Variation in the wheat AP2 homoeologs, the genes underlying lodicule development

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The bread wheat genome harbors three homoeologs of the barley gene HvAP2, which determines the cleistogamous/non-cleistogamous flowering. The three homoeologs, TaAP2-A, TaAP2-B and TaAP2-D, are derived from the A, B and D genomes. The importance of lodicule swelling in assuring non-cleistogamous flowering in a range of wild and domesticated wheat accessions of varying ploidy level was established. Re-sequencing of wheat AP2 homoeologous genes was carried out to identify natural variation at both the nucleotide and polypeptide level. The sequences of wheat AP2 homoeologs are highly conserved even across different ploidy levels and no functional variants at the key miR172 targeting site were detected. These results indicate that engineering of cleistogamous wheat will require the presence of a functional TaAP2 modification at each of the three homoeologs.

Key Words: Triticum aestivum L., cleistogamy, lodicule, microRNA172.

Introduction

Wheat is grown over a larger area than any other crop and, along with maize and rice, provides a large proportion of the human calorific intake. The two major forms of wheat in cultivation are durum, an AABB tetraploid (Triticum durum) and bread, an AABBD hexaploid (T. aestivum). The diploid progenitor of the A genome is known to be T. urartu (Chapman et al. 1976) and that of the D genome Aegilops tauschi (Kihara 1944, McFadden and Sears 1946). The progenitor of the B genome has yet to be established, but is likely to have been an extant or extinct species belonging to the Sitopsis section of Aegilops (Kilian et al. 2007, Petersen et al. 2006, Riley et al. 1958).

In barley, the major form of cleistogamy is determined by homozygosity for a recessive allele at the cleistogamy1 (cly1) locus on the telomeric region of chromosome 2HL (Kurauchi et al. 1994, Turuspekov et al. 2004). The Cly1 gene encodes an ortholog of Arabidopsis thaliana AP2 and thus was renamed HvAP2 by Nair et al. (2010). The sequence of the recessive HvAP2 allele differs from that of the wild type allele by a single nucleotide change at its miR172 targeting site, with the result that its transcript escapes cleavage. In the presence of sufficient intact HvAP2 transcript, the lodicules fail to develop properly, preventing normal opening of the floret.

Three bread wheat orthologs of HvAP2 have been identified, residing on the chromosomes homeologous to its site in barley (chromosome 2H) (Ning et al. 2013). The three genes, TaAP2-A, TaAP2-B and TaAP2-D, are all abundantly expressed in the wheat flower and particularly in the lodicule and they are all cleaved by miR172. As the role of the lodicule in exposing the stigma and the style at anthesis is the same in wheat as it is in barley, these genes are rational targets for engineering cleistogamy in wheat (Ning et al. 2013). These data proved the high likelihood that wheat three homoeologs are wheat AP2 genes, specifying the lodicule.

Although bread wheat three homoeologous orthologs (TaAP2) of the barley cleistogamy gene cly1 (HvAP2) had been described in a previous work (Ning et al. 2013), it is still a long way before successful application of wheat AP2 homoeologs in wheat breeding. The point mutations at the miR172 targeting site of cly1 produce the cleistogamous barley. A rational strategy for inducing cleistogamy in wheat would be to identify naturally occurring or induced mutants at the miR172-targeting site for wheat AP2 genes. Here, we report a survey of the natural variation in potential wheat germplasm for wheat AP2 homoeologous sequence across a diverse panel of wild and domesticated wheats, including the (likely) diploid progenitors of the bread wheat A, B and D genomes.

Materials and Methods

Assessment of flowering phenotype

The germplasm panel consisted of 63 accessions, of which 24 were diploids, 23 tetraploids and 16 hexaploids (Table 1). The material was grown in the field at Tsukuba,
Table 1. Source of germplasm utilized

| Species | Type | Genome | Line | Origin or Source | Lodicules before anthesis | Lodicules at anthesis | Haplotypes | GenBank Accession No. |
|---------|------|--------|------|------------------|--------------------------|-----------------------|------------|----------------------|------------------|
|         |      |        |      |                  | Depth (mm) | Width (mm) | Depth (mm) | Width (mm) | Hap-A | Hap-B(S) | Hap-D | AP2-A | AP2-B-S | AP2-D |
| T. monococcum L. subsp. aegilopoides (Link) Thell. | Wild | A/A | KU-101-1 | Collection of College of Agr., Hokkaido Univ., Japan | 0.51 | 0.52 | 0.97 | 0.63 | ND | – | – | ND | – | – |
| T. monococcum L. subsp. aegilopoides (Link) Thell. | Wild | A/A | KU-101-2 | Balaklava, Crimea, USSR | 0.54 | 0.54 | 1.00 | 0.62 | ND | – | – | ND | – | – |
| T. monococcum L. subsp. aegilopoides (Link) Thell. | Wild | A/A | KU-103 | Collection of Agr. Exp. Station., Tehran, Iran | 0.49 | 0.49 | 0.83 | 0.61 | ND | – | – | ND | – | – |
| T. urartu Tumanian ex Gandilyan Wild AA | KU-104-2 | Japan | 0.43 | 0.59 | 0.77 | 0.64 | – | ND | – | – | – |
| T. urartu Tumanian ex Gandilyan Wild AA | PI428186 | Mardin, Turkey | 0.42 | 0.49 | 0.83 | 0.61 | – | B5 | – | – | AB774246 |
| T. urartu Tumanian ex Gandilyan Wild AA | KU-199-15 | Kerak, Lebanon | 0.46 | 0.52 | 0.77 | 0.64 | – | ND | – | – | ND |
| T. urartu Tumanian ex Gandilyan Wild AA | KU-189-15 | Kerak, Lebanon | 0.43 | 0.49 | 0.73 | 0.57 | – | B6 | – | – | AB774247 |
| T. urartu Tumanian ex Gandilyan Wild AA | PI428230 | Derbent, Caucasus, Dagestan, USSR | 0.41 | 0.71 | 1.01 | 0.91 | – | D3 | – | – | AB774243 |
| T. urartu Tumanian ex Gandilyan Wild AA | PI428253 | Urfa, Turkey | 0.42 | 0.59 | 0.78 | 0.66 | – | A11 | – | – | AB774271 |
| T. urartu Tumanian ex Gandilyan Wild AA | PI428254 | Mus, Turkey | 0.42 | 0.59 | 0.77 | 0.64 | – | ND | – | – | AB774266 |
| T. urartu Tumanian ex Gandilyan Wild AA | PI428257 | Armenia | 0.42 | 0.59 | 0.77 | 0.64 | – | ND | – | – | AB774246 |
| Ae. speltoides Tausch Wild SS | KU-104-2 | Lebanon | 0.43 | 0.59 | 0.77 | 0.64 | – | ND | – | – | – |
| Ae. speltoides Tausch Wild SS | PI487231 | Halah, Syria | 0.43 | 0.49 | 0.83 | 0.61 | – | ND | – | – | AB774246 |
| Ae. speltoides Tausch Wild SS | PI499261 | China | 0.42 | 0.49 | 0.73 | 0.57 | – | B6 | – | – | AB774247 |
| Ae. tauschii subsp. tauschii Wild DD | AS60 | Middle East | 0.35 | 0.59 | 0.55 | 0.76 | – | D2 | – | – | AB774238 |
| Ae. tauschii subsp. tauschii Wild DD | AS64 | Canada-2 | 0.32 | 0.58 | 0.55 | 0.75 | – | D2 | – | – | AB774239 |
| Ae. tauschii subsp. tauschii Wild DD | AS68 | USA | 0.37 | 0.64 | 0.63 | 0.81 | – | D2 | – | – | AB774240 |
| Ae. tauschii subsp. tauschii Wild DD | KU-199-1 | Lebanon | 0.27 | 0.65 | 0.69 | 0.84 | – | D3 | – | – | AB774244 |
| Ae. tauschii subsp. tauschii (morphological variety 'typica') Wild DD | KU-20-1 | Derbent, Caucasus, Dagestan, USSR | 0.49 | 0.71 | 1.01 | 0.91 | – | D3 | – | – | AB774243 |
| Ae. tauschii subsp. tauschii (morphological variety 'meyeri') Wild DD | KU-20-9 | 9 km NW of Ramsar (Chalus-Rasht), Iran | 0.27 | 0.65 | 0.69 | 0.84 | – | D3 | – | – | AB774244 |
| Ae. tauschii subsp. strangulata (Eig) Tzevelev Wild DD | KU-20-9 | 5 km W of Behshahr (Sari-Behshahr), Iran | 0.35 | 0.72 | 0.64 | 0.93 | – | D4 | – | – | AB774245 |
| Ae. tauschii subsp. strangulata (Eig) Tzevelev Wild DD | AS2386 | Iran | 0.36 | 0.64 | 0.59 | 0.91 | – | D3 | – | – | AB774241 |
| Ae. tauschii subsp. strangulata (Eig) Tzevelev Wild DD | AS2396 | Israel | 0.34 | 0.59 | 0.62 | 0.77 | – | D3 | – | – | AB774242 |
| T. turgidum L. subsp. dicoccoides (Korn. ex Asch. & Graebn.) Thell. | Wild | AABB | KU-108-2 | 20 km NW of Suweida (Cheikh Meskine-Suweida), Syria | 0.45 | 0.57 | 1.05 | 0.76 | A2 | B7 | – | AB774284 | AB774259 |
| T. turgidum L. subsp. dicoccoides (Korn. ex Asch. & Graebn.) Thell. | Wild | AABB | KU-8817 | North slope of Jabal Sinjar, N of Kursi, Iraq | 0.64 | 0.84 | 1.13 | 0.99 | A4 | ND | – | AB774283 | ND |
| T. turgidum L. subsp. dicoccoides (Korn. ex Asch. & Graebn.) Thell. | Wild | ABB | KU-198 | Collected in Mt. Canaan (Israel) by Dr. Aaronsohn (1906), Israel | 0.53 | 0.73 | 0.94 | 0.90 | A14 | B12 | – | AB774288 | AB774260 |
| T. turgidum L. subsp. dicoccoides (Schrank ex Schübl.) Thell. | Domesticated | AABB | KU-112 | Peiping, China | 0.62 | 0.81 | 1.33 | 1.00 | A3 | B4 | – | AB774274 | AB774257 |
| T. turgidum L. subsp. dicoccoides (Schrank ex Schübl.) Thell. | Domesticated | AABB | KU-113 | Collection of Agr. Exp. Sta. of Koonosu, Japan | 0.67 | 0.85 | 1.45 | 1.10 | A3 | B4 | – | AB774275 | AB774258 |
Table 1 (continued)

| Species Type | Genome Line | Origin or Source | Liodecules at anthesis | Haplogroup | Accession No. |
|--------------|-------------|------------------|------------------------|------------|---------------|
| T. turgidum L. subsp. dicoccum (Schrank ex Schübl.) Thell. | AABB KU-114 | Collection of Ag. Exp. Sta. of Koonosu, Japan | 0.76 0.98 1.07 1.00 A6 B1 | AB774289 AB774248 | – |
| T. turgidum L. subsp. durum (Desf.) Husn. | AABB KU-125 | Collection of College of Agr., Hokkaido Univ., Japan | 0.58 0.78 1.25 1.00 A5 B1 C1 D3 | AB774217 AB774225 | – |
| T. turgidum L. subsp. durum (Desf.) Husn. | AABB KU-135 | Collection of Agr., Wash., Palman, USA | 0.57 0.74 1.02 0.95 A5 B8 | AB774214 AB774226 | – |
| T. turgidum L. subsp. durum (Desf.) Husn. | AABB KU-138 | Collection of College of Agr., Hokkaido Univ., Japan | 0.44 0.70 1.18 1.08 A6 B12 | AB774212 AB774226 | – |
| T. turgidum L. subsp. durum (Desf.) Husn. | AABB KU-188 | Collection of College of Agr., Hokkaido Univ., Japan | 0.39 0.70 0.99 0.99 A3 B5 | AB774215 AB774226 | – |
| T. turgidum L. subsp. durum (Desf.) Husn. | AABB KU-147 | Collection of College of Agr., Hokkaido Univ., Japan | 0.49 0.75 1.24 0.99 A6 B2 | AB774214 AB774226 | – |
| T. turgidum L. subsp. durum (Desf.) Husn. | AABB KU-148 | Collection of College of Agr., Hokkaido Univ., Japan | 0.49 0.75 1.18 1.07 A6 B2 | AB774214 AB774226 | – |
| T. turgidum L. subsp. durum (Desf.) Husn. | AABB KU-149 | Collection of College of Agr., Hokkaido Univ., Japan | 0.49 0.75 1.18 1.07 A6 B2 | AB774214 AB774226 | – |
| T. turgidum L. subsp. durum (Desf.) Husn. | AABB KU-141 | Collection of College of Agr., Hokkaido Univ., Japan | 0.49 0.75 1.24 0.99 A6 B2 | AB774214 AB774226 | – |
| T. turgidum L. subsp. durum (Desf.) Husn. | AABB KU-142 | Collection of College of Agr., Hokkaido Univ., Japan | 0.49 0.75 1.24 0.99 A6 B2 | AB774214 AB774226 | – |
| T. turgidum L. subsp. durum (Desf.) Husn. | AABB KU-143 | Collection of College of Agr., Hokkaido Univ., Japan | 0.49 0.75 1.24 0.99 A6 B2 | AB774214 AB774226 | – |
| T. turgidum L. subsp. durum (Desf.) Husn. | AABB KU-144 | Collection of College of Agr., Hokkaido Univ., Japan | 0.49 0.75 1.24 0.99 A6 B2 | AB774214 AB774226 | – |
| T. turgidum L. subsp. durum (Desf.) Husn. | AABB KU-145 | Collection of College of Agr., Hokkaido Univ., Japan | 0.49 0.75 1.24 0.99 A6 B2 | AB774214 AB774226 | – |
| T. turgidum L. subsp. durum (Desf.) Husn. | AABB KU-146 | Collection of College of Agr., Hokkaido Univ., Japan | 0.49 0.75 1.24 0.99 A6 B2 | AB774214 AB774226 | – |
| T. turgidum L. subsp. durum (Desf.) Husn. | AABB KU-147 | Collection of College of Agr., Hokkaido Univ., Japan | 0.49 0.75 1.24 0.99 A6 B2 | AB774214 AB774226 | – |
| T. turgidum L. subsp. durum (Desf.) Husn. | AABB KU-148 | Collection of College of Agr., Hokkaido Univ., Japan | 0.49 0.75 1.24 0.99 A6 B2 | AB774214 AB774226 | – |
| T. turgidum L. subsp. durum (Desf.) Husn. | AABB KU-149 | Collection of College of Agr., Hokkaido Univ., Japan | 0.49 0.75 1.24 0.99 A6 B2 | AB774214 AB774226 | – |
| T. turgidum L. subsp. durum (Desf.) Husn. | AABB KU-150 | Collection of College of Agr., Hokkaido Univ., Japan | 0.49 0.75 1.24 0.99 A6 B2 | AB774214 AB774226 | – |
Table 1. (continued)

| Species                      | Type         | Genome   | Line        | Origin or Source                        | Lodicules before anthesis | Lodicules at anthesis | Haplotype | GenBank Accession No. |
|------------------------------|--------------|----------|-------------|-----------------------------------------|---------------------------|-----------------------|-----------|-----------------------|
|                              |              |          |             |                                         | Depth (mm) | Width (mm) | Depth (mm) | Width (mm) | Hap-A | Hap-B(S) | Hap-D | AP2-A | AP2-B(S) | AP2-D |
| *T. aestivum* L. subsp. aestivum | Domesticated | AABBDD   | Chinese Spring | China                                   | 0.52        | 0.86       | 1.38       | 1.17       | A1   | B1       | D1   | AB749311 | AB749312 | AB749313 |
| *T. aestivum* L. subsp. compactum (Host) Mackey | Domesticated | AABBDD   | KU-150       | Collection of College of Agr., Hokkaido Univ., Japan | 0.51        | 0.74       | 0.91       | 0.93       | A7   | B13      | ND   | AB761170 | AB761184 | AB761195 |
| *T. aestivum* L. subsp. compactum (Host) Mackey | Domesticated | AABBDD   | KU-153       | Collection of Univ. Wash., Pullman, USA | 0.46        | 0.75       | 0.96       | 0.94       | A7   | B14      | D3   | AB761171 | AB761185 | AB761195 |
| *T. aestivum* L. subsp. macka (Dekapr. & A. M. Menabde) Mackey | Domesticated | AABBDD   | KU-154       | Unknown                                  | 0.54        | 0.84       | 1.14       | 1.07       | A6   | B1       | D3   | AB761167 | AB761174 | AB761196 |
| *T. aestivum* L. subsp. macka (Dekapr. & A. M. Menabde) Mackey | Domesticated | AABBDD   | KU-193       | Unknown                                  | 0.58        | 0.86       | 0.96       | 0.93       | A6   | B1       | D3   | AB761168 | AB761178 | AB761197 |
| *T. aestivum* L. subsp. spelta (L.) Thell. | Domesticated | AABBDD   | KU-197       | Collection of Ankara Univ. (Agri), Turkey | 0.51        | 0.94       | 1.39       | 1.15       | A6   | B16      | D3   | AB761169 | AB761187 | AB761198 |
| *T. aestivum* L. subsp. spelta (L.) Thell. | Domesticated | AABBDD   | KU-157       | Collection of College of Agr., Hokkaido Univ., Japan | 0.55        | 0.93       | 1.04       | 1.12       | A4   | B1       | D3   | AB761166 | AB761182 | AB761199 |
| *T. aestivum* L. subsp. sphaerococcum (Percival) Mackey | Domesticated | AABBDD   | KU-161       | Unknown                                  | 0.43        | 0.85       | 0.92       | 1.01       | A3   | B2       | D3   | AB761161 | AB761183 | AB761200 |
| *T. aestivum* L. subsp. sphaerococcum (Percival) Mackey | Domesticated | AABBDD   | KU-162-2     | Collection of Islamia College, Pakistan | 0.39        | 0.79       | 0.98       | 0.95       | A3   | B1       | D3   | AB761162 | AB761175 | AB761201 |
| *T. aestivum* varidivia Jakubz. | Domesticated | AABBDD   | KU-192       | Unknown                                  | 0.27        | 0.76       | 0.95       | 1.19       | A7   | B15      | D5   | AB761173 | AB761186 | AB761202 |

Accessions prefixed with KU provided by the Japanese National BioResource Project (NBRP), those with either PI and CIt by USDA-ARS, those with AS by Sichuan Agricultural University Triticeae Research Institute. ND: not determined. DNA sequences of *T. aestivum* L. subsp. *aestivum* were previously published (Ning et al. 2013). Taxonomic classification follows the recommendation of http://www.ars-grin.gov/cgi-bin/npgs/html/index.pl.
Re-sequencing of the wheat AP2 homoeologs

Japan. Just prior to anthesis, three spikes still attached to the peduncle were detached from each accession and the lemma from the first floret of spikelet in the middle portion of the spike was removed to allow imaging of the lodicules. Lodicle height and depth were obtained from these images using Makijaku v1.1 software (http://cse.naro.affrc.go.jp/iwatah/). Maintaining the spikes in a 100 mg/l solution of 2,4-D for 24 h at room temperature facilitated the assessment of the maximum lodicule width and depth attained.

DNA amplification and sequencing

Genomic DNA was extracted from young, freshly harvested leaves according to Komatsuda et al. (1998). We used A, B and D genome ortholog-specific PCR primers designed in our previous work (Ning et al. 2013). The three TaAP2 homoeologous sequences were amplified from their start to their stop codon using primers detailed in Table 2. Each 50 µl PCR targeting TaAP2-A fragment 1 contained 0.2 × GC Buffer II; amplification reactions targeting TaAP2-A fragment 2, TaAP2-B and TaAP2-D were based on 1 × Ex Taq polymerase buffer. All reactions contained 0.25U Ex Taq polymerase (Takara, Tokyo, Japan), 0.6 µM of each primer, 0.2 mM dNTP, 2.0 or 2.5 mM MgCl₂, 8 or 12% v/v dimethyl sulphoxide and 40 ng genomic DNA. The PCR regime comprised a denaturation step (94°C/5 min), followed by 30 cycles of 94°C/1 min, 57 or 65°C (primer-dependent)/1 min, 72°C/2 min and a final extension step (72°C/10 min). The resulting amplicons were electrophoresed through 1% agarose (Iwai Kagaku, Tokyo, Japan) in 0.5 × TBE buffer and were visualized using EtBr staining to check that amplification had been achieved. The amplicons were purified using a QIAquick PCR purification kit (QIAGEN, Germantown, MD, USA) in preparation for their cycle sequencing using a Big Dye Terminator kit (Applied Biosystem, Foster,

![Fig. 1. Variation in lodicule size across wheat ploidy levels. A: T. urartu (AA) PI428230, B: Ae. speltoides (SS) PI487231, C: Ae. tauschii (DD) KU-20-1, D: T. durum (AABB) KU-125, E: bread wheat (AABBDD) cv. ‘Chinese Spring’. Lodicule width and depth indicated by arrows. Left panel: prior to anthesis, right panel: at anthesis. Bar: 1 mm.](image)

| Target | Primer name | Sequence of primer (5′-3′) | Primer name | Sequence of primer (5′-3′) | PCR mixture |
|--------|-------------|-----------------------------|-------------|-----------------------------|-------------|
|        |             |                             |             |                             | Buffer      |
|        |             |                             |             |                             | MgCl₂ (mM)  |
|        |             |                             |             |                             | DMSO (%)    |
|        |             |                             |             |                             | Tm (°C)     |
|        |             |                             |             |                             | size (kb)   |
| AP2-A | U1005A23    | GCAGACAGAGAGAGGCTAGAGG      | 2223L20     | CTGCAAGGCAATTACAGG          | 0.2 × GC Buffer II |
|        | F695        | TGGCGCAAGCAGCTATCTGT        | A3794L19    | CCCATGCCTCCCTCCGTGATC       | 2.5         |
|        | F-est2      | AGAGCAGGGCAGGAGGGGCTAGGAGG | R-est1543   | GCTGCTGCTCCCTCGAGGTGATG    | 8           |
|        | 35U24       | GCAAGCAGGAGAGGGCAGCTAGCCA  | R1690       | GCCTCGAACTCCCTCGGCCG       | 57          |
|        | F695        | TGGCGCAAGCAGCTATCTGT        | 3897L20     | TGGAGCTGTGTCTTGATGATGTC     | 1.2         |

Table 2. PCR primer sequences used for the amplification of wheat AP2 homoeologs
CA, USA). The sequencing reactions were purified by Agencourt CleanSEQ (Beckman, Beverly, MA, USA) and analyzed using an ABI PRISM 3130 genetic analyzer (Applied Biosystem).

Sequence alignment and phylogenetic analysis
Multiple sequence alignments at both the nucleotide and predicted polypeptide level were performed using DNAMAN v6.0 software (Lynnon Biosoft, Quebec, Canada). Phylogenetic trees were constructed based on the neighbor-joining method, using MEGA v5 software (Tamura et al. 2011). Bootstrap analysis was based on 1,000 replicates.

Results
Flower opening and lodicule swelling in wheats of varying ploidy level
Anthesis was reached over the period early May to mid June, although six of the 63 accessions did not reach flowering. All plants formed normal lodicules and prior to anthesis, all florets were enclosed tightly by the palea and lemma. At anthesis, anther extrusion was driven by the swelling of the lodicules (Fig. 1). The range in lodicule width and depth displayed by the accessions prior to and at anthesis is given in Table 1. Their width prior to anthesis ranged from 0.40–0.72 mm among the diploid accessions, 0.57–0.95 mm among the tetraploids and 0.74–0.94 mm among the hexaploids; while at anthesis, the respective ranges were 0.57–0.93 mm, 0.76–1.10 mm and 0.90–1.17 mm. Similarly lodicule depth varied from 0.27–0.54 mm, 0.38–0.76 mm and 0.27–0.61 mm prior to anthesis and 0.55–1.01 mm, 0.79–1.45 mm and 0.91–1.39 mm at anthesis (Fig. 2). Overall, lodicule depth at anthesis was about double that prior to anthesis. Accession to accession variation in lodicule size was considerable (the largest was more than twice the size of the smallest) but continuous. In barley, variation in lodicule size was discontinuous, representing cleistogamous and non-cleistogamous phenotypes (Nair et al. 2010). Therefore the results in wheat were unlike in barley.

TaAP2: nucleotide sequences
The full set of wheat AP2 homoeologous sequences has been deposited in GenBank as accession AB761159 to AB761202, AB774238 to AB774291 (Table 1). All three homoeologs comprised ten exons, with a 21nt miR172 targeting site sited in the tenth exon.

A sample of 43 AP2 homoeologous sequences of the A genome, obtained from seven T. urartu, 18 T. turgidum (AABB tetraploid), two T. timopheevi (AAGG tetraploid) and 16 T. aestivum accessions, revealed 16 haplotypes (Fig. 3). The most common haplotype (Hap-A3) was present in 14 accessions (nine T. turgidum and five T. aestivum accessions) and bread wheat cv. Shinchunaga (Ning et al. 2013), followed by Hap-A6 (six accessions: three T. turgidum and three T. aestivum) and Hap-A7 (four T. aestivum accessions). Both Hap-A1 and Hap-A2 were present in three accessions (the former all T. aestivum and the latter all T. urartu). Hap-A4 and Hap-5 each were represented by two accessions (the former comprising one T. turgidum and one T. aestivum accession and the latter two T. turgidum accessions). The remaining nine haplotypes were unique to a single accession: Hap-A8 to -A11 were specific to T. urartu, Hap-A12 to -A14 to T. turgidum and A15 to -A16 to T. timopheevi. Exon variation involved four single nucleotide substitutions and four indels; five of these eight polymorphisms induced an altered peptide sequence; the intron variation involved 20 single nucleotide substitutions and three indels (Fig. 3).

AP2 homoeologous sequences of B and S genome were recovered from two accessions of Ae. speltoides (SS), 17 of T. turgidum (eight subspecies) and 16 of T. aestivum. A total of 17 haplotypes was recognized (Fig. 4). The most common haplotype (Hap-B1) was present in four T. turgidum and nine T. aestivum accessions, and bread wheat cv. Shinchunaga (Ning et al. 2013), Hap-B2 in two T. turgidum and two T. aestivum accessions. The authors conclude that the results provide valuable insights into the evolutionary history of AP2 and suggest potential applications in breeding programs.
Re-sequencing of the wheat AP2 homoeologs

T. aestivum accessions, Hap-B3 in three T. turgidum accessions and Hap-B4 in two T. turgidum accessions. The remaining haplotypes were unique to a single accession: Hap-B5 and -B6 to Ae. speltoides, -B7 to -B12 to T. turgidum and -B13 to -17 to T. aestivum. The variation in intron sequence involved 65 single nucleotide substitutions and 25 indels and that in the exon sequence 34 single nucleotide substitutions and 25 indels. Of the latter, 24 induced an altered peptide sequence (Fig. 4).

The AP2 homologous sequences of D genome were obtained from eight Ae. tauschii and 15 T. aestivum accessions. The most common of the five haplotypes recognized was Hap-D3, which was present in four Ae. tauschii and 13 of the 15 T. aestivum accessions and bread wheat cv. Shinchunaga (Ning et al. 2013). Hap-D2 was represented in three Ae. tauschii accessions, while Hap-D1 and -D5 each had one T. aestivum accession and Hap-D4 one Ae. tauschii accession. There were 16 single nucleotide substitutions and five indels in the intronic sequence and nine single nucleotide substitutions and three indels in the exonic sequence; five of the latter induced an altered peptide sequence (Fig. 5).

With respect to the miR172 targeting sites of wheat AP2 homoeologs, the second nucleotide was uniformly a cytosine in B(S) genome and a thymine in A and D genomes (Fig. 8).

Wheat AP2 homoeologs: peptide sequences

At the polypeptide sequence level, the 16 haplotypes from AP2 homoeologous sequences of A genome collapsed into seven distinct polypeptides: the products translated from Hap-A1 and -A7 were indistinguishable from one another, as were those from -A2, -A8, -A9, -A10 and -A11, those from -A3, -A6 and -A13, those from -A15 and -A16 and those from -A5 and -A14. The gene products of Hap-A4 and -A12 were different from the other five, as well as from one another. For B(S) genome, the 17 haplotypes collapsed into ten distinct polypeptides: the products translated from Hap-B1, -B4 and -B17 were indistinguishable from one another, as were those from -B2 and -B13 and those from -B3, -B8, -B10, -B11 and -B15. The remaining haplotypes (Hap-B5, -B6, -B7, -B9, -B12, -B14 and -B16) each produced a unique polypeptide. For D genome, the five haplotypes collapsed into four distinct polypeptides: the products translated from Hap-D3 and -D5 were indistinguishable from one another, while the others each produced a unique polypeptide. A comparison of the three AP2 homoeologous protein sequences is shown in Fig. 6. Their structural organization was identical and they shared several conserved sequence features, namely the central core of the AP2 polypeptide (residues 118–276; boxed in Fig. 6), including AP2 domains R1 and R2 and the α-helix, the highly basic ten residue domain thought to be a nuclear localization signal (residues 108–117, boxed in Fig. 6), the AASSFGF box (residues 460–477, boxed in Fig. 6) and the three motifs (residues 1–5, 50–56 and 289–294, boxed in Fig. 6) (Jofuku et al. 1994, Tang et al. 2007). The only variant detected involved the homoeologous sequence of A genome present in T. timopheevi KU-1901 and KU-8735 and the homoeologous sequence of B genome in KU-197, both of which had a single residue substitution at, respectively, positions 271 and 274 (Fig. 6).

Wheat AP2 homoeologs phylogeny

The phylogeny of the wheat AP2 homoeologs based on full-length nucleotide sequences is shown as Fig. 7. Each of
Fig. 4. Haplotype variation in wheat AP2 homoeolog of B(S) genome in a sample of diploid, tetraploid and hexaploid wheats. Variation from the bread wheat cv. Chinese Spring (CS) is indicated. Hap-B1: KU-114, -125, -141, -147, -154, -162-2, -163, -165, -193, -265, -515, CS, cv. Norin 61 and cv. Shinchunaga (Ning et al. 2013); Hap-B2: KU-156, -157, -190-1; Hap-B3: KU-138, -146 and -185; Hap-B4: KU-112, -113; Hap-B5: PI487231; Hap-B6: PI542238; Hap-B7: KU-108-2; Hap-B8: KU-135; Hap-B9: KU-137; Hap-B10: KU-187; Hap-B11: KU-188; Hap-B12: KU-198; Hap-B13: KU-150; Hap-B14: KU-153; Hap-B15: KU-192; Hap-B16: KU-197; Hap-B17: cv. Fukuho. Genomic sequences between start and stop codon were aligned. Exonic polymorphisms indicated in bold and those generating a changed peptide by asterisks.
the three homoeologs formed a distinct clade. The homoeologs from A and D genome clades appeared to be more closely related to one another than to the homoeologs from B(S) genome clade. Within the homoeolog from A genome clade, two sub-groups were recognizable: one comprised the *T. urartu* sequences Hap-A2, -A8, -A9, -A10 and -A11 and the other the A genome sequences present in *T. timopheevi*, *T. turgidum* and *T. aestivum*. For homoeolog from B(S) genome, all but three of the haplotypes clustered into one large clade; Hap-B14 (present in *T. aestivum* ssp. *compactum*) was an outlier, as were the two haplotypes Hap-B5 and -B6, both present in *Ae. speltoides*. The phylogeny of the sequences of homoeologs from D genome was less easy to interpret, because only five haplotypes were representative. However, the sequences Hap-D3 (*Aegilops tauschii* ssp. *tauschii* morphological varieties ‘typica’: *KU*-20-1 and morphological varieties ‘meyeri’ *KU*-20-10, two lines of ssp. *strangulata* and most of wheats), Hap-D4 (ssp. *strangulata* KU-20-9) and -D5 (*T. aestivum* vavilovii *KU*-192) formed a clear subgroup, which was not closely related to either the -D1 (Chinese Spring) or the -D2 (*Aegilops tauschii* ssp. *tauschii*: AS60, AS64 and AS68) sequence.

**Discussion**

Lodicule swelling at anthesis is ubiquitous in wheat and its near relatives at all ploidy levels; the mechanical pressure which this process generates is sufficient to prise apart the lemma and the palea and allow the stamens to be extruded. The identical mechanism is used by non-cleistogamous barley cultivars (Nair et al. 2010). All three homoeologous versions of *TaAP2* were structured into ten exons, and their sequences were highly homologous with one another. The sequence variation between them was concentrated more in the intronic than in the exonic DNA (Figs. 3, 4, 5). The sequence of the miR172 targeting site was very highly conserved throughout, with only the variants detected among the 101 re-sequenced wheat *AP2* homologous genes being at the second nucleotide (Fig. 8). The miR172 targeting site sequences matched those present in the *TaAP2* homologous of the bread wheat cv. Shinchunaga, which are all successfully cleaved by miR172 (Ning et al. 2013), while the T/C polymorphism (which discriminated the *AP2*-B version from that of both *AP2*-A and -D) in barley is known to have no effect on cleistogamy (Nair et al. 2010). At the polypeptide level, all of the *AP2* homologous products shared the same structural organization and retained the key features of the protein, namely the *AP2* domain, the putative nuclear localizing signal, the AASSFGF box and the three motifs (Jofuku et al. 1994, Tang et al. 2007). The only departures from this conservation related to *AP2*-A homolog in *T. timopheevi* accessions *KU*-1901 and KU-8735 and *AP2*-B homolog in accession *KU*-197, but in both of these accessions, the lodicules became swollen at anthesis, meaning that their *TaAP2* polymorphism was non-functional in terms of cleistogamy – presumably because it had no effect on *TaAP2* transcript abundance. Thus, the indication is that all three *AP2* homologous genes are regulated by miR172 cleavage, in the same way as occurs in barley, and that as a result, non-cleistogamy in wheat is determined by the successful cleavage of *AP2* homologous mRNA (Nair et al. 2010, Ning et al. 2013).

Although the germplasm studied here was extremely diverse, covering a range of ploidy levels and ranging from cultivated varieties to wild relatives, the plants were uniformly non-cleistogamous, and their *AP2* homologous sequences were highly conserved. No functional mutations at the miR172 targeting site were identified. Because of failure of amplification, the *AP2* homologous sequences of individual materials were not determined in Table 1, possibly by the variations in primer binding sites. Non-cleistogamy is the norm in barley as well, but two naturally occurring cleistogamous types carrying distinct alleles at *HvAP2* have been identified (Nair et al. 2010). As both of these alleles are recessive to the wild type non-cleistogamous allele, the implication is that in polyploid wheat, functional wheat *AP2* homologous mutants are unlikely to display a cleistogamous phenotype, because their effect will be hidden by the presence of a wild type allele at the other homoeologous loci. Engineering a cleistogamous hexaploid wheat will require the presence of a functional wheat *AP2* homologous mutant at each of the three homoeologs. Given the absence of any such alleles among the 101 re-sequenced wheat *AP2* homologous genes, the likelihood of finding a naturally

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**Fig. 5.** Haplotype variation in wheat *AP2* homoeolog of D genome in a sample of diploid, tetraploid and hexaploid wheats. Variation from the bread wheat cv. Chinese Spring (CS) is indicated. Hap-D1: CS; Hap-D2: AS60, AS64 and AS68; Hap-D3: KU-20-1, -20-10, AS2386, AS2396 and all the hexaploid wheat accessions including cv. Shinchunaga (Ning et al. 2013) except KU-192; Hap-D4: KU-20-9; Hap-D5: KU-192. Exonic polymorphisms indicated in bold and those generating a changed peptide by asterisks.
Fig. 6. Alignment of polypeptide sequences of wheat AP2 homoeologs. Accessions within each of the haplotypes given in the legends to Figs. 3, 4, 5. The various key features of AP2 proteins are shown boxed, and the α-helix present in the core region of each AP2 domain shown delimited by arrows.
Re-sequencing of the wheat AP2 homoeologs

The D genome donor *Ae. tauschii* has been taxonomically divided on the basis of its morphology into four types, of which three (‘typica’, ‘meyeri’ and ‘anathera’) have been grouped together to form subsp. *tauschii*, while *strangulata* forms its own subspecies. It was indicated that the donor of the bread wheat D genome belonged to subsp. *strangulata* (Dvorak et al. 1998, Jaaska 1980, Xiang et al. 2009). The two *strangulata* accessions and the single accessions of *typica* and *meyeri* all shared the same AP2 homoeologous sequence, which was also present in all bar two of the *T. aestivum* accessions. The two subsp. *strangulata* lines and the ‘typica’ and ‘meyeri’ representatives proved to be phylogenetically close to *T. aestivum* but distinct from the other subsp. *tauschii* accessions, providing evidence to support the hypothesis that ‘typica’ and ‘meyeri’ are equidistant from subsp. *strangulata*, although both belong to the *strangulata* genepool (Dvorak et al. 1998).

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**Literature Cited**

Chapman, V., T.E. Miller and R. Riley (1976) Equivalence of the A genome of bread wheat and that of *Triticum urartu*. Genet. Res. 27: 69–76.

Dvorak, J., M.-C. Luo, Z.-L. Yang and H.-B. Zhang (1998) The structure of *Aegilops tauschii* genepool and the evolution of hexaploid wheat. Theor. Appl. Genet. 97: 657–670.

Henikoff, S., B.J. Till and L. Comai (2004) TILLING. Traditional mutagenesis meets functional genomics. Plant Physiol. 135: 630–636.

Jaaska, V. (1980) Electrophoretic survey of seedling esterases in wheats in relation to their phylogeny. Theor. Appl. Genet. 56: 273–284.

Jofuku, K.D., B.G. den Boer, M.V. Montagu and J.K. Okamuro (1994) Control of Arabidopsis flower and seed development by the homeotic gene APETALA2. Plant Cell 6: 1211–1225.

Kihara, H. (1944) Discovery of the DD-analyser, one of the ancestors of *Triticum vulgare*. Agric. Hortic. 19: 889–890.

Kilian, B., H. Özkan, O. Deusch, S. Effgen, A. Brandolini, J. Kohl, W. Martin and F. Salamini (2007) Independent wheat B and G genome origins in outcrossing *Aegilops* progenitor haplotypes. Mol. Biol. Evol. 24: 217–227.

Komatsuda, T., I. Nakamura, F. Takaiwa and S. Oka (1998) Development of STS markers closely linked to the *vrs1* locus in barley, *Hordeum vulgare*. Genome 41: 680–685.

Kurauchi, N., T. Makino and S. Hirose (1994) Inheritance of cleistogamy-chasmogamy in barley. Barley Genet. Newsl. 23: 19.

McFadden, E.S. and E.R. Sears (1946) The origin of *Triticum spelta* and its free-threshing hexaploid relatives. J. Hered. 37: 81–89.

Miller, J.C., S. Tan, G. Qiao, K.A. Barlow, J. Wang, D.F. Xia, X. Meng, D.E. Paschon, E. Leung, S.J. Hinkley *et al.* (2011) A TALE nuclease architecture for efficient genome editing. Nat. Biot. 29: 143–148.

Nair, S.K., N. Wang, Y. Turuspekov, M. Pourkheirandish, S. Sinsuwongwat, G. Chen, M. Sameri, A. Tagiri, J. Honda, Y. Watanabe *et al.* (2010) Cleistogamous flowering in barley arises from the suppression of microRNA-guided *HvAP2* mRNA cleavage. Proc. Natl. Acad. Sci. USA 107: 490–495.

Ning, S., N. Wang, S. Sakuma, M. Pourkheirandish, J. Wu, T. Matsumoto, T. Koba and T. Komatsuda (2013) Structure, transcription and post-transcriptional regulation of the bread wheat orthologs of the barley cleistogamy gene *Cly1*. Theor. Appl. Genet. 126: 1273–1283.

Petersen, G., O. Seberg, M. Yde and K. Berthelsen (2006) Phylogenetic relationships of *Triticum* and *Aegilops* and evidence for the origin of the A, B, and D genomes of common wheat (*Triticum aestivum*). Mol. Phylogenet. Evol. 39: 70–82.

Riley, R., J. Unrau and V. Chapman (1958) Evidence on the origin of the B genome of wheat. J. Hered. 49: 91–98.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28: 2731–2739.

Tang, M., G. Li and M. Chen (2007) The phylogeny and expression pattern of *APETALA2*-like genes in rice. J. Genet. Genomics 34: 930–938.

Turuspekov, Y., Y. Mano, J. Honda, N. Kawada, Y. Watanabe and T. Komatsuda (2004) Identification and mapping of cleistogamy genes in barley. Theor. Appl. Genet. 109: 480–487.

Urnov, F.D., E.J. Rebar, M.C. Holmes, H.S. Zhang and P.D. Gregory (2010) Genome editing with engineered zinc finger nucleases. Nat. Rev. Genet. 11: 636–646.

Xiang, Z.-G., L.-Q. Zhang, S.-Z. Ning, Y.-L. Zheng and D.-C. Liu (2009) Evaluation of *Aegilops tauschii* for heading date and gene location in a re-synthesized hexaploid wheat. Agricultural Sciences in China 8: 1–7.
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