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APETALA 2-like genes AP2L2 and Q specify lemma identity and axillary floral meristem development in wheat

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SUMMARY

The spikelet is the basic unit of the grass inflorescence. In tetraploid (Triticum turgidum) and hexaploid wheat (Triticum aestivum), the spikelet is a short indeterminate branch with two proximal sterile bracts (glumes) followed by a variable number of florets, each including a bract (lemma) with an axillary flower. Varying levels of miR172 and/or its target gene Q (AP2L5) result in gradual transitions of glumes to lemmas, and vice versa. Here, we show that AP2L5 and its related paralog AP2L2 play critical and redundant roles in the specification of axillary floral meristems and lemma identity. AP2L2, also targeted by miR172, displayed similar expression profiles to AP2L5 during spikelet development. Loss-of-function mutants in both homeologs of AP2L2 (henceforth ap2l2) developed normal spikelets, but ap2l2 ap2l5 double mutants generated spikelets with multiple empty bracts before transitioning to florets. The coordinated nature of these changes suggest an early role of these genes in floret development. Moreover, the flowers of ap2l2 ap2l5 mutants showed organ defects in paleas and lodicules, including the homeotic conversion of lodicules into carpels. Mutations in the miR172 target site of AP2L2 were associated with reduced plant height, more compact spikes, promotion of lemma-like characters in glumes and smaller lodicules. Taken together, our results show that the balance in the expression of miR172 and AP2-like genes is crucial for the correct development of spikelets and florets, and that this balance has been altered during the process of wheat and barley (Hordeum vulgare) domestication. The manipulation of this regulatory module provides an opportunity to modify spikelet architecture and improve grain yield.

Keywords: Triticum aestivum, Triticum turgidum, spikelet development, floral meristem, miRNA, AP2, floral organs, lodicules.

INTRODUCTION

Cereal inflorescence architecture is a major determinant of grain yield and, not surprisingly, it has been extensively modified by human selection during crop domestication. Modifications in cereal inflorescence development facilitated increases in grain number and size, and fine-tuned factors limiting shattering while improving threshability (Doebley, 2006; Debernardi et al., 2017). A better understanding of the molecular mechanisms that control inflorescence development may allow the engineering of new architectures with enhanced grain productivity.

Inflorescence development begins when the shoot apical meristem (SAM) transitions from the vegetative to the reproductive phase. In the ancestral grass inflorescence, the panicle, the inflorescence meristem (IM) generates lateral primary and secondary branches, each ending in a spikelet (‘little spike’). In tetraploid (Triticum turgidum) and hexaploid wheat (Triticum aestivum), the primary and secondary branches of the inflorescence are absent, resulting in spikelets attached directly to the rachis, forming a derived structure called a spike. The wheat IM generates a determined number of lateral spikelet meristems (SMs) in an alternating distichous pattern along the central rachis before forming a terminal spikelet (Kellogg et al., 2013).

The spikelet is the basic unit of the grass inflorescence and comprises a series of overlapping bracts arising distichously from a short axis called the rachilla (Clifford, 1987). In wheat, the two proximal bracts lack axillary meristems and are designated as glumes, whereas the next bracts, called lemmas, have axillary meristems that develop into
short reproductive shoots. In the floral axis, the floral meristem generates the palea (a membranous two-keeled structure), two scales called lodicules that can swell to spread the lemma and palea, three stamens and a terminal ovary. These lateral shoots with their subtending lemmas are designated as florets (Clifford, 1987). In some grass species the SM produces a determinate number of florets, e.g. in Hordeum vulgare (barley), Oryza sativa (rice), Sorghum sp. and Zea mays (maize), whereas wheat generates an indeterminate number of lateral florets with only the most basal florets surviving to support grains (Kellogg, 2001; Guo et al., 2017; Sakuma et al., 2019).

The grass inflorescence architecture is determined by the maintenance or termination and the identities acquired by the IM and lateral meristems, which in turn depend on the expression and interactions of developmental regulatory genes in the meristem or in adjacent signaling centers (Whipple, 2017; Bommert and Whipple, 2018). In wheat, it was recently shown that the MIKC-type MADS-box proteins of the APETALA 1 (AP1)-like family (VRN1, FUL2 and FUL3) play central roles controlling the activity and determinacy of the IM and the specification of the SMs and their subtending bracts (Li et al., 2019). These and other MIKC-type MADS-box proteins play conserved roles in the specification of SM fate and floral organ identity, which are well documented in the ABCDE model of flower development (Callens et al., 2018; Wu et al., 2018; Chongloi et al., 2019). MADS-box genes act as tetrameric complexes and different protein combinations result in the specification of different floral organ identities (Theissen et al., 2016).

In contrast, the mechanisms and genes that control the transition of the SM, from producing sterile glumes to florets, are not entirely clear. Members of the APETALA 2 (AP2)-like family of transcription factors (TFs) are good candidates for this function. Combined mutations in two closely related AP2-like genes from maize, INDETERMINATE SPIKELET 1 (IDS1) and SISTER OF INDETERMINATE SPIKELET 1 (SID1) (Chuck et al., 2008), or in the two orthologs from rice, OsIDS1 and SUPERNUMERARY BRACT (SNB) (Lee and An, 2012), result in spikelets that generate multiple bract-like structures before producing one or more florets (except for the maize tassel, where no florets are formed). In polyploid wheat, the orthologs of IDS1 include the well-known gene Q on chromosome 5A. This gene played a critical role during wheat domestication by conferring the square spike and free-threshing characteristics (Simons et al., 2006). Loss-of-function mutants in the Q gene in tetraploid wheat also resulted in the formation of additional sterile bracts with characteristics intermediate between glumes and lemmas (Debernardi et al., 2017).

Studies of the conserved microRNA172 (miR172), which targets AP2-like mRNAs for cleavage (Huijser and Schmid, 2011; Zhu and Hellwell, 2011), provide additional evidence for the roles of AP2-like genes in inflorescence and spikelet development. In maize, disruption of the miR172e locus by a transposon insertion in the mutant tasselseed 4 (ts4) or a single mutation within the miR172 binding site of IDS1 (Ts6 allele) resulted in the production of additional florets in the spikelet and a lack of pistil abortion in the tassel (Chuck et al., 2007). Similarly, disruption of barley miR172 by a transposon insertion showed abnormal spikelet development, including the conversion of glumes to partially developed florets in apical regions of the spikes (Brown and Bregitzer, 2011). The domesticated Q allele in wheat originated from a point mutation in the miR172 target site that reduced miR172 activity. Further reduction in miR172 activity (generated by target mimicry, henceforth MIM172) or an extra point mutation in the miR172 binding site of Q showed a similar conversion from glumes to florets in spikelets located in distal positions of the spike (Debernardi et al., 2017; Greenwood et al., 2017). In spikelets located in subterminal positions, glumes were converted into sterile florets consisting of only a lemma and a palea. In proximal spikelets, the glumes subtended no axillary meristems but had longer awns and reduced keels relative to the wild type, indicative of a partial transition to lemmas. This gradient of homeotic conversions correlated with a decrease of miR172 and an increase of Q expression levels from basal to apical regions of the spike (Debernardi et al., 2017).

Overexpression of an miR172 precursor driven by the maize UBIQUITIN promoter (Ubi::miR172) also results in alterations in inflorescence and spikelet development. Rice Ubi::miR172 panicles showed reduced branching and additional glume-like bracts similar to those observed in snb osids1 double mutants. The Ubi::miR172 plants with the highest miR172 expression levels showed more severe effects than the double mutants, however, suggesting that additional miR172 targets were likely involved in the

Figure 1. Wheat AP2-like genes. (a) Representative spikes of a wild-type plant (Wt), ap2S null mutant and T2 transgenic lines transformed with a vector expressing the miR172d precursor under the maize UBIQUITIN promoter (Ubi::miR172). Scale bar: 1 cm. (b) Genomic structure of a typical AP2-like gene, showing the exons in blue, the AP2 domains in pink and the miR172 target site in red (sequences for different wheat AP2-like genes below, with variants in red). (c) Neighbor-joining molecular phylogenetic analysis of Arabidopsis, Brachypodium, rice, maize, barley and wheat AP2-like proteins (based on alignments of the two AP2-domains). (d) RNA-seq heat map showing the expression of different AP2-like homeologs from hexaploid wheat in different tissues (data from the web tool available at http://www.wheat-expression.com). Each row corresponds to a different developmental stage (from younger to older) of the tissue listed on the left. (e) Relative expression of AP2-like genes in apices from the tetraploid variety Kronos at different developmental stages on the Waddington scale (W1, vegetative apex; W2, early double-ridge stage; W3, glume primordium; W3.25, lemma primordium; W3.5, floret primordium). Expression data were determined by qRT-PCR using the ΔΔCT method and ACTIN as endogenous control. Expression was normalized to W1 stage for each gene. Bars represent means ± SEMs of three or more biological replicates, and different letters indicate statistically significant differences (P < 0.05).
Floret development in wheat

(a) Wt  ap2l5  Ubi::miR172

(b) AP2L1 (A,B,D)  CTGACGCATCAGGATTCT
    AP2L2 (A,D)  CTGACGCATCAGGATTC
    AP2L5 (Aq,B,D)  CTGACGCATCAGGATTCC
    AP2L7 (B,D)  CTGACGCATCAGGATTT
    AP2L-B2  CTGACGCATCAGGATTCT
    AP2L-A5Q  CTGACGCATCAGGATTC
    AP2L7 (B,D)  CTGACGCATCAGGATTCC

(c) W1  W2  W3  W3.25  W3.5

(d) Spike  Grain  Leaves/Shoots  Roots

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regulation of these phenotypes (Lee and An, 2012). A similar result was observed in wheat transgenic plants overexpressing miR172. Most wheat Ubi::miR172 plants showed similar phenotypes to the Q-null mutants, with one or two florets transformed into sterile glume-like structures (Debernardi et al., 2017). However, two out of the 14 independent wheat transgenic plants showed an even stronger phenotype, with a large number of sterile glume-like organs (Figure 1a). These plants failed to produce seeds, limiting further analyses and precluding their inclusion in our previous study.

We hypothesized that the more severe spikelet phenotypes observed in the strong Ubi::miR172 plants relative to the wheat Q-null and rice snb osids1 double mutants (Lee and An, 2012) could result from the downregulation of additional AP2-like paralogs. In this study, we combined gene expression data with transgenic and genetic approaches to show that another AP2-like gene, an ortholog to barley HvAP2 Cly1/Zeo1 and rice SHAT1 genes (Nair et al., 2010; Zhou et al., 2012; Houston et al., 2013; Wang et al., 2015a), plays an important and overlapping role with Q in wheat floret development.

RESULTS

Wheat AP2-like family

To prioritize which mutant AP2-like genes to combine with the Q loss-of-function mutant, we characterized the A, B and D homeologs for the three other wheat AP2-like genes harboring a miR172 target site (Figure 1b; Table S1, from Wheat Genome RefSeqv1.1). We designated these genes as AP2L1, AP2L2, AP2L5 (synonymous with Q) and AP2L7, with numbers corresponding to their chromosome locations. Henceforth, and to avoid confusion, we will use the symbols Q and q to refer specifically to the A-genome alleles with or without the mutation in the miR172 binding site, respectively, AP2L5 when referring to the overall function of the different homeologs, and ap2l5 when referring to the loss-of-function mutants for all homeologs.

A phylogenetic analysis including all AP2-like genes targeted by miR172 from wheat, barley, rice, maize, Brachypodium and Arabidopsis showed that the wheat AP2L5 gene belongs to the IDS1/SID1 cluster (Figure 1c), and that the ortholog of SID1/SNB is absent in wheat and barley genomes. We also failed to detect an ortholog of SID1/SNB in the available genomic sequences of Secale cereal (rye), Triticum urartu (einkorn wheat, an A-genome progenitor), Aegilops tauschii (a D-genome progenitor) and Triticum turgidum ssp. dicoccoides (wild emmer), accession Zavitan (Avni et al., 2017) (Table S2). These observations suggest that the ortholog of SID1/SNB was probably lost in the tribe Triticaceae.

The wheat gene most closely related to AP2L5 is AP2L1, a homolog of the flowering repressor TOE1 from Arabidopsis and maize (Aukerman and Sakai, 2003; Salvi et al., 2007). The RNA-seq data available (Borrill et al., 2016; Ramirez-Gonzalez et al., 2018) show that AP2L1 is expressed at very low levels in the spike (Figure 1d), making it an unlikely candidate for inflorescence or flower development. Wheat AP2L7, the ortholog of maize GLOSSY15 (Moose and Sisco, 1996) (Figure 1c), was nearly undetectable in all tissues, whereas AP2L2 showed a very similar expression profile to AP2L5, with high transcript levels in the spikes (Figure 1d).

Wheat AP2L2 belongs to the same clade as the rice SHAT1, barley Cly1/Zeo1 and Arabidopsis AP2 genes (Jofuku et al., 1994; Nair et al., 2010; Zhou et al., 2012; Houston et al., 2013) (Figure 1c), which are all important regulators of inflorescence and/or flower development.

To confirm the published RNA-seq data, we performed quantitative reverse transcription PCR (qRT-PCR) on cDNA derived from the vegetative SAM and early stages of spike development identified using the Waddington scale (Waddington et al., 1983) (Figure 1e). All four AP2-like genes were expressed in vegetative apices (W1), but AP2L1 and AP2L7 expression decreased after the reproductive transition (from W2 to W3.5). Expression of AP2L5 and AP2L2 did not change significantly in the different developmental stages and were expressed at the floret primordia stage (W3.5), when floral meristems and floral organs are specified (Figure 1e). Based on the expression data and the phylogenetic proximity to other AP2-like genes involved in inflorescence development, we prioritized AP2L2 for further functional characterization.

AP2L2 and AP2L5 function redundantly in the specification of lemma identity and the development of axillary floral meristems

Using a sequenced mutant population in the tetraploid wheat variety Kronos (Krasileva et al., 2017), we identified 100 and 79 mutations in the coding regions of AP2L-A2 and AP2L-B2, respectively. For the A-genome copy, we selected line K2233 that has a mutation in the splicing donor site of the fourth intron, and for the B-genome copy we selected line K3634 with a mutation in the splicing acceptor site of the fourth intron (Figure S1a). Sequencing of K2233 ap2l-A2 transcripts revealed that the splice site mutation causes the use of a nearby intronic GT site, resulting in four additional nucleotides and a frame-shift mutation (Figure S1b). The encoded protein (393 amino acids) lacks the two critical AP2 domains and is most likely not functional. Transcripts from the K3634 ap2l-B2 allele were not detected in the expression experiments (see Experimental procedures), suggesting that the mutation may affect transcript stability. We backcrossed the individual mutant lines with wild-type Kronos twice to reduce background mutations, and then intercrossed the ap2l-A2 and ap2l-B2 mutants to generate an ap2l2-null mutant, which for simplicity will be referred to hereafter as ap2l2.
We next compared the phenotypes of \textit{ap2l2} and \textit{ap2l5} mutants in a growth-chamber experiment. As previously observed, the spikes of the \textit{ap2l5} mutant plants displayed reduced spikelet number and density and a higher number of florets per spikelet than wild-type spikes (Figure 2a,b,d,e). In contrast, \textit{ap2l2} mutant plants produced spikes that were no different from wild-type spikes, both in spikelet density and in number of florets (Figure 2a,b,d,e). We observed that the \textit{ap2l2} mutants produced a reduced number of grains per spike (Figure S1c), although we did not detect clear developmental defects in floret organs to explain this observation.

The most important result was observed when we combined the \textit{ap2l2} and \textit{ap2l5} mutations. The \textit{ap2l2 ap2l5} mutant plants (four homozygous mutations) displayed more severe spike phenotypes than the single-gene mutants (Figure 2a), which were reminiscent of the strongest Ubi::miR172 overexpression plants (Figure 1a). The spikelet density of \textit{ap2l2 ap2l5} was significantly reduced relative to \textit{ap2l5} (Figure 2d), although the spikelet number per spike was unchanged (Figure S2a). Furthermore, the spikelets of \textit{ap2l2 ap2l5} produced an increased number of organs, but instead of florets, we observed mostly empty bracts with no axillary floral organs (Figure 2a,b,e).

In mature spikes of Kronos and the \textit{ap2l2} mutant, glumes had short awns and strong keels, whereas lemmas had elongated awns and less pronounced or no keels (Figure 2b,c). In the spikelets of the \textit{ap2l5} mutant, the first lemma looked like a third glume, as it was always empty, and it had a shorter awn and a more pronounced keel than the wild-type lemma (Figure 2b,c,f,h). The second lemma had a longer awn, reduced keel and most of the time developed an axillary meristem. In the spikelets of \textit{ap2l2 ap2l5}, all the empty lemmas resembled glumes (Figure 2b), with significantly shorter awns and longer keels than in the \textit{ap2l5} mutant (Figure 2b,c,f-i).

To describe the phenotypes in more detail, we dissected and compared immature reproductive apices from the

![Figure 2. \textit{ap2l2 ap2l5} mutant spikelets produce multiple glume-like lemmas instead of florets. (a–d) Representative pictures showing the phenotypes observed in the primary spike of Kronos (Wt), \textit{ap2l2}, \textit{ap2l5} and \textit{ap2l2 ap2l5} mutants. (a) Primary spike 3 weeks after heading. Scale bar: 1 cm. (b) Mature central spikelets with separated organs to show the higher number of empty glume-like lemmas (white asterisks) in the \textit{ap2l2 ap2l5} mutant. Scale bar: 1 cm. (c) Transverse sections of the second glume and the second lemma from central spikelets. A white arrowhead points to the more pronounced keel in the second lemma of the \textit{ap2l2 ap2l5} mutant. Scale bar: 200 μm. (d) Spikelet density in the primary spike. (e–i) Number of organs (\(n \geq 20\)) (e), length of the awn (\(n \geq 10\)) and keel (\(n \geq 8\)) in the first (f and h) and in the second (g and i) lemma in the central spikelet of the primary spike. Bars represent means ± SEMs and different letters indicate statistically significant differences (\(P < 0.05\)) by the Student–Newman–Keuls mean comparison test.](https://example.com/figure2.png)
different genotypes (Figures 3 and S3). At the double-ridge stage, we did not observe differences between the wild type and the mutants (Figure S3a); however, differences became evident during the differentiation of the floral meristems (Figure 3). Scanning electron microscopy (SEM) of the wild type and ap2l2 showed the normal development of glumes and lemmas with their axillary floral meristems (Figure 3a). In the ap2l5 mutants, the developing floral meristems were also visible, but the first floret primordium was always replaced by a lemma primordium without an axillary meristem. This phenotype was more severe in the developing spikelets of ap2l2 ap2l5, where all initial lemma primordia lacked axillary meristems (Figure 3a). At a later developmental stage, we observed developing floral organ primordia in the spikelets of wild-type and single-mutant plants (Figure 3b). At this stage, the ap2l2 ap2l5 spikelets contained mostly empty bracts, although we observed a floral meristem developing in the axil of some of the late-developing lemmas (Figure 3b). In a more advanced developmental stage (Figure S3b), the awns of the lemmas were elongated in the wild type, ap2l2 and ap2l5 spikes, but not in the ap2l2 ap2l5 mutant, where the lemma primordia were similar to glume primordia.

Taken together, our phenotypic observations indicate that both AP2L2 and AP2L5 promote the transition from glumes to lemmas and the formation of floral meristems in the axil of the lemmas in the developing spikelets.

**AP2L2 and AP2L5 regulate floral organ identity and modulate the expression of floral organ identity genes**

The conversion from florets to sterile glume-like lemmas in ap2l2 ap2l5 spikes was not complete, and most spikelets were still able to develop axillary flowers, generally in the distal positions of the spikelets (Figure 2e). Those flowers exhibited many developmental defects and did not produce grains, however. Flowers from the wild-type plants include a carpel surrounded by three stamens, two lodicules and one palea, all subtended by one lemma (Figure 4a,g), whereas in the ap2l2 ap2l5 florets the lodicules were absent, the number of stamens was reduced, and occasionally the palea was missing (Figure 4d–f). Interestingly, we observed that in ~40% of the flowers a carpelloid organ replaced the lodicules and the adjacent anterior stamen (Figure 4e–g). In addition, we also observed that ~45% of the flowers developed only carpel-like structures (Figure 4d). The frequency of these changes in organ number per flower is presented in Table 1 (note that empty glume-like lemmas were not included in this analysis). Similar phenotypes, but at a lower frequency, were observed in the flowers of the ap2l5 spikelets (Table 1). The flowers of ap2l2 had a normal number of organs (Figure 4b), but with larger lodicules compared with wild-type plants (Figure S1d–f).

To further describe the mutant phenotypes, we measured the expression of the wheat orthologs of several MIIK-type MADS-box genes previously described as members of the ABCE flowering model (Paolacci et al., 2007; Theissen et al., 2016; Chongloi et al., 2019) in the different genotypes by qRT-PCR (Table S3). We extracted RNA from developing spikes when the lemma, palea and floral meristem primordia were visible at the terminal spikelet (Figure 5a, W3.5–W4.25). As a reference, we compiled the expression levels of the same genes during spikelet development from a published RNA-seq study (Li et al., 2018) and summarized the data in Figure S4.

**VRN1, FUL2 and FUL3, which belong to the A-class genes, are the earliest to be expressed and their...**
expression increases through spike development, except for VRN1 that decreases after the double-ridge stage (Figure S4a). B-class TaPI1 and TaAP3 (Figure S4b) and C-class genes TaAG1 and TaAG2 (Figure S4c) are both expressed mainly after the glume primordium differentiation stage. Finally, E-class genes can be divided into two groups based on their expression profiles, an earlier group including TaSEP5 and TaSEP6 that is expressed at the double-ridge stage (Figure S4d) and a later expressing group including TaSEP2, TaSEP3 and TaSEP4, which is upregulated at or after the glume primordium differentiation stage (Figure S4e).

We then compared the expression levels of the same genes between Kronos, ap2l2, ap2l5 and ap2l2 ap2l5 mutants. No significant differences in expression among genotypes was detected for the A-class genes VRN1, FUL2 and FUL3 (Figure 5b). By contrast, transcription levels of the B-class genes were significantly reduced in ap2l5 and in ap2l2 ap2l5, with the latter showing a stronger reduction (Figure 5c). The C-class gene TaAG2 was upregulated in ap2l5 and ap2l2 ap2l5, whereas TaAG1 was only upregulated in ap2l2 ap2l5 (Figure 5d). The early expressing TaSEP5 and TaSEP6 showed no significant differences in expression among genotypes (Figure 5e), whereas the late expressing TaSEP2, TaSEP3 and TaSEP4 were significantly downregulated in ap2l5 and ap2l2 ap2l5, with the latter showing a stronger reduction (Figure 5f).

Taken together, the expression results described above show that the young spikes of ap2l5 ap2l2, and less dramatically the ap2l5 single-gene mutant, have reduced expression of genes involved in floral organ identity (B-class and late-expressing E-class genes), but increased expression of AG-like genes (C-class).

Mutations in the miR172 target site of AP2L2 result in pleiotropic effects on plant height, spike architecture and lodicule size

After testing the effect of the ap2l2 loss-of-function mutations in Kronos, we explored the effect of mutations in the miR172 binding site of AP2L2 in tetraploid Kronos and in hexaploid wheat. First, we identified a tetraploid wheat tillering mutant (K2236) with a point mutation in the miR172 target site of the AP2L-A2 homeolog (henceforth resistant Ap2l-A2 or rAp2l-A2), in exactly the same position as the one that generated the Q allele in AP2L-A5 (Figures 6a and 1b). This mutation is silent but significantly affects the repression mediated by miR172 (Debernardi et al., 2017).

We backcrossed K2236 with wild-type Kronos and genotyped and phenotyped the F2 segregating population. F2 plants homozygous for the rAp2l-A2 allele had more compact spikes (Figure 6b,c) and were 14% shorter than the wild type (Figure 6d). Plants heterozygous for the K2236 mutation showed intermediate spikelet density and plant height.

Figure 4. AP2L2 and AP2L5 control floral organ identity. (a) Wild-type first floret from a central spikelet. The lemma and palea were separated to show the inner floral organs. Scale bar: 2 mm. (b) First floret from an ap2l2 mutant central spikelet. Scale bar: 2 mm. (c) First floret from an ap2l5 mutant central spikelet. Scale bar: 2 mm. Note that in this mutant background the first floret is generated after one or two glume-like lemmas are produced. (d–f) Representative florets from ap2l2 ap2l5 mutants. Scale bar: 2 mm. (f) Magnification of (e) to show the floral organs. Scale bar: 0.5 mm. A red arrow points to a carpelloid organ replacing the lodicules, and white arrows point to immature stamens. (g) Floral diagrams for the wild type (left) and the ap2l2 ap2l5 mutant (right). Ca, carpel; Le, lemma; Lo, lodicule; Pa, palea; St, stamen. Note the replacement of lodicules and the adjacent stamen by a carpel.
A second induced mutation in the miR172 target site of AP2L2 was identified in the hexaploid winter wheat variety Wedgetail. We had previously identified a dwarf compact spike mutant in a mutagenized population of this variety (Figure 6f). Sequencing of the miR172 target site of the AP2L-A5 and AP2L-D5 homeologs did not reveal any polymorphisms (AP2L-B5 is not functional). Sequencing of the miR172 target site of the AP2L2 homeologs revealed an SNP at a different position in the miR172 target site of the AP2L-B2 gene (henceforth rAp2l-B2, Figure 6e), however. This mutation produces an Asp→Asn change in the encoded protein, which is predicted to have limited effect on protein structure and function (BLOSUM 62 score = 1). This mutation is also predicted to have a stronger effect on miR172 activity than the mutation previously described for K2236, as reflected by a higher free energy of interaction (Figure 6e). The mutant line was backcrossed with the wild-type parental line and the F2 population was genotyped and phenotyped. F2 plants homozygous for the rAp2l-B2 allele showed increased spikelet density (even higher than the K2236 mutant; Figure 6f,g) and a 28% reduction in plant height (Figure 6h), both of which co-segregated with the rAp2l-B2 mutant allele.

Reducing miR172 activity in wheat by a MIM172 approach or by mutations in the miR172 target site of AP2L-A5 promoted glume-to-lemma transitions that were recognized by reduced keels and increased awn length, and in the distal spikelets by the formation of axillary flowers in the glumes (Debernardi et al., 2017; Greenwood et al., 2017). Interestingly, distal spikelets of the spike of the F2 plants carrying the rAp2l2 alleles have glumes with longer awns and reduced keel compared with the lines carrying the wild-type alleles both in tetraploid Kronos (Figure 6i–l) and in hexaploid wheat (Figure S5a–c).

In barley, point mutations in the miR172 target site of the AP2L2 ortholog reduce lodicule swelling (Nair et al., 2010), so we examined the lodicules in the wheat F2 populations. Lodicules in the tetraploid and hexaploid plants carrying the rAp2l2 allele were still able to swell at anthesis (Figures 6m and S5d). A detailed inspection showed that the swollen area of the lodicules in the plants carrying the rAp2l2 alleles was significantly reduced (45% in rAp2l2-A2 and 30% in rAp2l2-B2) when compared with the wild type (Figures 6n and S5d), however. These results indicate that the regulation of AP2L2 expression by miR172 is important for lodicule swelling in wheat, as was observed in barley.

Taken together, the results from two independent mutant lines show that mutations in the miR172 site of AP2L2 homeologs induce phenotypes that are similar to the effects generated by the domesticated Q allele (reduced plant height, compact spike and glume-to-lemma transitions), with the exception of the specific effects on lodicules.

**DISCUSSION**

Wheat AP2L2 and AP2L5 genes have overlapping roles in the regulation of homeotic changes between glumes and lemmas, the development of axillary floral meristems, spike compactness and plant height. The simultaneous absence of both genes results in spikelets with multiple sterile bracts that form a few distal florets with no lodicules and with other altered floral organs. In addition to their overlapping functions, AP2L2 affects lodicule size and AP2L5 affects spikelet number, heading time and floret number. We discuss first the traits for which both genes have overlapping effects and then the specific effects of each gene.

**AP2L2 and AP2L5 reduce plant height and increase spike compactness**

Mutations in the miR172 binding site of AP2L2 resulted in reduced plant height in tetraploid and hexaploid wheat, with a stronger effect in hexaploid wheat. This stronger effect is likely to be associated with the more disruptive effect of the rAp2l-B2 mutation in hexaploid wheat relative to the rAp2l-A2 mutation in tetraploid wheat. An induced mutation in the miR172 binding site of AP2L-A5, which already had the Q mutation, also resulted in a severe reduction in plant height (Greenwood et al., 2017). These results suggest that both AP2L2 and AP2L5 have overlapping roles in the regulation of plant height in wheat. This

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**Table 1** Percentage of plants with different numbers of floral organs per floret in wild-type (Wt) plants, and in ap2l2, ap2l5 and ap2l2 ap2l5 mutants. 0 indicates absence. For ap2l5 and ap2l2 ap2l5, empty lemmas were not considered as florets and were not included in the analysis. More than 35 florets from central spikelets were analyzed for each genotype.

| Genotype | Lemma | Palea | Lodicules | Stamen | Carpel |
|----------|-------|-------|-----------|--------|--------|
|          | Wt    | 100   | 100       | 100    | 100    |
|          | ap2l2 | 100   | 100       | 100    | 100    |
|          | ap2l5 | 100   | 100       | 97.3   | 97.1   |
|          | ap2l2 ap2l5 | 100 | 2.7   | 35.3  | 44.1  |
|          |       |       |           |        |        |
|          | Wt    | 100   | 100       | 100    | 100    |
|          | ap2l2 | 100   | 100       | 21.6   | 2.7    |
|          | ap2l5 | 100   | 100       | 8.1    | 16.2   |
|          | ap2l2 ap2l5 | 100 | 2.7   | 35.3  | 44.1  |
|          |       |       |           |        |        |
|          | Wt    | 100   | 100       | 100    | 100    |
|          | ap2l2 | 100   | 100       | 2.7    | 2.9    |
|          | ap2l5 | 100   | 100       | 21.6   | 29.7   |
|          | ap2l2 ap2l5 | 100 | 2.7   | 35.3  | 44.1  |
|          |       |       |           |        |        |
|          | Wt    | 100   | 100       | 100    | 100    |
|          | ap2l2 | 100   | 100       | 91.9   | 91.9   |
|          | ap2l5 | 100   | 100       | 8.1    | 8.1    |
|          | ap2l2 ap2l5 | 100 | 1.5   | 20.6  | 41.2  | 26.5  | 7.4  | 2.9  |
function seems to be conserved in other grass species because rice MIM172 plants with increased levels of AP2-like genes also exhibited reduced plant height (Wang et al., 2015a). The molecular mechanisms responsible for the height changes remain unknown, however.

Both ap2l2 and ap2l5 single mutants showed more lax spikes (with a lower number of spikelets per cm), but the differences were significant only for ap2l5. However, the ap2l2 ap2l5 mutant showed a significant reduction in spikelets per cm relative to the ap2l5 mutant (Figure 2), confirming that both genes have overlapping roles in the regulation of this trait. The role of AP2L2 in this trait was further demonstrated by the increase in spikelets per cm in rAp2l2 mutants (Figure 6). Similar observations have been

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**Figure 5.** Expression analysis of MIKC-type MADS-domain genes of the ABCE flowering model in developing spikes of the wild type (Wt), and the ap2l2, ap2l5 and ap2l2 ap2l5 mutants. (a) Dissected apices from Wt and ap2l2, ap2l5 and ap2l2 ap2l5 mutants at the W3.5–W4.25 stage (Waddington scale). Scale bars: 250 μm. Magnified developing terminal spikelets at the W3.5 stage are shown (bottom row). Scale bars: 200 μm. The red arrows indicate floral meristems. (b–f) Transcript levels relative to the ACTIN gene. (b) A-class genes VRN1, FUL2 and FUL3. (c) B-class genes TaPI1 and TaAP3. (d) C-class genes TaAG2 and TaAG1. (e) Early expressed E-class genes TaSEP5 and TaSEP6. (f) Late expressed E-class genes TaSEP2, TaSEP3 and TaSEP4. The expression data were determined by quantitative reverse transcription PCR and normalized against the wild type. Bars represent means ± SEMs of four biological replicates, and different letters above error bars indicate statistically significant differences (P < 0.05).
made in barley, where synonymous and non-synonymous mutations in the miR172 binding site result in spikes that were more compact (Houston et al., 2013).

**AP2L2 and AP2L5 promote floret development**

Homologies between grass-specific spikelet organs and floral organs in non-grass species have been widely debated and controversies persist. Glumes are generally interpreted as bracts, but lemmas have been interpreted either as floral bracts or as sepals (Clifford, 1987; Prasad et al., 2001; Malcomber et al., 2006). Comparative studies and mutants are helping to resolve this controversy. In many grass species, sterile lemmas (with no axillary meristem) are located between glumes and lemmas, suggesting a developmental gradient between these two organs (Malcomber et al., 2006). A similar gradient has been observed in tetraploid wheat ap2l5 mutants and transgenic plants overexpressing miR172. These plants have one or two empty lemmas between the glumes and the fertile lemmas, which are not observed in the wild-type plants.

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**Figure 6.** Mutations in the miR172 target site of AP2L2 (rAp2l2) alter spike and floret development and plant height. (a-d) rAp2l-A2 in Kronos K2236. (a) Schematic representation of the interaction between miR172 and AP2L-A2 target sites in the wild type and rAp2l-A2 (mutation in red). The predicted energy of the interactions is indicated beneath the sequences. (b) Primary spike of a homozygous wild-type plant (left) and a homozygous rAp2l-A2 plant (right), 3 weeks after heading. Scale bar: 1 cm. (c) Spikelets per cm in the primary spike (c) and plant height (d) in the F2 population segregating for rAp2l-A2 (n ≥ 8). (e-h) rAp2l-B2 in hexaploid wheat Wedgetail. (e) Schematic representation of the interaction between miR172 and AP2L-B2 target sites with and without mutation. The predicted energy of the interactions is indicated beneath the sequences. (f) Primary spike of a wild-type plant (left) and a homozygous rAp2l-B2 plant (right). Scale bar: 1 cm. (g) Spikelets per cm in the primary spike and (h) plant height in cm in an F2 population (n ≥ 17). (i-j) rAp2l-B2 in Kronos K2236. (i) Typical penultimate spikelets from a wild-type plant and a homozygous rAp2l2-B2 plant. Red arrows point to glume-1 (G1) and glume-2 (G2) awn tips. Scale bars: 1 cm. (j) Length of the awn in the F2 population segregating for rAp2l-B2 (n ≥ 8). (k-l) Transverse sections and (l) length of the keel of the second glumes in the penultimate spikelet from segregating homozygous wild-type and rAp2l-B2 plants (n = 15). (m) Dissected lodicules from homozygous wild-type and mutant florets. Scale bar: 0.5 mm. (n) Area of the swollen region of lodicules from wild-type, heterozygous and homozygous rAp2l-A2 florets (n = 40). Bars represent means ± SEMs. Different letters indicate statistically significant differences (P < 0.05) by the Student-Newman-Keuls mean comparison test. **Significant difference (P < 0.001) by Student’s t-test.**
In this study, we show that the determination of the axillary floral meristem (Lee and An, 2012). In addition, miR172 target genes participate in the determination of axillary floral meristems in the spikelets (Chuck et al., 2007; Debernardi et al., 2017; Greenwood et al., 2017; Zhao et al., 2018), suggesting a negative and conserved role of miR172 in the specification of axillary floral meristems.

**AP2-like genes in both grasses and Arabidopsis share a common role in promoting the establishment and differentiation of floral meristems.** In Arabidopsis, AP2 controls the establishment of floral meristem identity in addition to its later role in the specification of floral organ identity (Bowman et al., 1993). Under SD conditions, ap2 mutant flowers showed enhanced inflorescence-like characteristics (Oka-muro et al., 1997). We speculate that AP2-like genes might have an ancestral role promoting floral meristem establishment in Angiosperms.

**AP2-like genes alone are not sufficient to establish floral meristem identity, however.** These genes are expressed in many other tissues (including root, leaf and shoot; Figure 1d) and AP2 overexpression does not produce ectopic flowers in vegetative tissues. These observations indicate that in order to promote floral meristems, the AP2 genes require the activation of additional genes involved in the reproductive phase. For example, the Arabidopsis triple mutant ap1 cal ful shows a non-flowering phenotype in which plants continuously generate leafy shoots in place of flowers (Ferrándiz et al., 2000). Combined mutations in the homologous wheat genes vrn1 ful2 ful3 resulted in spikes where the lateral spikelets were replaced by vegetative tillers (Li et al., 2019). In rice, the triple mutant of the SEP-like genes osmds1 osmds5 osmds34 also showed a significant increase in the number of sterile bracts (Wu et al., 2018). These results suggest that the expression of A-class and some early E-class MADS-box genes may be a prerequisite for the AP2-like genes to promote the differentiation of axillary floral meristems.

**AP2L2 and AP2L5 affect floral organ identity**

The ap2i2 ap2i5 mutant was still able to produce distal florets that featured various floral organ abnormalities, including an absence of palea, homoeotic transformations of lodicules and the adjacent stamen into carpelloid structures, and a reduced number of stamens. Floral abnormalities were also observed in the ap2i2 mutant, but at a lower frequency (Table 1). The only abnormality detected in the ap2i2 mutant was an enlargement of the lodicules (Figure S1). To understand better the floral phenotypes observed in the AP2-like mutants, we characterized the transcript levels of several MADS-box genes known to be involved in the ABCE model, for the determination of floral organ identity (Coen and Meyerowitz, 1991; Theissen et al., 2016).

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The transcript levels of A-class genes VRN1, FUL2 and FUL3, which control early stages of spike and spikelet development (Li et al., 2019), did not significantly differ between ap2l2 ap2l5 and the wild type control (Figure 5b). This result suggests that the A-class MADS-box genes operate upstream of AP2L2 and AP2L5 genes. The B-class genes TaAP3 and TaAP3, the orthologs of which control lodicule and stamen development in rice and maize (Ambrose et al., 2000; Nagasawa et al., 2003; Whipple et al., 2004; Yao et al., 2008), showed a greater than 10-fold reduction in transcript levels in the ap2l5 and ap2l2 ap2l5 mutants relative to the control (Figure 5c). This result may explain the developmental defects observed in the lodicules and stamens in these two mutants. Although no significant differences in transcript levels between ap2l2 and the wild type were detected for TaPI1 and TaAP3, their transcript levels were consistently lower in ap2l2 ap2l5 relative to ap2l5 (not significant), suggesting a limited role of AP2L2 in the regulation of B-class genes. This result may explain the increase in lodicule size observed in ap2l2 (Figure S1d–f) and the decrease in lodicule size in the rAP2l2 plants (Figure 6m).

Two closely related C-class AGAMOUS-like genes, TaAG1 (OsMADS58) and TaAG2 (OsMADS3), have been identified in monocots (Yamaguchi et al., 2006), and both were highly upregulated in the wheat ap2l2 ap2l5 mutant (Figure 5d). The rice homologs have partially sub-functionalyzed roles in the specification of stamens and carpels (Yamaguchi et al., 2006; Dreni et al., 2011), so their increased expression in the ap2l2 ap2l5 wheat mutant may explain the generation of ectopic carpelloid organs replacing the lodicule and adjacent stamen. In Arabidopsis, the negative regulation of AG by AP2 and the expansion of the AG expression domain in ap2 mutants is a central concept in the classical ABC model (Bowman et al., 1991; Coen and Meyerowitz, 1991; Drews et al., 1991). Our data suggest that this interaction also persists in wheat.

The SEP-like genes (E class), are divided into two subfamilies (Malcomber and Kellogg, 2005). TaSEP4 and TaSEP3 belong to the SEP3 subfamily, which controls lodicule, stamen and carpel identity in rice (Cui et al., 2010). The other SEP-like genes, including TaSEP2, TaSEP6 and TaSEP5, belong to the LOFSEP subfamily and are involved in the specification of most spikelet and floral organs (Cui et al., 2010; Wu et al., 2018). Two of the LOFSEP genes, TaSEP5 (OsMADS34) and TaSEP6 (OsMADS5) are expressed earlier than the other SEPALLATA genes in both rice (Wu et al., 2018) and wheat (Figure S4d). In rice, OsMADS34 (PAP2) is the earliest expressed among the SEP-like genes and regulates the transition of branches and spikelet meristems (Kobayashi et al., 2010). Interestingly, mutations in ap2l5 and ap2l5 ap2l2 only affect the expression of the three SEP-like genes expressed later in flower development (Figure S4c).

This result is consistent with the effect of AP2L2 and AP2L5 in the regulation of B- and C-class but not A-class genes, as described above. The 10-fold downregulation of TaSEP2 (OsMADS1) in ap2l5 and ap2l5 ap2l2 may contribute to the conversion of lemmas to glumes, as overexpression of the rice ortholog OsMADS1 results in the conversion of rudimentary glumes to lemmas (Prasad et al., 2001).

Genetic studies in rice and maize have shown that the functions of the B-, C- and E-class genes are relatively well conserved between eudicots and grasses, but that the role of A-class genes is less clear (Litt, 2007; Causier et al., 2010). Mutations in Arabidopsis AP2, an A-class gene (Theissen et al., 2016), affect development of the first (sepall) and second (petal) whorls. In strong ap2 mutants, the number of organs in the third whorl (stamens) is also reduced, whereas the fourth whorl is normal in all ap2 mutant alleles (Kunst et al., 1989; Drews et al., 1991). In the wheat ap2l2 ap2l5 mutant, lemmas resemble glumes and the development of lodicules and paleas are affected, indicating that these genes also control the identity/development of the perianth. Moreover, the reduced number of stamens in whorl 3 is similar to the phenotype of the strongest ap2 alleles in Arabidopsis.

The homeotic conversion of lodicules to carpels observed in the ap2l2 ap2l5 mutants is different from the petal-to-stamen conversion observed in Arabidopsis ap2 mutants. We propose that the floret phenotypes observed in the ap2l5 and ap2l2 ap2l5 mutants probably result from a failure to specify floral meristem fate (Litt 2007; Causier et al. 2010) combined with the misregulation of B-class and C-class genes (Figure 5). In the Arabidopsis ap2 mutants, the unchanged activity of B-class genes and the expansion of the AG expression domain converts petals into stamens. By contrast, the reduced expression of B-class genes and increased expression AG-like (C-class) genes in the wheat ap2l2 ap2l5 mutant result in the conversion of lodicules into carpelloid organs.

**AP2L2 affects the swollen area of lodicules**

The swelling of the lodicules is necessary to force apart the lemma and palea at anthesis, allowing the stamen filaments to extrude the anthers that release the pollen. There are natural variants of barley where the palea and lemma remain tightly closed throughout the period of pollen release, a character known as cleistogamy (Nair et al., 2010; Ning et al., 2013). In barley the locus regulating cleistogamy (Cly1/Zeo) was mapped to the distal region of chromosome arm 2H (Turuspekov et al., 2004). Cloning of this gene revealed that it was a homolog of Arabidopsis AP2 (Nair et al., 2010) that belongs to the same clade as AP2L2 in wheat (henceforth, HvAP2L2; Figure 1). Cleistogamous flowering in barley is caused by a mutation in the binding site of miR172 in HvAP2L2 (cly1.b), which reduces
mRNA cleavage (Nair et al., 2010) and results in a higher accumulation of HvAP2L2 protein in the lodicules and reduced lodicule size (Anwar et al., 2018). An epigenetic modification in a regulatory region has been postulated to explain the reduced expression of HvAP2L2 and the increased swelling of the lodicules (although still insufficient to open the floret) in barley accession SV235 relative to cly1.b, in spite of having the same miR172 target sequence (Wang et al., 2015b).

In wheat, the ap2l2 mutant generated larger lodicules than in wild-type plants (Figure S1). By contrast, wheat lines with the rAp2l-A2 and rAp2l-B2 alleles developed florets with smaller lodicules in both tetraploid (Figure 6m–n) and hexaploid wheat (Figure S5i). Thus, similarly to barley, the swollen area of the lodicules seems to be inversely correlated with the AP2L2 levels. The lodicules were always present in the ap2l2 mutant, but they were missing in 21.6% of ap2l5 flowers and 100% of ap2l2 ap2l5 flowers (Table 1). These results suggest that both AP2L2 and AP2L5 play critical and redundant roles in lodicule development.

A previous characterization of natural variation in AP2L2 in 63 wheat accessions found no natural variation in the miR172 binding site within the AP2L2-A2, AP2L-B2 or AP2L-D2 homeologs (Ning et al., 2013). Although there is a polymorphism in the second position between a thymine in the A and D genomes and a cytosine in the B genome, these mutations are in the 5’ end of the target site and are predicted to have little to no effect on miRNA activity (Liu et al., 2014). Moreover, a similar mutation in the 5’ end of the miR172 target site of HvAP2L2 from barley Morex does not have phenotypic effects (Nair et al., 2010).

We looked at 72 additional wheat accessions comprising two diploid, 11 tetraploid and 59 hexaploid accessions (Table S4), and we failed to detect variation in the miR172 binding site of the different AP2L2 homeologs. Therefore, the two rAp2l2 alleles identified in this study in tetraploid and hexaploid wheat represent useful tools to modulate plant height, spike compactness and lodicule function. In wheat, reduced anther extrusion and closed flowering has been associated with a lower risk of Fusarium head blight infections (Kubo et al., 2010, 2013). It would be interesting to combine rAp2l-A2 and rAp2l-B2 to see if they are sufficient to induce cleistogamy in polyplody wheat.

**AP2L5, but not AP2L2, affects spikelet number and heading time**

AP2L5 increases spikelet number. The ap2l5 mutants showed a significant reduction in spikelet number per spike (SNS), which indicates a premature transition of the inflorescence meristem to a terminal spikelet. This effect was not observed in the ap2l2 mutant, and was not enhanced in ap2l2 ap2l5 relative to ap2l5 (Figure S2a). Mutations in the miR172 binding site of AP2L5 resulted in increased SNS, indicating a delay in the transition between IM and the terminal spikelet (Greenwood et al., 2017). Similar effects were reported in rice, where snb osids1 double mutants showed fewer branches (Zhu et al., 2009; Lee and An, 2012) whereas MIM172 plants had increased branching (Wang et al., 2015a). The tassels of maize sid1 sid1 mutants also showed reduced branching (Chuck et al., 2008), whereas the de-repression of AP2-like genes in ts4 and Ts6 (rIDS1) resulted in increased tassel branching (Chuck et al., 2007). These results suggest a conserved role of grass AP2L5/IDS1 SID orthologs in delaying the transition of inflorescence meristems (or branch meristems) into spikelets.

The previous results seem related to the role of AP2 in Arabidopsis in maintaining the stem cell niche (= apical initials) and the proliferative nature of the shoot meristem (Wurschum et al., 2006; Balanza et al., 2018). In the Arabidopsis IM, the MADS-box protein FUL directly and negatively regulates the accumulation of AP2, and ful mutants produce more fruits than the wild type. (Balanza et al., 2018). Interestingly, loss-of-function mutations in wheat FUL2 or VRN1 (homologs of Arabidopsis FUL) result in significant increases in SNS and number of grains per spike (Li et al., 2019), suggesting the potential conservation of a similar regulatory module in wheat and Arabidopsis inflorescence meristems.

**AP2L5 controls floret number.** The ap2l5 mutant (but not the ap2l2 mutant) showed a significant increase in floret number per spikelet (Figure 2e). The ap2l2 ap2l5 mutants also exhibited a large number of organs per spikelet, but in this case, most of them were sterile bracts (Figure 2b,e). In maize and rice, the ids1 sid1 and osids1 snb mutants produced multiple sterile bracts before the development of a terminal spikelet (Chuck et al., 2007; Lee and An, 2012). These observations indicate a role of AP2L5 and its orthologs in reducing the meristematic activity of the spikelet meristem and the number of florets that can be generated. We currently do not know why mutations in the ap2-like genes in grasses operate differently in the IM (reducing the number of lateral organs) than in the SM (increasing the number of lateral organs).

**AP2L5 delays heading time.** If spikelets are generated by the IM at the same rate in different genotypes, a reduction in SNS is expected to accelerate heading time. This was observed in the ap2l5 mutants, which flowered approximately 4 days earlier than the wild-type controls (Figure S2); however, Ubi::miR172 plants produce one fewer leaf than the wild type, suggesting that one or more AP2-like genes also affect the transition of the SAM from the vegetative to the reproductive stage. AP2-like genes are known repressors of the flowering promoting gene FT in many species, and mutation in several ap2-like genes or
overexpression of miR172 produce early flowering (Huijser and Schmid, 2011; Zhu and Helliwell, 2011). The flowering phenotype of wheat ap2-like mutants suggests that AP2L5, but not AP2L2, may have a conserved role in the regulation of FT expression.

In summary, AP2L5 in wheat seems to have retained a broader role controlling reproductive development (spikelet and floret number and heading time) than AP2L2, which seems to be more restricted to spikelet and floret development.

CONCLUSION

The results from this and previous studies show that the balance in the expression of miR172 and AP2-like genes is crucial for the correct development of the grass spikelet, and that this balance has been altered during the domestication of wheat and barley. Both the domesticated allele of wheat gene Q, a major determinant of the free-threshing and compact spike character, and the barley Cly1/Zeo1 gene, which confers compact spike and cleistogamy, resulted from spontaneous mutations in their miR172 target sites that reduce miR172 cleavage activity. These examples show the potential for the modulation of the activity of the AP2-like genes to control important agronomic traits. The two rAp2l2 alleles identified in this study provide tools to explore the value of the resulting modifications in plant height and spike compactness in different wheat classes and/or in different environments.

In addition to its potential practical applications, this study provided insights on the critical and redundant roles of AP2L2 and AP2L5 in the development of axillary floral meristems and the differentiation of lemma characteristics in the subtending bract. Finally, our study showed an essential role of these genes in the development of lodicules and on the regulation of B-, C- and late E-class MADS-box floral genes.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

The tetraploid wheat variety Kronos used in this study has a spring growth habit determined by the Vrn-A1c allele. Kronos also has the Q allele, which confers the subcompact spike phenotype and the free-threshing character. TILLING populations of Kronos mutagenized with ethyl methane sulphonate (EMS) (Uauy et al., 2009) were used to screen for mutants of the AP2L2 gene. The two selected truncation mutations and the mutation in the miR172 target site were confirmed in M4 grain using the genome-specific primers described in Table S5.

The effect of the mutations on the AP2L2 transcripts was verified by RT-PCR on RNA extracted from leaves of the ap2l2 mutant. The genome-specific primers are described in Table S5. For the K3634 mutation we also tested nested PCR, but we were unable to detect the transcript in the mutant. For all experiments, grains were first cold imbibed for 2–4 days at 4°C. The plants were grown in cones in PGR15 growth chambers (Convirion, http://www.convirion.com) adjusted to 16 h of light (22°C) and 8 h of darkness (18°C). The intensity of the sodium halide lights measured at the height of plant heads was (260 µm2 s−1). The line with the mutation in the miR172 target site of the AP2L-B2 mutant was obtained in the winter hexaploid variety Wedgetail, which was mutagenized using sodium azide (Chandler and Harding, 2013). Primers used to genotype the mutant line are listed in Table S5. Phenotyping for co-segregation analysis was performed in a glasshouse with 16 h of light (22°C) and 8 h of dark (18°C), after 7 weeks of vernalization.

qRT-PCR

RNA samples were extracted using the Spectrum Plant Total RNA Kit (Sigma-Aldrich, https://www.sigmaaldrich.com). We followed Protocol A that allows for the purification of total RNA including small RNA molecules. Total RNA was treated with RNase-free DNase (Promega, https://www.promega.com). cDNA synthesis was carried out using SuperScript II Reverse Transcriptase (Invitrogen, now ThermoFisher Scientific, https://www.thermofisher.com). mRNAs were reverse transcribed starting from 1 µg of total RNA and using Oligo(dT) primer. The product from the first-strand synthesis was diluted 1 in 20, and 5 µl of diluted cDNAs was used in the qRT-PCR reaction, which was performed using SYBR Green and a 7500 Fast Real-Time PCR system (Applied Biosystems, a branch of ThermoFisher Scientific). The ACTIN gene was used as an endogenous control for mRNAs. Primers are listed in Table S5.

Morphological traits

To study the anatomical changes in the glumes and lemmas of the different genotypes we made transverse sections of dry glumes and lemmas of fully developed spikes. We boiled the organs in water and then sectioned them by hand using a razor blade. Transverse sections were stained with toluidine blue O solution for 30 s. Images of the stained sections and dissected floret organs were digitally captured using a stereo-dissecting scope.

Scanning electron microscopy (SEM)

Spikes at different developmental stages were dissected, fixed for a minimum of 24 h in FAA (50% ethanol, 5% v/v acetic acid, 3.7% v/v formaldehyde), rinsed twice in the same buffer, and dehydrated through a graded ethanol series to absolute ethanol. Samples were critical-point dried in liquid CO2 (tousimis.com) adjusted to 16 h of light (22°C) and 8 h of dark (18°C). The intensity of the sodium halide lights measured at the height of plant heads was (260 µm2 s−1). The line with the mutation in the miR172 target site of the AP2L-B2 mutant was obtained in the winter hexaploid variety Wedgetail, which was mutagenized using sodium azide (Chandler and Harding, 2013). Primers used to genotype the mutant line are listed in Table S5. Phenotyping for co-segregation analysis was performed in a glasshouse with 16 h of light (22°C) and 8 h of dark (18°C), after 7 weeks of vernalization.

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To study the anatomical changes in the glumes and lemmas of the different genotypes we made transverse sections of dry glumes and lemmas of fully developed spikes. We boiled the organs in water and then sectioned them by hand using a razor blade. Transverse sections were stained with toluidine blue O solution for 30 s. Images of the stained sections and dissected floret organs were digitally captured using a stereo-dissecting scope.

Phylogenetic analysis

The complete protein sequences of the different AP2-like genes from Brachypodium, maize, rice and Arabidopsis were obtained from the Phytozone web resource (https://phytozone.jgi.doe.gov/pz/portal.html). Sequences from barley were obtained from the International Barley Sequencing Consortium (https://webblast.ipskateslerben.de/barley_ibsc/viroblast.php). Protein sequences from wheat were obtained from Wheat Genome RefSeqv 1.1. Evolutionary analysis was conducted in MEGA X. For analysis, we used a region that included the two AP2 domains.

Wheat transformation

Transgenic wheat plants were generated at the UC Davis Plant Transformation Facility (http://ucdptf.ucdavis.edu/) using the
Japan Tobacco (JT) technology licensed to UC Davis. Immature embryos from Kronos were transformed using Agrobacterium EHA105. The selection of transgenic plants was conducted using hygromycin, and transgene insertion was validated by DNA extraction and PCR.

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CONFLICT OF INTEREST

The authors of this manuscript declare that they do not have any conflicts of interest.

AUTHOR CONTRIBUTIONS

JMD and JD conceived the study. JMD performed most of the experimental work. JMD and JD analysed the data. JJ was responsible for the scanning electron microscopy studies. JRG and EJF. identified and characterized the hexaploid wheat variety Wedgetail with an induced mutation in the experimental work. JRG and EJF. identified and characterized the hexaploid wheat variety Wedgetail with an induced mutation in the experimental work. JRG and EJF. identified and characterized the hexaploid wheat variety Wedgetail with an induced mutation in the experimental work. JRG and EJF. identified and characterized the hexaploid wheat variety Wedgetail with an induced mutation in the experimental work. JRG and EJF. identified and characterized the hexaploid wheat variety Wedgetail with an induced mutation in the experimental work. JRG and EJF. identified and characterized the hexaploid wheat variety Wedgetail with an induced mutation in the experimental work. JRG and EJF. identified and characterized the hexaploid wheat variety Wedgetail with an induced mutation in the experimental work. JRG and EJF. identified and characterized the hexaploid wheat variety Wedgetail with an induced mutation in the experimental work. JRG and EJF. identified and characterized the hexaploid wheat variety Wedgetail with an induced mutation in the experimental work. JRG and EJF. identified and characterized the hexaploid wheat variety Wedgetail with an induced mutation in the experimental work. JRG and EJF. identified and characterized the hexaploid wheat variety Wedgetail with an induced mutation in the experimental work. JRG and EJF. identified and characterized the hexaploid wheat variety Wedgetail with an induced mutation in the experimental work. JRG and EJF. identified and characterized the hexaploid wheat variety Wedgetail with an induced mutation in the experimental work. JRG and EJF. identified and characterized the hexaploid wheat variety Wedgetail with an induced mutation.

DATA STATEMENT

This article does not include large data sets, but all the data and genetic materials are available from the authors upon request.

SUPPLEMENTARY INFORMATION

Additional Supporting Information may be found in the online version of this article.

Table S4. Natural variation in miR172 target site of AP2L2.

Table S5. Primers used in this study.

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