

## RESEARCH REPORT

# New alleles of the wheat domestication gene *Q* reveal multiple roles in growth and reproductive development

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## ABSTRACT

The advantages of free threshing in wheat led to the selection of the domesticated *Q* allele, which is now present in almost all modern wheat varieties. *Q* and the pre-domestication allele, *q*, encode an AP2 transcription factor, with the domesticated allele conferring a free-threshing character and a subcompact (i.e. partially compact) inflorescence (spike). We demonstrate that mutations in the miR172 binding site of the *Q* gene are sufficient to increase transcript levels via a reduction in miRNA-dependent degradation, consistent with the conclusion that a single nucleotide polymorphism in the miRNA binding site of *Q* relative to *q* was essential in defining the modern *Q* allele. We describe novel gain- and loss-of-function alleles of *Q* and use these to define new roles for this gene in spike development. *Q* is required for the suppression of ‘sham ramification’, and increased *Q* expression can lead to the formation of ectopic florets and spikelets (specialized inflorescence branches that bear florets and grains), resulting in a deviation from the canonical spike and spikelet structures of domesticated wheat.

**KEY WORDS:** Wheat, Spike, Inflorescence, AP2, Domestication, microRNA

## INTRODUCTION

The causal molecular mechanism for the domestication of *Q* is thought to be an amino acid change in the predicted *Q* protein (Simons et al., 2006) and/or a single nucleotide polymorphism (SNP) present in a presumed miRNA binding site of *Q* (Sormacheva et al., 2015; Chuck et al., 2007). Unlike the domesticated homeoallele *Q* (chromosome 5A) (Faris et al., 2003), the B and D homeoalleles of hexaploid bread wheat are thought to be a pseudogene and expressed at a low level, respectively (Zhang et al., 2011). *Q* is a member of the AP2 class of transcription factors, which are known to influence many traits associated with floral transition, including both flowering time and the definition of floral organs (Aukerman and Sakai, 2003; Chuck et al., 2007; Lauter et al., 2005; Lee and An, 2012; Lee et al., 2007; Brown and Bregitzer, 2011; Varkonyi-Gasic et al., 2012). Generally, gain-of-function mutations and overexpression of *AP2* genes result in delayed flowering (Mlotshwa et al., 2006; Aukerman and Sakai, 2003; Schmid et al., 2003; Jung et al., 2007) and additional florets in the *Tasselseed6* mutant of corn (Chuck et al., 2007). Loss-of-

function mutations and reduced expression can cause early flowering and disruptions in floral patterning and determinacy (Chuck et al., 1998, 2008; Lee and An, 2012; Mlotshwa et al., 2006; Jung et al., 2007; Mathieu et al., 2009); these effects can be masked by redundant function of other *AP2* genes (Yant et al., 2010). *AP2* genes can be regulated by miR172, and mutations affecting the expression of miR172 or SNPs in either *miR172* or in its conserved target site in *AP2* genes can lead to misregulation, with the potential to increase or reduce regulatory targeting by the miRNA (Aukerman and Sakai, 2003; Chuck et al., 2007; Varkonyi-Gasic et al., 2012; Zhu et al., 2009).

## RESULTS AND DISCUSSION

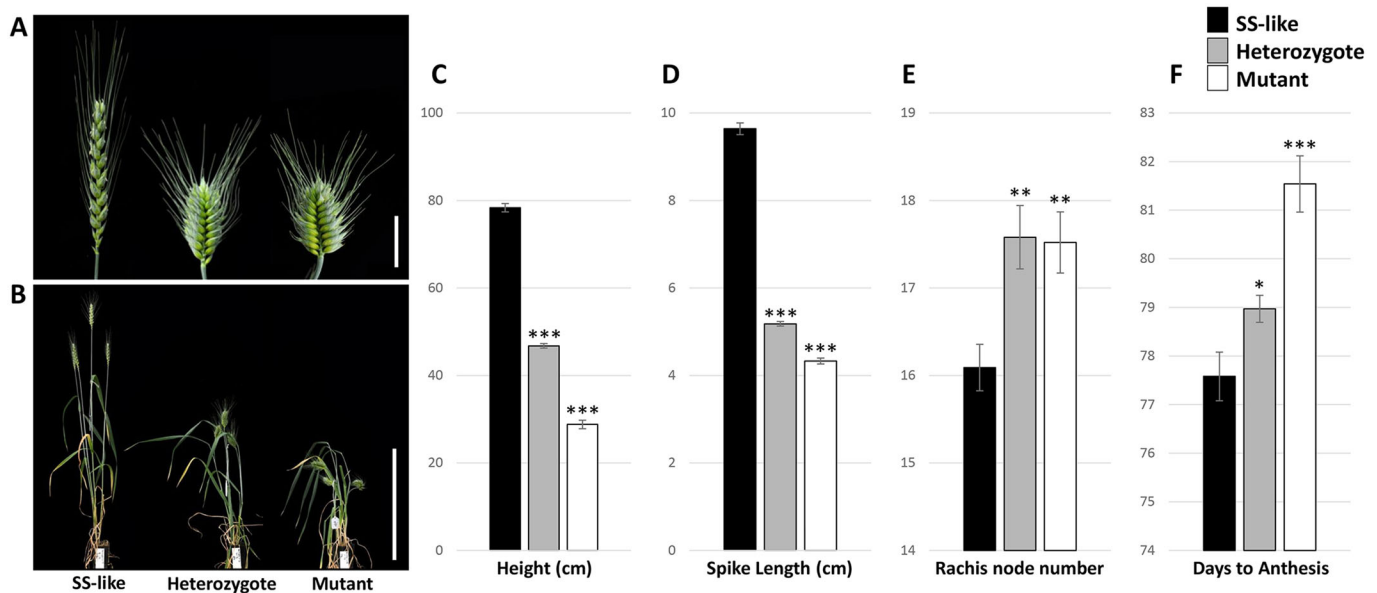
A dwarf, compact spike mutant was identified in an  $M_2$  mutant population derived from the Australian wheat cultivar Sunstate (SS) and backcrossed to the progenitor line SS. The  $F_2$  progeny of this cross could be separated into three distinct height classes, namely SS-like, intermediate (heterozygous) and short (homozygous mutant) (Fig. 1A,B), which were subsequently confirmed by genetic analysis (see below). Differences in height were substantial (Fig. 1B,C) and unambiguously separated plants into the three classes. Both heterozygous and homozygous mutant plants were characterized by a reduction in internode length relative to SS-like siblings, resulting in spike compaction and reduced overall height (Fig. 1C,D; Fig. S1). Mutant plants were also late flowering and possessed a small increase in rachis node number (nodes along the spike that potentially bear a fertile spikelet) (Fig. 1E,F).

The compact mutant resembled transgenic wheat lines with increased copy number and expression of *Q* (Simons et al., 2006; Förster et al., 2012, 2013). The mutant (hereafter called *Q'*) contained a novel single nucleotide change in the miRNA binding site of *Q* that causes an additional mismatch when aligned to the targeting miRNA Ta-miR172 (Fig. 2A; Fig. S2). No other sequence changes were observed in the coding region. Expression of *Q*, as measured by qPCR, was higher in developing inflorescences of *Q'* plants than in their SS-like siblings (Fig. 2B,C). Modified 5' RACE detected multiple *Q* cleavage products in mRNA from SS-like plants, whereas only a single cleavage product was detected from *Q'* mRNA (Fig. 2D). The most abundant class of *Q* cleavage products matched the expected product from miRNA-directed cleavage between the tenth and eleventh nucleotides within the miRNA, whereas the single *Q'* cleavage product detected was shifted by a single base. Combined with our expression data, the reduced levels of cleavage product in *Q'* indicate that the induced mismatch to Ta-miR172 results in reduced targeted mRNA degradation, and ultimately higher *Q* protein abundance. Based on its partial genetic dominance and increased mRNA expression, *Q'* appears to be a gain-of-function allele relative to *Q*. We cannot exclude the possibility that the amino acid change (G to E) in the predicted *Q* protein resulting from the *Q'* SNP also contributes to the observed phenotypes. However, similar compact spike phenotypes have been

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**Fig. 1. Identification and characterization of a compact spike mutant.** (A) Adult wheat inflorescence (spike) of the Sunstate (SS)-like sibling line, heterozygous line and homozygous mutant line. Scale bar: 3 cm. (B) SS-like, heterozygous and homozygous mutant plants at maturity. Scale bar: 30 cm. (C–F) Plant height (C), spike length (D), rachis node number (number of nodes along the spike, E) and days to anthesis (flowering time, F) of SS-like, heterozygous and compact spike mutant plants. Data are presented as mean $\pm$ s.e.m.  $n=33$  SS-like,  $n=35$  heterozygous,  $n=22$  mutant. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ , compared with SS-like plants.

reported in transgenic plants containing an miR172 binding site mimic (MIM172) and reduced levels of miR172 (Debernardi et al., 2017), suggesting that reduced miR172 cleavage in  $Q'$  is sufficient to induce the observed phenotypes. For the barley paralog *HvAP2* (*2H*), both synonymous and non-synonymous mutations causing mismatches to miR172 result in compact spikes, further supporting that nucleotide mismatches without amino acid changes can affect this trait (Houston et al., 2013).

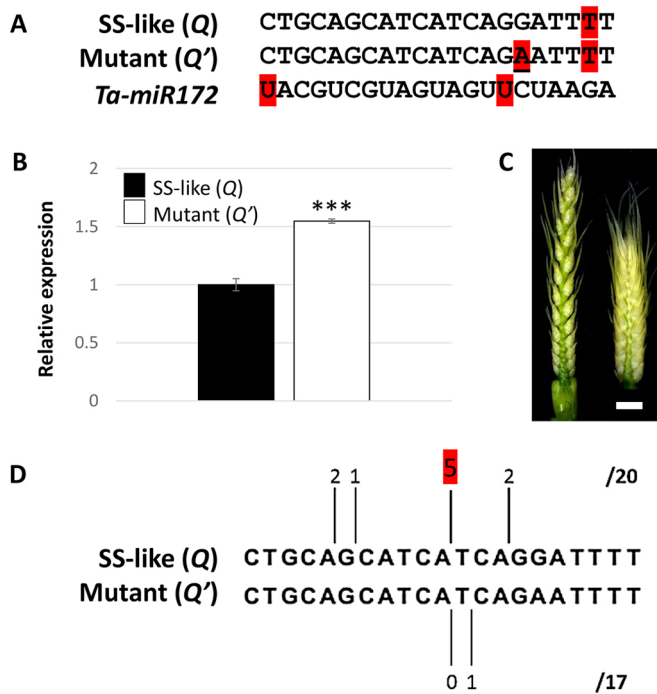
To establish that the  $Q'$  mutation was causal, we first confirmed complete genetic linkage between the  $Q'$  SNP and the reduced height/compact spike phenotypes (Fig. 3B). Second, we investigated additional alleles. Two allelic dwarf mutant lines with compact spikes, ANBW5C Dwarf (5CD) and ANBW5B Dwarf (5BD) (Fig. 3A), have previously been described and mapped to chromosome 5AL (which contains  $Q$ ), although the genetic basis was not determined (Kosuge et al., 2012). Sequencing revealed that both mutant lines contained SNPs within the miRNA binding site of  $Q$ , with 5CD containing the exact same mutation as  $Q'$  and 5BD ( $Q'$ -like) featuring a unique SNP in the miRNA binding site of  $Q$  (Fig. 3C; Fig. S2). The similar phenotypes of the two independent mutants support the hypothesis that the causal effect of the  $Q'$  mutation is associated with a reduction in miR172 repression rather than with the change in the encoded amino acid. Consistent with impaired regulation by miR172, all compact spike mutants showed higher expression of  $Q$  than their sibling or parent lines in both developing inflorescence and elongating peduncle internode tissue (Fig. 3G,H).

To formally confirm that the  $Q'$  mutation was causal to the observed phenotypes, we performed a second round of mutagenesis in the  $Q'$  background. Two unique revertant alleles were isolated with SNPs in the first exon of the  $Q'$  gene:  $Q'$ -Rev1, a presumed complete loss-of-function revertant with an introduced stop codon; and  $Q'$ -Rev2, a partial revertant with an amino acid change immediately before the first predicted AP2 domain (Fig. 3C). Both 'revertants' retained the  $Q$  and  $Q'$  mutations, as expected. The  $Q'$  revertants completely ( $Q'$ -Rev1) or partially ( $Q'$ -Rev2) suppressed the phenotypic changes in  $Q'$  (see below),

confirming that changes in  $Q$  are responsible for the  $Q'$  gain-of-function phenotypes.

The presence of independent gain- and loss-of-function  $Q$  alleles in a common background allows the function of  $Q$  to be analyzed with a precision not previously possible. In contrast to  $Q'$ ,  $Q'$ -Rev1 plants showed a reduction in rachis node number compared with SS-like plants, demonstrating an earlier (in terms of nodes) transition from inflorescence meristem to terminal spikelet meristem (Fig. 3F). Whereas plant height was increased in  $Q'$ -Rev1 plants compared with SS-like ( $Q$ ) plants, spike length did not differ significantly (Fig. 3D,E). Reduced rachis node number in  $Q'$ -Rev1 compared with  $Q$  meant that the average internode length between each spikelet was greater, resulting in reduced spikelet density, also known as a lax spike. Compared with SS-like and  $Q'$ , the lax spikes of  $Q'$ -Rev1 were difficult to hand thresh (Fig. S3), consistent with observations of plants containing pre-domestication  $q$ , or 5A deletions that lack domesticated  $Q$  (Faris et al., 2003; Simons et al., 2006; Förster et al., 2012), and with  $Q$  playing an important role in wheat domestication. Partial reversion of the  $Q'$  mutant phenotype in  $Q'$ -Rev2 was characterized by an increase in height and spike length relative to  $Q'$ , although not to the extent of SS-like plants (Fig. 3A).

Given that *AP2* genes in other species have diverse roles in spikelet and floret development, we examined whether increased  $Q$  activity resulted in additional, previously undescribed changes in reproductive development. The two independent  $Q'$  mutants and  $Q'$ -like all exhibited several alterations in spikelet and floret development, although we focused on detailed analysis of the original  $Q'$  allele in the SS background.  $Q'$  plants produced fully formed floret-containing spikelets usually by the second rachis node from the base of the spike, whereas  $Q$  and  $Q'$ -Rev1 plants typically produced three or four rudimentary spikelets at the basal rachis nodes before producing fertile floret-bearing spikelets, as often occurs for modern wheat varieties (Fig. 4D; Fig. S4). Thus, increased  $Q$  activity can promote basal spikelet fertility as well as increase total rachis node number (Fig. 3F). A role for  $Q'$  in delaying conversion of the inflorescence to spikelet meristem is consistent



**Fig. 2. The compact spike mutant *Q'* contains a novel SNP in the miRNA binding site that leads to changes in transcriptional regulation.**

(A) Sequence alignment showing the miRNA binding site sequence of SS-like with the domesticated *Q* allele and the mutant (*Q'*), and the complementary wheat Ta-miR172 sequence. The miRNA mismatch underlying the domestication *Q* allele (C to T) is indicated by the 'T' highlighted in red, and is present in both *Q* and *Q'*. The 'U's highlighted in red in the miR172 sequence represent mismatches with all known *Q* sequences. The *Q'* mutation is highlighted in red and underlined. (B) Expression of *Q* in SS-like and mutant inflorescence tissue harvested at the beginning of internode elongation (~10 mm inflorescences at terminal spikelet stage). Data are presented as mean  $\pm$  s.e.m. of four biological replicates. \*\*\* $P < 0.001$ . (C) Developing inflorescence of SS-like (left) and mutant (right) plants at the early internode elongation stage when the compaction phenotype first becomes apparent. Scale bar: 2 mm. (D) Cleavage products as determined by sequencing of 5' RACE products using RNA pooled from the biological samples used for the expression analysis in B. The number of clones sequenced and their detected cleavage site location are presented (expected cleavage site of *Q* is marked in red). The total number of clones presented (20 for SS-like and 17 for *Q'*) is from sequenced clones which contained *Q* transcript.

with the recently proposed role of miR172 and *AP2* genes in regulating panicle development in rice (Wang et al., 2015).

Wheat spikelets comprise two basal glumes (always sterile) followed by an indeterminate number of florets (Fig. 4A). *Q'* plants deviated from this fundamental pattern, with spikelets often possessing floret structures in place of glumes (Fig. 4B,C; Fig. S5). In the basal and apical portion of the spike, glumes were often replaced either by rudimentary florets with only a lemma and palea, or – with increasing frequency towards the terminal spikelet – complete fertile florets (Fig. 4B,C). Florets occupying typical positions in *Q'* formed normally. SEM analysis revealed floret organs forming early in spikelet development adaxial to glume-lemma organs in *Q'* (Fig. S5), with no additional lemma-like organ visible. These ectopic florets contributed to an increase in visible florets per spikelet along the spike of *Q'* plants (Fig. 4D). Spikelets in the central portion of *Q'* spikes were less likely to form florets (partial or complete) in place of glumes (Fig. 4C). In *Q'* plants there was a tendency for the glume-like structures of the spikelet to be elongated and produce lemma-like awns, with awn length

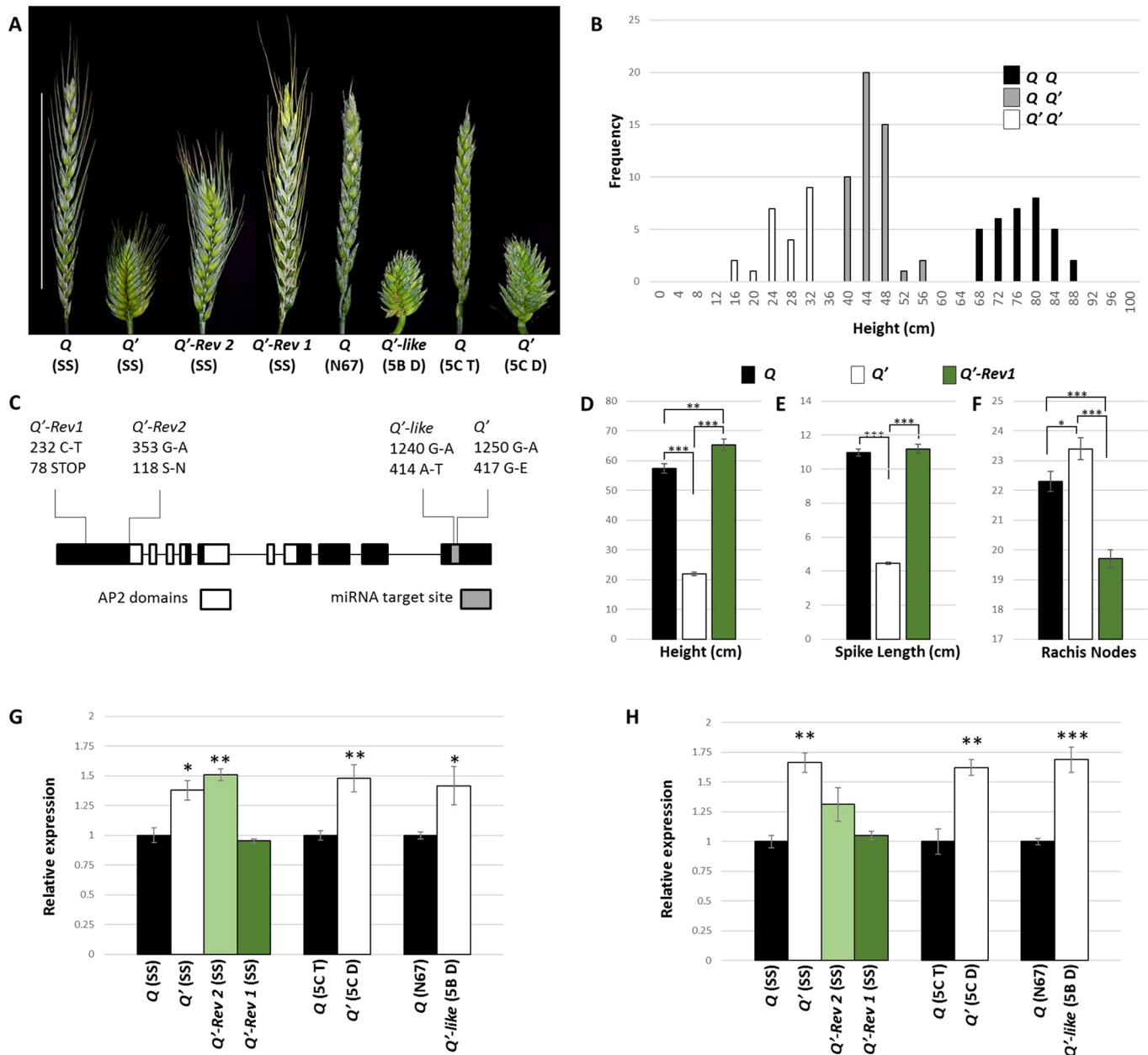
increasing along the spike from the base to the terminal spikelet (Figs S6 and S7) independently of whether floret structures were visible. Similar phenotypes were observed in MIM172 plants with increased *Q* expression (Debernardi et al., 2017). The simplest interpretation of these *Q'* phenotypes is a replacement of glumes with partially or fully developed florets, including awned glumes. This in turn suggests that increased *Q* activity promotes ectopic floret formation during spikelet development and, remarkably, that it can alter one of the defining features of the grasses, namely two sterile glumes at the base of each spikelet.

Another characteristic of wheat spikes is that a single spikelet is usually present at each rachis node. However, in some genetic backgrounds or under appropriate environmental conditions, two spikelets can form at a single node to generate a 'paired spikelet', which might be the equivalent of a 'spikelet pair' in plants such as corn (Boden et al., 2015 and references therein). Although absent in *Q* and *Q'-Rev1* plants, *Q'* spikes contained paired spikelets, with their frequency peaking around the central rachis nodes of the spike (Figs S8 and S9).

While increased *Q* activity has multiple effects on spike, spikelet and floret development, the loss-of-function *Q'-Rev1* allele also reveals that *Q* possesses broader, previously unidentified roles (see also Debernardi et al., 2017). Unlike *Q* and *Q'*, *Q'-Rev1* plants intermittently produced spikelets with elongated rachilla internodes and many florets (also known as 'sham ramification', reminiscent of the *shr1/exg* locus on 5A (Amagai et al., 2014, 2015) (Fig. 4D,E). This trait was more severe in tillers (data not shown) than in the main spike, but the 'extra' florets in these spikelets did not produce grains. The sham ramification trait has been mapped to chromosome 5AL, in a similar position to *Q*, and has been shown to be repressed by the presence of the D genome in some backgrounds (Alieva and Aminov, 2013; Amagai et al., 2014). In the absence of a D genome, sham ramification and extra florets were observed in tetraploid wheat lines with loss-of-function alleles of *Q* and in lines overexpressing miR172 (Debernardi et al., 2017). Debernardi et al. (2017) also report extra sterile glumes in place of florets associated with *Q* loss-of-function, although we did not observe these traits in our *Q'-Rev1* line. Our observations suggest that *Q* activity must be tightly regulated, as both increases (ectopic florets in place of glumes) and decreases (sham ramification) in expression can lead to increases in floret number, similar to reports in maize *AP2* mutants (Chuck et al., 2007, 2008).

Many of the inflorescence architecture defects of *Q'* are confined to, or more severe in, certain regions of the spike. Most notably, spike compaction (Fig. S10), replacement of glumes with florets and increased awn length all become more severe in nodes closer to the terminal spikelet (Fig. 4; Figs S6 and S7). *Q* expression exhibited temporal and spatial variation during spike development (Fig. S11), with *Q'* typically more highly expressed than *Q*, and a somewhat reciprocal relationship between *Q* and miR172 expression, consistent with the results of Debernardi et al. (2017). Increased expression of *Q* in the peduncle internode and severe reduction in the size of this internode suggest that targeted degradation of *Q* by miR172 is broadly required to ensure correct elongation of internodes (stem, rachis and rachilla) in the wheat plant, demonstrating that *Q* plays an important role throughout wheat reproductive development.

In summary, using mutagenesis and a candidate gene approach we have generated a series of gain- and loss-of-function *Q* alleles that have allowed us to identify previously unknown aspects of *Q* gene function in wheat reproductive development. The presumed miRNA mismatches in the gain-of-function mutants we have isolated confirm that the common miRNA regulation of *AP2* genes



**Fig. 3. Co-segregation analysis, loss-of-function mutants derived from *Q* and additional *Q* miRNA mutants confirm that the novel SNP is causal for *Q* phenotypes.** (A) Adult wheat inflorescences of SS-like sibling (*Q*), *Q'* and secondary induced mutants *Q'-Rev2* and *Q'-Rev1* in the SS background, as well as Novosibirskaya 67 (N67) and *Q'-like* mutant ANBW5B Dwarf (5BD) in the N67 background, and sibling lines of ANBW5C Tall (5CT) and ANBW5C Dwarf (5CD, *Q'*). Scale bar: 10 cm. (B) Co-segregation of *Q* and *Q'* in the SS background showing the frequency of plants grown from heterozygous parents that fell within specific height ranges (bins covering 4 cm). Bars are shaded according to the genotype of plants within those height ranges as determined by cleaved amplified polymorphic sequence (CAPS) marker analysis. Plants segregated in agreement with a 1:2:1 ratio as determined by a chi-squared test ( $P=0.281$ ,  $n=104$  progeny). (C) *Q* gene schematic showing exons (black boxes), introns (thin lines), miRNA target site and AP2 domains. The location of *Q'* and *Q'-like* mutations and derived revertant mutations in *Q'* are shown, including nucleotide changes and predicted translational changes. (D–F) Plant height (D), spike length (E) and rachis node number (F) of *Q*, *Q'* and *Q'-Rev1* plants. Data are presented as mean $\pm$ s.e.m.  $n=10$ . (G,H) Relative expression of *Q* transcript in compact mutant lines normalized to their sibling or parent line in developing inflorescences ( $\sim 10$  mm inflorescences at terminal spikelet stage) (G) and in elongating peduncle internode tissue (H). Data are presented as mean $\pm$ s.e.m. of three biological replicates. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ .

also extends to wheat and that correct regulation of *Q* expression is required for normal formation of the wheat spike and spikelets.

## MATERIALS AND METHODS

### Plant materials and mutagenesis

The spring habit, bread wheat cultivar Sunstate (SS) was mutagenized using sodium azide as described by Chandler and Harding (2013). SS contains the domesticated *Q* allele. The same mutagenesis was performed on *Q'* grain

when generating revertant alleles. Further information regarding additional *Q* alleles, threshing and growth conditions is provided in the supplementary Materials and Methods.

### Expression analysis by qRT-PCR

Developing inflorescence tissue was harvested for qRT-PCR at terminal spikelet stage. Five developing inflorescences were harvested per biological replicate. Peduncle internode tissue was harvested when the peduncle

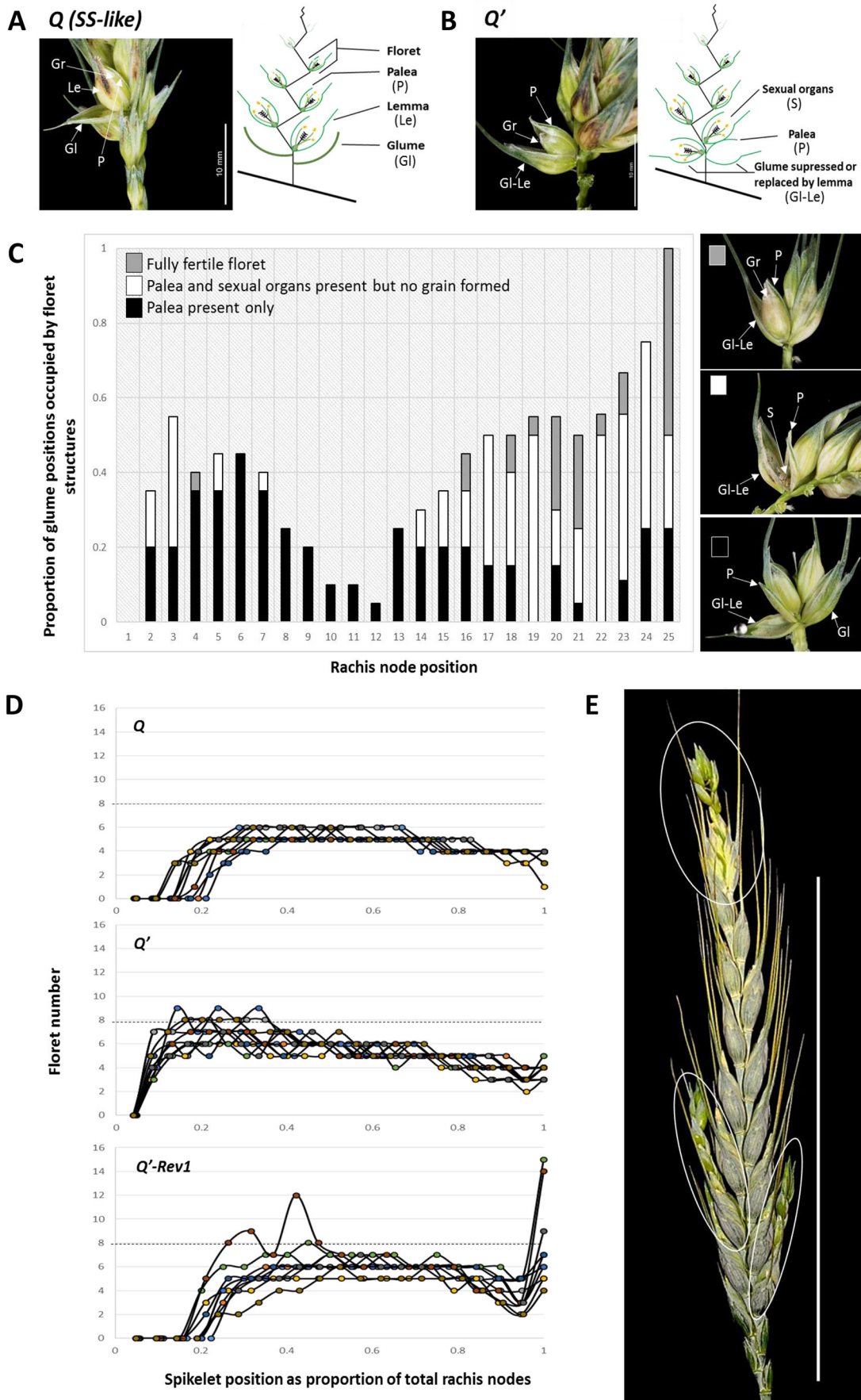


Fig. 4. See next page for legend.

**Fig. 4. Detailed phenotyping of Q, Q' and Q'-Rev1 plants reveals inflorescence architecture defects.** (A) SS-like (Q) spikelet image and schematic showing typical wheat spikelet structure in which no ectopic florets form. (B) Q' spikelet, by contrast, shows a severe ectopic floret phenotype in which complete florets replace glumes. Scale bars: 10 mm in A,B. (C) Q' plants exhibit complete ectopic florets or floret-like structures in place of glumes, most frequently in the base and apical portions of the spike. Data are presented as the proportion of ectopic florets at each rachis node position across ten plants, where the total number of possible glumes is 2 per rachis node per plant. P, palea; Le, lemma; Gl, glume; Gr, grain; Gl-Le, glume-lemma; S, sexual organs. (D) Floret numbers of sequential spikelets from the base (left) to top (right) of spikes, where total spike length is set at 1 for Q, Q' and Q'-Rev1 plants. Data are presented for each of ten individuals per genotype. Plot points mark individual spikelets (terminal spikelet at distance =1), with colors indicating separate spikes scored. Dotted line is for ease of comparison between genotypes. (E) Q'-Rev1 spike illustrating elongated spikelets (sham ramification) containing many florets (circled). Scale bar: 10 cm.

internode of lines containing the Q domestication allele were 10 mm in length. A single peduncle internode was harvested for each biological replicate. Details of sample preparation and the qPCR protocol, including primers, are provided in the supplementary Materials and Methods and Table S1.

### Modified 5' RACE

For 5' RACE, mRNA was purified from the same inflorescence RNA samples as used for the initial qRT-PCR analysis of Q in SS-like and Q' plants (Fig. 2). A GeneRacer Kit (Invitrogen) was used, except the de-capping protocol was not carried out, and the adapter was ligated directly to mRNA. Amplification of cleaved and ligated Q transcript was performed using gene-specific and GeneRacer adapter-specific primers (Table S1). Amplicon of the expected size was ligated into pGEM<sup>®</sup>-T Easy (Promega) before transformation, selection and sequencing of individual clones to determine cleavage location and frequency. See the supplementary Materials and Methods for more details.

### Scanning electron microscopy (SEM)

Developing inflorescence samples were prepared for SEM with a Zeiss Evo LS15 scanning electron microscope as described in the supplementary Materials and Methods.

### Sequence information

The miR172 sequence shown in this study is that of Ta-miR172a obtained from Yao et al. (2007). Although other isoforms have been reported and might contribute to regulation of Q, Ta-miR172a was used as a reference sequence for alignment purposes. Q sequence is available through GenBank accession AY702956.1.

### Statistical analysis

Two-tailed Student's *t*-test was employed to compare means. Sample sizes (*n*) and *P*-values are given in figure legends.

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

Conceptualization: J.R.G., E.J.F., B.T., S.M.S.; Methodology: J.R.G., E.J.F., B.T., S.M.S.; Validation: J.R.G., E.J.F.; Formal analysis: J.R.G., E.J.F.; Investigation: J.R.G., E.J.F.; Resources: J.R.G., N.W., B.T., S.M.S.; Writing - original draft: J.R.G., E.J.F., B.T., S.M.S.; Writing - review & editing: J.R.G., E.J.F., S.M.S.; Visualization: J.R.G.; Supervision: B.T., S.M.S.; Project administration: B.T., S.M.S.; Funding acquisition: S.M.S.

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### Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/doi/10.1242/dev.146407.supplemental>

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