene has been shown to govern some aspects of inflorescence architecture such as free-threshing character, square spike phenotype and rachis fragility (Faris *et al.*, 2003; Simons *et al.*, 2006). The *Q* gene encodes an *AP2* family transcription factor. Sequencing and Southern analysis of this locus indicated that the dominant *Q* allele most likely arose through a gain-of-function mutation of *q*.

Bread wheat (*Triticum aestivum*) is a hexaploid species with the genome constitution AABBDD that originated from three diploid ancestral species: the A genome came from *T. urartu*, the B genome from *Aegilops speltoides* or another species classified in the *Sitopsis* section, and the D genome from *Ae. tauschii* (Feldman, 2001). It is known that hexaploid wheats originated on multiple occasions from crosses of the domesticated tetraploid wheats (*T. turgidum* ssp. *dicoccum*), and the diploid D genome donor, *Aegilops tauschii*. During the polyploidization step, polyploid wheats acquired environmental adaptability and high yield potential. Polyploidy has a distinctive effect on inflorescence morphology through influencing floret number per spikelet. As the ploidy level increases, so does the floret number per spikelet: one or two florets per spikelet in diploid, two or three in tetraploid, and more then three in hexaploid wheats. It is estimated that 30–80 % of the angiosperms are polyploids (Leitch and Leitch, 2008). Generally, polyploids exhibit growth vigour and wide adaptation to environment compared with diploid species, indicating that polyploidy is a driving force in plant evolution and speciation (Otto and Whitton, 2000). Diploid, tetraploid and hexaploid wheats should be good material for addressing the question of how mechanisms contribute to the growth vigour and adaptive traits in polyploids.

In this study, the mechanisms that regulate the number of florets per spikelet by a comparative morphological analysis of diploid (*T. monococcum*), tetraploid (*T. turgidum* ssp. *durum*) and hexaploid (*Triticum aestivum*) wheats were investigated. It was found that the number of floret primordia differed between these species. Furthermore, a comparison of the meristematic tissue distribution showed that the difference in the number of floret primordia was due to the heterochronic development of FM initiation from the SM. Heterochrony is defined as a change in the timing of developmental events. In general, it leads to variation in size and shape, which are important traits for speciation.

MATERIALS AND METHODS

Plant materials

The diploid wheat (*Triticum monococcum*, genome constitution AA) strain KU104-2, tetraploid wheat (*T. turgidum* ssp. *durum*, AABB) 'Langdon' and hexaploid wheat (*T. aestivum*, AABBDD) 'Chinese Spring' were grown in an experimental field, and were used for light microscope, scanning electron microscope (SEM) and *in situ* hybridization analyses. A tetraploid mutant, which was designated as *branching head* (*bh*) (*T. turgidum* ssp. *turgidum* strain KT10-2), is a classic natural mutant of emmer wheat; genetic and mapping analyses indicated it is controlled by single gene locus located on the short arm of chromosome 2A (Klindworth *et al.*, 1990*a*, *b*, 1997). The *bh* mutant has a highly branched spike (inflorescence).

Light microscopy

Spikes at the heading stage and at 3 d after flowering from diploid, tetraploid and hexaploid wheats were sampled from the field, and all the florets of a spikelet were analysed by light microscopy. The spikes at the two different stages were sampled from the same spike of the same plant. The analysis was performed using an OLYMPUS SZX12 light microscope.

Scanning electron microscope (SEM)

A low-vacuum SEM (S-3000N, Hitachi Co., Ltd, Japan) was used to observe the morphological features of inflorescences. Young spikes were chilled at −15 °C on a cool stage and examined at under low vacuum conditions (30 Pa) and an accelerating voltage of 15 kV or 20 kV.

In situ *hybridization analysis*

In situ hybridization was performed as described previously (Shitsukawa *et al.*, 2006). Spikes at different developmental stages (from pre-double ridge to floral organ differentiation stages) were sampled from CS and *bh* mutant plants, and fixed with FAA solution (3·7 % paraformaldehyde, 5 % acetic acid, 50 % ethanol) at 4 °C overnight. The fixed tissues were dehydrated and embedded in Paraplast Plus (Oxford Labware). The tissues were cut into 20-μm sections and dried overnight. Hybridization was performed overnight at 55 °C. A DIG-labelled probe was made by *in vitro* transcription of a plasmid containing the full-length *Wknox1* (Takumi *et al.*, 2000) using a DIG RNA labeling kit (Roche Diagnostics). After hybridization, the sections were washed

twice with 0·5 × SSC at 52 °C. Immunological detection of the hybridized probe was performed as described by Hama *et al.* (2004). As controls for specificity, sections were hybridized with sense and antisense probes of the same region of the *Wknox1* gene.

RESULTS

Inflorescence structure of diploid, tetraploid and hexaploid wheats revealed by light microscopy

The early stages of inflorescence development and the inflorescence structures of hexaploid wheat were described in previous studies (Murai *et al.*, 2002; Shitsukawa *et al.*, 2006). The wheat inflorescence (spike, ear or head) develops at the tip of a stem and is composed of spikelets. The spikelets are arranged as two opposite rows on the main axis, the rachis. The number of spikelets per spike is determined by the timing of terminal spikelet initiation, which depends upon genotype and environmental conditions. In this respect, the rachis meristem in wheat is determinate. The spikelet is composed of florets joined at the axis (rachilla) alternately on opposite sides, and encompassed by two small bract leaves called glumes (Fig. 1A). There are multiple florets in each spikelet: hexaploid wheat usually has four to six fertile florets. In contrast to barley, rice and maize, the wheat rachilla meristem is classified as indeterminate. In each floret, the reproductive organs are enveloped by two leaf-like structures, a lemma and a palea. The lemma and palea have one and two midribs, respectively (Fig. 1B). The lemma and palea correspond to the bract, but their origin is still unclear. An individual wheat flower contains one pistil, three stamens and two lodicules (Fig. 1B, C). The lodicules are formed on the lemma side and are considered as an analogous organ to the petal of other angiosperms.

The anatomy of florets of diploid, tetraploid and hexaploid wheats were analysed by light microscopy to identify morphological differences in their development at different ploidy levels (Fig. 2). The number of florets per spikelet increased with ploidy level: two florets in diploid, four in tetraploid and five in hexaploid wheats. In the heading stage spikelets analysed here, one fertile floret was found in diploid (Fig. 2A), three in tetraploid (Fig. 2D–F) and four in hexaploid wheats (Fig. 2I–L). Hypoplasia, underdevelopment or incomplete development, was observed in the floret primordia at the tips of spikelets of diploid (Fig 2C), tetraploid (Fig. 2H) and hexaploid wheats (Fig. 2N). Thus, SM was similarly indeterminate at all ploidy levels.

In spikes at 3 d after flowering, fertile florets usually contain developing grains. Examples of spikes at this growth stage from diploid, tetraploid and hexaploid plants are shown in Fig. 3A, E and J, respectively. One developing grain was found in diploid (Fig. 3B), three in tetraploid (Fig. 3F–H) and four in hexaploid wheats (Fig. 3K–N). In contrast to fertile florets with developing grains, sterile florets regressed in size (Fig. 3C, D, I, O, P). These observations indicate that the number of fertile florets per spikelet is determined by hypoplasia and abortion of florets in the distal segment of the spikelet.

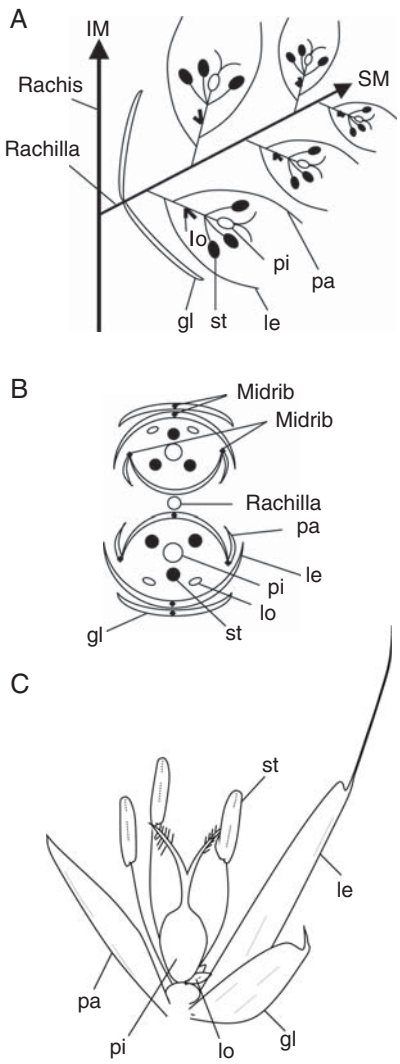

FIG. 1. Schematic illustrations of the wheat inflorescence. (A) A spikelet of hexaploid wheat (*Triticum aestivum*). The spikelets are arranged as two opposite rows of lateral branches from the main axis (rachis). Each spikelet is comprised of florets, joined at the axis (rachilla) alternately on opposite sides, encompassed by two glumes. (B) Cross-sectional diagram of a spikelet showing the first and second florets. The lemma and palea have one and two midribs, respectively. (C) Each floret is comprised of a lemma, a palea, two lodicules, three stamens and a pistil. Abbreviations: IM, inflorescence meristem; SM, spikelet meristem; gl, glume; le, lemma; pa, palea; lo, lodicule; st, stamen; pi, pistil.

Inflorescence development in diploid, tetraploid and hexaploid wheats revealed by SEM

Morphological differences in inflorescence development in diploid, tetraploid and hexaploid wheats were analysed further using SEM. The early steps of inflorescence development were similar at all ploidy levels (Fig. 4A, E, I). The inflorescence meristem forms the spikelet primordia in two opposite rows, and the glume is developed as the first organ of the spikelet (Fig. 4B, F, J). At the floret differentiation stage, the SM produces the FM at a lateral branch (Fig. 4C, G, K). There were clear differences among the diploid, tetraploid and hexaploid wheats at the floret differentiation to floral organ development stages. In diploid wheat, four floret primordia were initiated (Fig. 4D), whereas

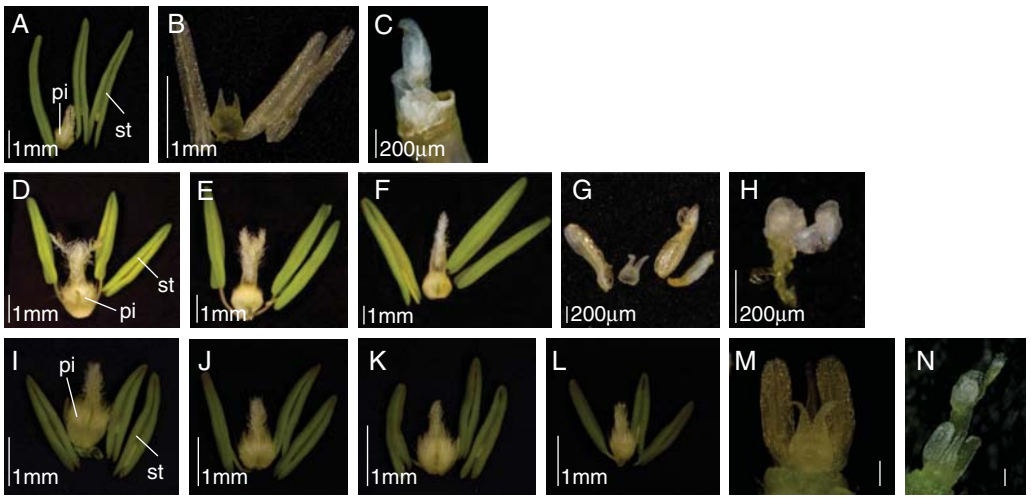

FIG. 2. Light microscope images of florets at the heading stage. (A–C) Florets of diploid wheat (*T. monococcum*): the first floret (A), the second floret (B), and the tip of the spikelet (C). (D–H) Florets of tetraploid wheat (*T. turgidum* ssp. *durum*): the first floret (D), the second floret (E), the third floret (F), the fourth floret (G), and the tip of the spikelet (H). (I–N) Florets of hexaploid wheat (*T. aestivum*): the first floret (I), the second floret (J), the third floret (K), the fourth floret (L), the fifth floret (M), and the tip of the spikelet (N). Hypoplasia occurs similarly at the tip of the spikelet in diploid, tetraploid and hexaploid wheats. Abbreviations: st, stamen; pi, pistil.

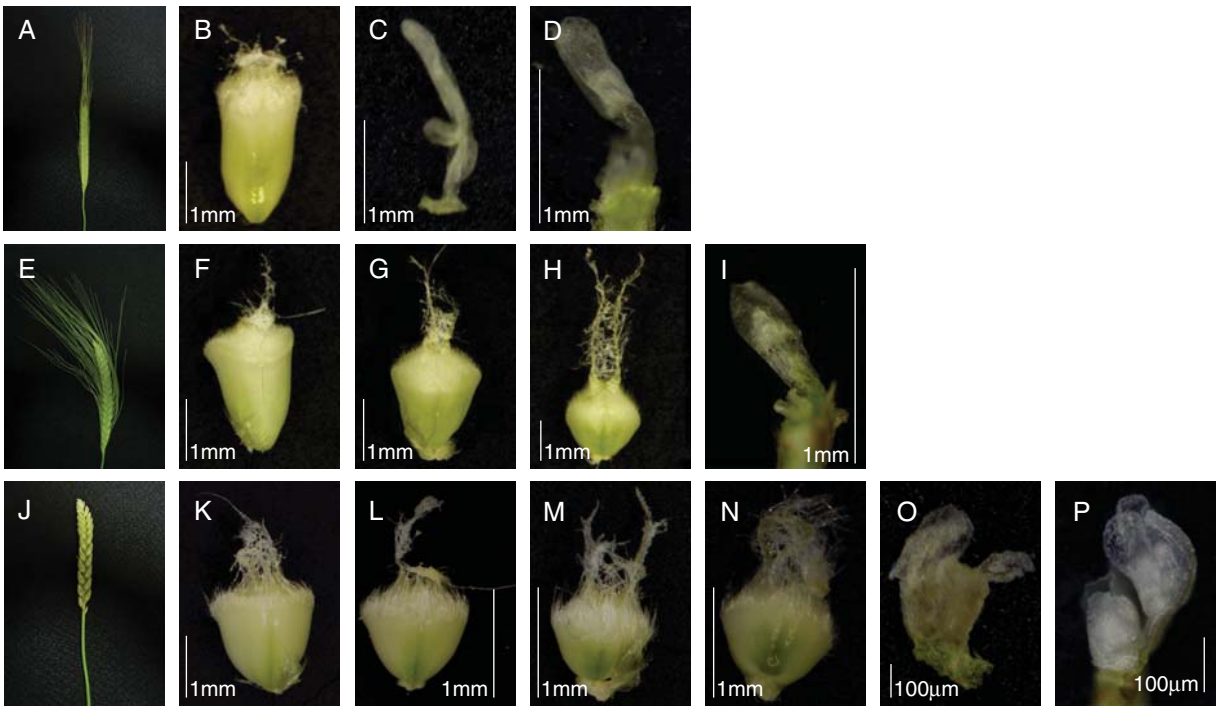

FIG. 3. Light microscope images of spikes and developing grains at 3 d after flowering. (A) A spike of diploid wheat (*T. monococcum*). (B–D) Developing grains of diploid wheat at the first floret (B), aborted in the second floret (C), and the tip of the spikelet (D). (E) A spike of tetraploid wheat (*T. turgidum* ssp. *durum*). (F–I) Developing grains of tetraploid wheat at the first floret (F), the second floret (G), the third floret (H) and the tip of the spikelet (I); (J) A spike of hexaploid wheat (*T. aestivum*). (K–P) Developing grains of hexaploid wheat at the first floret (K), the second floret (L), the third floret (M), the fourth floret (N), aborted in the fifth floret (O) and the tip of the spikelet (P). Floret abortion and hypoplasia occurred similarly at the tip of the spikelet in diploid, tetraploid and hexaploid wheats.

five and six floret primordia were initiated in tetraploid (Fig. 4H) and hexaploid wheats (Fig. 4L), respectively. These results are in agreement with those from the light microscope analysis and indicate that the number of floret primordia is correlated with ploidy level in wheat.

Characterization of bh *mutant confirms the utility of* Wknox1 *as a molecular marker for meristematic tissue*

The wheat *KNOX1* gene, *Wknox1*, belongs to the *knotted1 (kn1)*-type class I homeobox (*KNOX*) genes, such as maize

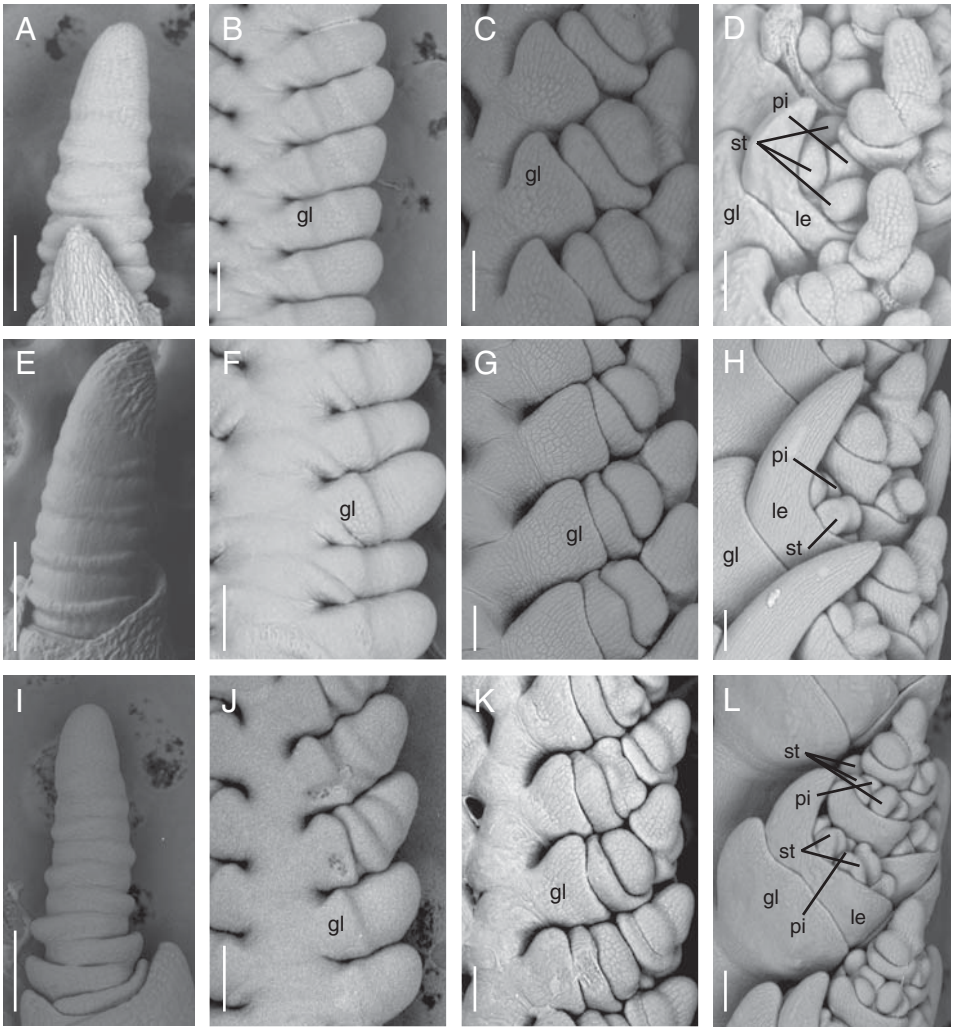

FIG. 4. Scannning electron microscope (SEM) images of diploid, tetraploid and hexaploid wheats. (A–D) SEM images of the developing inflorescence of diploid wheat (*T. monococcum*) at the pre-double ridge stage (A), spikelet differentiation stage (B), early (C) and late (D) floret differentiation stage. (E–H) SEM images of the developing inflorescence of tetraploid wheat (*T. turgidum* ssp. *durum*) at the pre-double ridge stage (E), spikelet differentiation stage (F), early (G) and late (H) floret differentiation stage. (I–L) SEM images of the developing inflorescence of hexaploid wheat (*T. aestivum*) at the pre-double ridge stage (I), spikelet differentiation stage (J), early (K) and late (L) floret differentiation stage. At the spikelet differentiation stage, the spikelets are differentiated to form two opposite rows at the rachis, and glume primordia are initiated at the base of each spikelet (B, F, J). Floret primordia are initiated in each spikelet at the early floret differentiation stage (C, G, K), and floral organs are differentiated in each floret at the late floret differentiation stage (D, H, L). Abbreviations: gl, glume; le, lemma; st, stamen; pi, pistil. Scale bars = 100 μm.

kn1 and rice *OSH1*, and is strongly expressed in the meristem (Takumi *et al.*, 2000; Morimoto *et al.*, 2005). *OSH1* has been reported to be a useful molecular marker for meristematic tissues (Kerstetter *et al.*, 1994; Sentoku *et al.*, 1999). To determine whether *Wknox1* could likewise prove to be a robust molecular marker for meristematic tissues in wheat, its expression pattern was examined in the spikes of mutant *branching head* (*bh*) wheat. In normal wheat, the inflorescence is composed of spikelets and florets, and does not produce any lateral branches from the inflorescence axis (Fig. 1A). In *bh* wheat, a well-known natural variant of tetraploid wheat (*T. turgidum* ssp. *turgidum)*, long lateral branches form in the basal segment of inflorescences; these ectopic lateral branches bear spikelets with fertile florets (Fig. 5A, B). Although the *BH* gene has not yet been cloned, *bh* remains an important mutant for understanding inflorescence architecture in wheat because the non-branched inflorescence axis is the one of the most remarkable characteristics of wheat compared with rice and maize.

The inflorescence of *bh* wheat appears normal at the early stages of development (Fig. 5C). The inflorescence meristem produces an SM on the axis alternately on opposite sides (Fig. 5D). Abnormal morphological development compared with normal spikelets was only observed when the SM elongated and formed an ectopic branch after the initiation of glume primordia. Meristem arose from ectopic branches of the axis alternately on opposite sides (Fig. 5D). The meristem was not FM but produced a spikelet-like organ (ectopic spikelet; Fig. 5E) that consisted of a floret-like organ (ectopic floret; Fig. 5F). The ectopic florets had a normal morphology with three fertile stamens and a pistil. The ectopic branch derived from the SM had a pair of glumes (Fig. 5B,

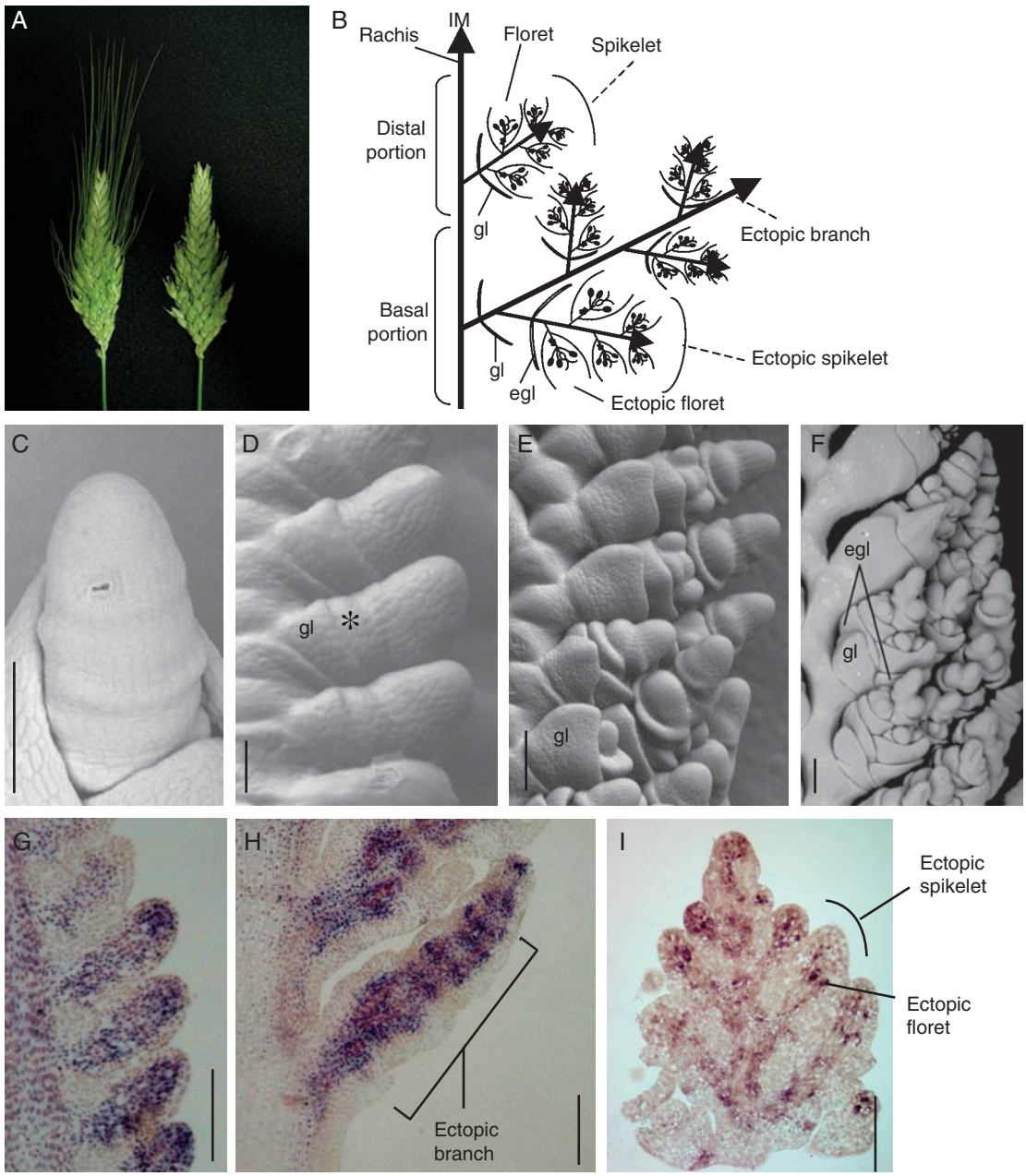

FIG. 5. (A) Spikes of a *branching head* (*bh*) mutant at the heading stage (right: awns were removed to allow the spike structure to be seen more easily). (B) Illustration of the *bh* inflorescence. Spikelets in the basal portion are transformed into ectopic branches with two glumes. Each ectopic branch differentiates ectopic spikelets in two opposite rows from the main axis. The ectopic spikelet is composed of ectopic florets, joined alternately on opposite sides to the axis, and encompassed by ectopic glumes. (C–F) SEM images of a *bh* inflorescence at the pre-double ridge stage (C), at the spikelet differentiation stage (D), at the stage when the ectopic floret is differentiated (E) and the stage when ectopic floral organs are differentiated (F) in ectopic spikelets of an ectopic branch. The asterisk indicates the ectopic meristem that produces the ectopic spikelet. Each ectopic branch continues to elongate and differentiate ectopic spikelets in a rectangular arrangement to produce ectopic florets. The ectopic floret is normal. (G–I) *in situ* hybridization patterns of *Wknox1* in sections of the basal portion of the *bh* inflorescence at the spikelet differentiation stage (G), and at the stage when ectopic florets are differentiated (H, longitudinal section; I, horizontal section). *Wknox1* expression was detected in the primordia of the ectopic branch, the ectopic spikelet, and the ectopic floret. Abbreviations: IM, inflorescence meristem; gl, glume; egl, ectopic glume. Scale bars = 100 μm.

D–F) suggesting that it was analogous to a spikelet. These observations indicate that the *bh* phenotype is caused by the transformation of the FM into an SM-like meristem.

To confirm that *Wknox1* is a functional orthologue of other *KNOX* homeobox genes, an *in situ* analysis was performed. *Wknox1* expression was detected in longitudinal sections of the ectopic branch at the primordia (Fig. 5G) and elongating stages (Fig. 5H) in the *bh* inflorescence. In horizontal sections of the ectopic branch, the *Wknox1* signal showed an expanded distribution across a large part of the ectopic spikelet and also into ectopic floret primordia (Fig. 5I). The pattern of *Wknox1* localization corresponded to meristem patterning, suggesting

that *Wknox1* has functional orthology to the *KNOX* homeobox genes and that its meristem-specific expression pattern is a valuable tool for studying the distribution of meristematic tissue in wheat inflorescences.

Expression patterns of the meristem marker gene, Wknox1*, in diploid, tetraploid and hexaploid wheat inflorescences*

To investigate the meristem patterning of the wheat inflorescence, an *in situ* hybridization analysis of *Wknox1* expression was performed in diploid, tetraploid and hexaploid wheats. At the spikelet differentiation stage, the *Wknox1* signal showed a similar localization pattern in the SM of diploid (Fig. 6A), tetraploid (Fig. 6C) and hexaploid wheats (Fig. 6E). As shown in the figure, it is notable that the SM appears to be similar in size at the different ploidy levels. The SM elongates gradually and produces FM as a lateral branch. At the floret differentiation stage, we observed three signals corresponding to the FM in diploid wheat (Fig. 6B), four in tetraploid wheat (Fig. 6D) and six in hexaploid wheat (Fig. 6F). Notably, although the *Wknox1* signal was similarly persistent at the tip of the SM in diploid, tetraploid and hexaploid wheats, the numbers of FM per spikelet were correlated with ploidy level. This observation is in agreement with the results of the morphological studies (Figs 2 and 3). The length of the floret differentiation stage did not differ in the diploid, tetraploid and hexaploid wheats, i.e. from the middle of April to the beginning of May in the experimental field. This suggests that the timing of FM initiation varied with ploidy level: the FM initiated slowly from the SM in diploid wheat but rapidly in hexaploid wheat. Based on the results of this study, it is concluded that the heterochronic development of the FM at different ploidy levels causes the difference in the number of florets in diploid, tetraploid and hexaploid wheats.

DISCUSSION

Hypoplasia of florets in the wheat spikelet

The inflorescence of grass species is composed of a unique unit called the spikelet. The spikelet is encompassed by two small bract leaves called glumes, and composed of florets joined at the rachilla. In barley, rice and maize, the number of florets per spikelet is determinate as one, one (originally three) and two, respectively (Schmidt and Ambrose, 1998). In contrast, wheat has an indeterminate SM, and there are multiple florets in each spikelet: usually four to six fertile florets in hexaploid wheat (Murai *et al.*, 2002). In this study, hypoplasia was observed in the florets in the distal portion of the spikelet (Figs 2 and 3). The florets shown in Figs 2 and 3 were sampled from the same spike; therefore, floret positions in the spikelet in Fig. 2 correspond to those in Fig. 3. The present results indicate that there are two types of floret hypoplasia. In the first type, floral organs such as stamens and pistil are differentiated but they are sterile and aborted (e.g. in diploid wheat; compare Figs 2B and 3C). In the second type, the FM initiates but no floral organs develop (e.g. in diploid wheat; compare Figs 2C and 3D). The number of florets with hypoplasia might be affected by genotype and growth conditions, such as use of fertilizer. A better understanding of the mechanism of floret hypoplasia would provide useful information for increasing the grain number per spikelet in crop species.

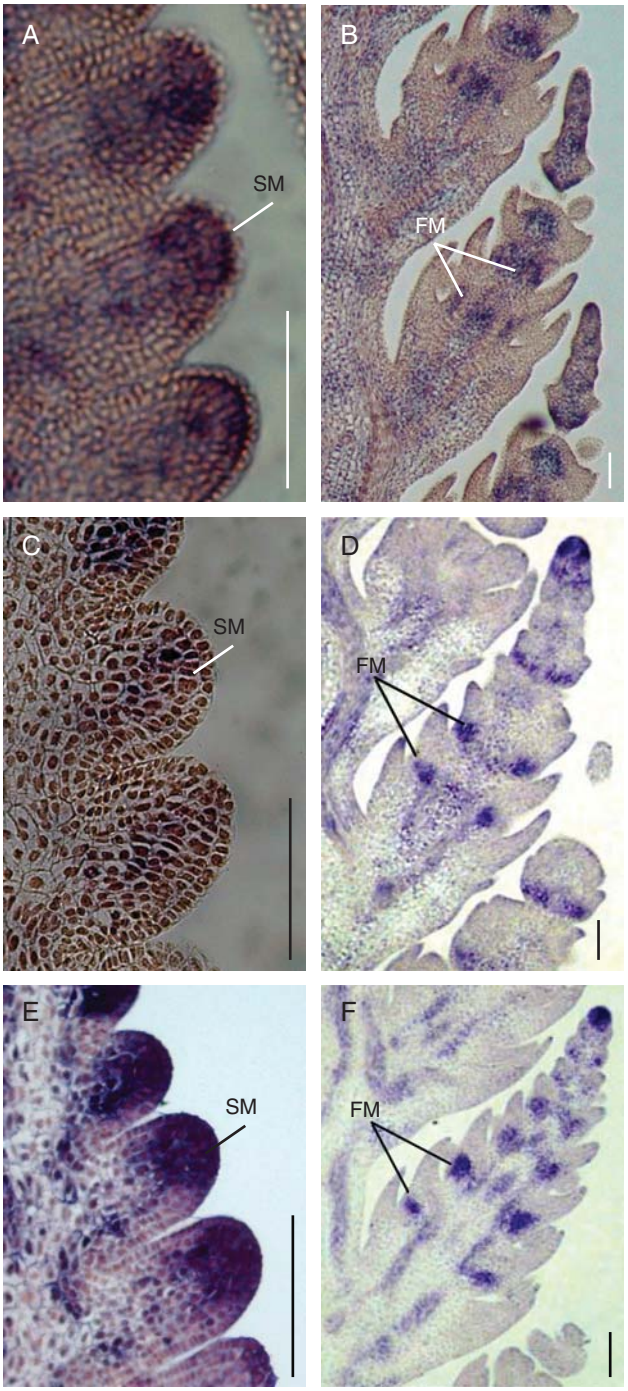

FIG. 6. *In situ* hybridization patterns of *Wknox1* in longitudinal sections of spikelets. (A, B) *Wknox1* expression in a young spike of diploid wheat (*T. monococcum*) at the spikelet differentiation stage (A) and the floret differentiation stage (B). (C, D) *Wknox1* expression in a young spike of the tetraploid wheat (*T. turgidum* ssp. *durum*) at the spikelet differentiation stage (C) and the floret differentiation stage (D). *Wknox1* expression in the young spike of hexaploid wheat (*T. aestivum*) at the spikelet differentiation stage (E) and the floret differentiation stage (F). Abbreviations: SM, spikelet meristem; FM, floret meristem. Scale bars = 100 μm.

In this study, it was found that the number of floret primordia increased with the ploidy level. Furthermore, the comparison of floret hypoplasia in diploid, tetraploid and hexaploid

wheats showed that hypoplasia occurred in florets in the distal portion of spikelets at all ploidy levels (Figs 2 and 3). Therefore, the present study indicates that the difference in number of fertile florets among diploid, tetraploid and hexaploid wheats is associated with the number of floret primordia initiated in spikelets. Why do the numbers of florets differ in diploid, tetraploid and hexaploid wheats? The SEM and *in situ* analyses indicated that heterochronic development of the FM at different ploidy levels might cause the difference in the number of florets (Figs 4 and 6). This conclusion then raises the question of what causes the heterochronic development of the FM in diploid, tetraploid and hexaploid wheats. This may be related to a heterosis effect in the polyploid species; however, the mechanism is unclear at present.

Indeterminacy of the SM in wheat

In this study, it was confirmed that the wheat SM is indeterminate (Fig. 6) and it was found that the number of fertile florets per spikelet was determined by the numbers of floret primordia and of florets with hypoplasia (Figs 2 and 3). In maize, the *IDS1* gene is a major candidate for mediating the number of florets per spikelet (Chuck *et al.*, 1998). In both male and female inflorescences of maize *ids1* mutants, the SM becomes indeterminate and produces additional florets, suggesting that *IDS1* is required either to suppress FM differentiation as lateral branches of SM or to promote the conversion of the SM to FM. *IDS1* encodes an *APETALA2* (*AP2*)-like transcription factor. Another *AP2*-like gene, *SID1*, also acts to promote determinacy and produce sex organs in maize (Chuck *et al.*, 2008). A different class of mutant, *tasselseed4* (*ts4*), produces highly branched meristems. *TS4* encodes a *mir172* microRNA that targets both *IDS1* and *SID1* in maize (Chuck *et al.*, 2007, 2008). It is possible that the loss-of-function mutations of these genes are involved in the indeterminacy of the SM. To date, wheat orthologues of *IDS1* and *SID1* have not been identified. In wheat, the *AP2*-like gene *Q* is a major regulator of spike formation, for example, of the square-headed phenotype and free-threshing character. Notably, hexaploid wheat mutants and natural variants that possess the *q* allele have a speltoid-type spike in which the number of florets per spikelet is reduced, especially in the spikelet in the distal portion of the spike. Diploid wheat (*T. monococcum*) carries the *q* allele and has a speltoid-type spike. In contrast, cultivated tetraploid (*T. turgidum* ssp. *durum*) and hexaploid (*T. aestivum*) wheats have the *Q* allele. This may be one of the reasons why the number of florets per spikelet in the diploid is lower than that in polyploid wheats. Furthermore, it is known that the *Q* gene shows dosage effects, suggesting that the effect of *Q* is stronger in hexaploid than tetraploid wheat. Although it is possible that *Q* influences the number of florets per spikelet, it is not clear whether *Q* might control determinacy of the SM or heterochronic development of the FM.

In rice, the *SUPERNUMERARY BRACT* (*SNB*) gene regulates the timing of meristem development and the pattern of formation of floral organs (Lee *et al.*, 2007). The *SNB* gene encodes a protein that belongs to the *IDS1* sub-group of *AP2*-like genes. *SNB* knockout plants have extra bract-like structures in the basal portion of the spikelet and show pleiotropic effects on floral organ formation. In addition, *snb* plants produce extra florets in the tip of the rachilla due to the heterochronic development of the meristem. The phenotype of *snb* rice plants suggests the possibility that heterochronic development of the FM in the wheat spikelet is associated with an *SNB*-like gene.

Polyploidization and heterochronic development of the wheat floret

The *in situ* analysis showed that *Wknox1* transcripts were present in the tips of the SMs in diploid, tetraploid and hexaploid wheats (Fig. 6), indicating that the indeterminacy of the SM is maintained in polyploid wheats, i.e. indeterminacy of the SM is not affected by polyploidization. Therefore, it is concluded that differences in the numbers of grains per spikelet in diploid, tetraploid and hexaploid wheats are due to the heterochronic development of FM initiation from the SM. What is the relationship between polyploidization and heterochrony of FM development? Which genes might regulate heterochrony? Polyploidization leads to the generation of duplicated homoeologous genes (homoeologues), as opposed to paralogous genes (paralogues). There are three possible evolutionary fates for homoeologous genes in polyploids: functional diversification, gene silencing, and retention of original or similar function (Wendel, 2000). Functional diversification of homoeologues is one of the important factors in the evolutionary success of polyploid species. Furthermore, the evolutionary success of allopolyploids is due to the retention of the function of all homoeologues at many loci. This facilitates positive inter-genomic interactions that are maintained in a self-pollinating plant such as wheat to produce permanent heterosis. The tetraploid and hexaploid wheat genomes contain duplicated and triplicated homoeologues derived from their respective ancestral diploid species. Dosage effects and/or functional divergence of the genes for heterochrony, e.g. an *SNB*-like gene (Lee *et al.*, 2007), might regulate FM development in polyploids. This may be one factor causing heterosis in polyploids.

Polyploidy: a driving force in plant evolution and speciation

Polyploidy often occurs throughout the evolution of flowering plants, including many important crops (Leitch and Bennett, 1997). Studies of natural and synthetic polyploids revealed that dynamic and stochastic changes occurred in genomic structure and gene expression in polyploids, which maybe provided growth vigour and adaptive traits to polyploids (Chen and Ni, 2006; Chen, 2007). In this study, it was shown that heterochronic development of the FM is associated with the difference in floret number per spikelet among diploid, tetraploid and hexaploid wheats. Of course, it has to be noted that there are wide variations in floret number per spikelet within diploid, tetraploid or hexaploid species. However, heterochronic development of the FM should be one of the important mechanisms to determine the difference in floret number among diploid, tetraploid and hexaploid wheats. In developmental biology, heterochrony is defined as a change in the timing of developmental events, leading to variation in size and shape, which are important

traits for speciation. The identification of the genes for the heterochronic development of the FM and a functional analysis of the homoeologous genes is a future goal for research. Increase in the number of florets is one of the important characters that have contributed to the success of polyploids in nature and in agriculture, since it leads directly to an increase in seed production. This topic is therefore of interest both to applied and basic sciences.

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