Interactions between two QTLs for time to anthesis on spike development and fertility in wheat

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Earliness per se (Eps) genes are reported to be important in fine-tuning flowering time in wheat independently of photoperiod (Ppd) and vernalisation (Vrn). Unlike Ppd and Vrn genes, Eps have relatively small effects and their physiological effect along with chromosomal position are not well defined. We evaluated eight lines derived from crossing two vernalisation insensitive lines, Paragon and Baj (late and early flowering respectively), to study the detailed effects of two newly identified QTLs, Eps-7D and Eps-2B and their interactions under field conditions. The effect of both QTLs was minor and was affected by the allelic status of the other. While the magnitude of effect of these QTLs on anthesis was similar, they are associated with very different profiles of pre-anthesis development which also depends on their interaction. Eps-7D affected both duration before and after terminal spikelet while not affecting final leaf number (FLN) so Eps-7D-early had a faster rate of leaf appearance. Eps-2B acted more specifically in the early reproductive phase and slightly altered FLN without affecting the leaf appearance rate. Both QTLs affected the spike fertility by altering the rate of floret development and mortality. The effect of Eps-2B was very small but consistent in that -late allele tended to produce more fertile florets.

Wheat adaptation has been possible by coarse tuning time to anthesis12. Moreover, fine tuning time to anthesis is relevant to improve adaptation and performance within a specific environment13. The possibility of coarse and fine tuning of time to anthesis in wheat (and other winter cereals) has helped to maximise yield because of optimised anthesis time12,13; and will be instrumental in adapting better to climate change14,15.

Wheat development from sowing to anthesis encompasses various stages that make up two major component phases: time from sowing to terminal spikelet (TS), combining the vegetative and early reproductive phases of leaf and spikelet initiation, and time from TS to anthesis, i.e. the late reproductive phase (LRP) of floret initiation and survival10. The duration of each of these phases plays a key role in determining the generation and degeneration of various organs (shoots, spikes, spikelets, florets and grains) that define components of yield16,17. It has been hypothesised that developmental phases prior to anthesis can be adjusted with little or no changes in time to anthesis to keep improving adaptation along with stable or improved yield18,19.

Wheat responds to various environmental stimuli like vernalisation and photoperiod and genetic factors responsible for these sensitivities are mainly Vrn and Ppd genes, respectively20. Responses to photoperiod and vernalisation sensitivities can be found in all the three (vegetative, early and later reproductive) phases of wheat development10,11,15,16. The effects of different Ppd and Vrn genes on wheat development have been well studied. There have been detailed studies on the possibilities of various combinations of these genes under different environments to develop a tailored cultivar to achieve desired time to anthesis with different combinations of pre-anthesis phases (e.g. for Vrnp17,18, for Ppd19,20; for combination of Ppd with Vrn or Eps or both21-23). In addition, cultivars carry residual differences in phenology after the photoperiod and vernalisation requirements have been completely satisfied, ascribed as earliness per se (Eps). In the past, the process of selecting for Ppd and Vrn genes has fixed whole breeding pools for a particular combination of photoperiod sensitivity and growth habit but Eps genes continue to segregate24. Having much smaller effect than Ppd and Vrn, Eps genes are ideal for fine-tuning wheat development21. The direct effect of Eps is known to be on heading or anthesis time, although few studies25,27 (Eps-A′′′ 1-l of T. monococcum) have shown effects of Eps on the development of spikelets. Therefore, it can be speculated that the indirect effect of Eps on yield could be the reason for their indirect selection in the...
in the present study only the Eps allele was dependent upon the allelic status of the other. For that purpose, we evaluated the effect of Eps-7D on contrasting Eps-2B backgrounds and vice-versa on (1) phenology, not only determining time to anthesis but also quantifying whether they mainly affect development before or after TS; (2) rate of leaf appearance; (3) dynamics of leaf, spikelet and floret primordia development; and (4) spike fertility.

Results

Heading date QTL. Three heading date QTL were identified in the Paragon × Baj RIL population with genomic locations on chromosomes 2B, 2D and 7D (see Table 1 and Supplementary Fig. S1). In all cases early alleles were carried by Baj. The 2D QTL corresponds to the location of Ppd-D1 and Baj carries the common Ppd-D1a allele typical of most CIMMYT varieties. The 2B QTL is not Ppd-B1. On 7D we identified a major QTL with similar additive effect to Ppd-D1 (under UK conditions). Although specific tests of the genotypic differences in time to heading under saturating long days were not made, we assumed in principle that these genetic factors identified in chromosomes 2B and 7D are “earliness per se” (Eps) QTLs, simply based on the fact that they are definitively not Ppd genes and still produced significant differences in time to heading in a spring wheat background. With the aim to analyse the effect and possible interaction of the two Eps QTLs in detail, eight RILs were selected from the population, carrying all four possible combinations of alleles of the two genes (Table 2). The following analysis was conducted on these eight RILs.

Phenology. Despite that these two genes had been characterised as Eps factors modifying time to anthesis, in the present study only the Eps-7D affected this trait consistently, with a large direct effect (Table 2). The direct effect of Eps-2B was not significant; but there was interaction between the two loci (Table 3). This means that the magnitude of the effect on time to anthesis of these Eps was dependent upon the allelic status of the other Eps. Thus, although duration from sowing to anthesis was affected by Eps-7D always (i.e. in both seasons and with both Eps-2B alleles), the magnitude of effect was different depending on the Eps-2B allele in the background (Fig. 1a,b). The presence of the Eps-7D-early allele shortened the time to anthesis compared with Eps-7D-late by c. 90 °C day consistently across the two growing seasons when the background had the late allele of Eps-2B (Fig. 1a). Whereas when the background had the Eps-2B-early allele, the presence of the Eps-7D-early advanced anthesis only c. 45 °C day in comparison with the Eps-7D-late, the difference was in general smaller yet significant in both seasons (Fig. 1b). Regarding the Eps-2B the difference in time to anthesis between lines having the early and late allele was only significant in the first season if the Eps-7D in the background was the late allele (in the second season the difference was in the same direction but only a trend; Fig. 1c), with no difference at all between the lines with contrasting Eps-2B alleles when the Eps-7D-early was in the background (Fig. 1d).

When analysing the effect of Eps-7D on particular pre-anthesis phases (before and after TS) the results again provided evidence for an interaction with the allelic form of Eps-2B gene in the background (Fig. 2). The effect of Eps-7D strongly and consistently affected the duration of the period until TS (c. 60 °C day averaging across the seasons; p < 0.001) and only marginally affected the LRP in the second season when the Eps-2B gene in the background was the late allele (Fig. 2a,b). On the other hand, not only the overall effect of Eps-7D was smaller present cultivars. Current cultivars may have a reasonable variation of Eps alleles, providing opportunities to study these alleles in order to improve our understanding on their effects on phenology as well as on yield.

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There have been fewer studies on effects of Eps genes on time to anthesis, and even less on whether and how the rate of organ initiation is affected. These are areas that demand attention as Eps genes are quite numerous (virtually across the whole genome of wheat), and each potentially having a different mode of action (beyond that all Eps produce relatively minor changes in time to anthesis). Dissecting the effects of Eps into vegetative or/and reproductive phases could be crucial when considering likely effects on yield, more so on how development of organs are altered. The reports on effects of Eps have been inconsistent so far as some studies have reported effects limited to earlier phases and others to that of LRP. These studies have shown context dependent expression of traits to the extent that the same allele has differential responses based on the background it is introgressed into and epistatic interaction with respect to Ppd and Vrn genetic factors along with a different magnitude of interaction with the growing temperature. Furthermore, as different Eps genes may affect development differently, it may also be possible that they might interact in determining the effects on developmental traits. To the best of our knowledge there have been studies comparing in the same experiments different Eps genes, but the interaction between different Eps genes has never been analysed (i.e. whether the effect of a particular Eps gene is affected by presence of another Eps), which is important as many Eps genes are acting simultaneously in any genotype.

In the present study we aimed to analyse for the first time how different developmental traits as affected by two newly identified Eps QTLs on chromosomes 7D and 2B and also their interactions (i.e. to what degree the effects of each of them depend upon the allelic form of the other). For that purpose, we evaluated the effect of Eps-7D on contrasting Eps-2B backgrounds and vice-versa on (1) phenology, not only determining time to anthesis but also quantifying whether they mainly affect development before or after TS; (2) rate of leaf appearance; (3) dynamics of leaf, spikelet and floret primordia development; and (4) spike fertility.

| chr | LOD | %var | Add eff | Peak marker | Start marker | End marker | Trait | Increasing allele |
|-----|-----|------|--------|------------|-------------|------------|-------|-----------------|
| 2B  | 4.3 | 6.0  | ~ 2.12 | AX-94940971| AX-94408000 | AX-94940971| Heading | Baj             |
| 2D  | 16.4| 27.0 | ~ 3.87 | AX-9606120 | AX-95217264 | AX-95124335| Heading | Baj             |
| 7D  | 10.7| 16.2 | ~ 3.25 | AX-94545759| AX-94935560 | AX-94523269| Heading | Baj             |

Table 1. Summary of the QTL result for heading date in the UK environment. chr chromosome, %var percentage variance explained by the QTL, start and end marker border the QTL confidence interval.
when in the background the Eps-2B was the early allele (Fig. 1c) but also it was mainly due to its effect on the LRP (c. 44 °C d averaging across the seasons; p < 0.001) with no changes in time to TS at all (Fig. 2e,f). The Eps-2B gene only affected the measured phenology traits when the Eps-7D in the background was the late allele (Fig. 2c,d), with non-significant differences when the Eps-7D-early allele was in the background (Fig. 2g,h). In that case and consistently across both seasons, the effect was seen in early development up to TS (c. 52 °C day averaging across the seasons; p = 0.08) with no clear effects on the duration of LRP (Fig. 2c,d).

### Table 2

| Line                   | Eps allele (source) | Contrasts                  |
|------------------------|---------------------|----------------------------|
| Paragon x Baj 19       | late (Paragon)      | Eps-7D-late + Eps-2B-late  |
| Paragon x Baj 34       | late (Paragon)      | Eps-7D-late + Eps-2B-early |
| Paragon x Baj 93       | late (Paragon)      | Eps-7D-late + Eps-2B-early |
| Paragon x Baj 107      | late (Paragon)      | Eps-7D-late + Eps-2B-early |
| Paragon x Baj 132      | late (Paragon)      | Eps-7D-late + Eps-2B-early |
| Paragon x Baj 48       | early (Baj)         | Eps-7D-early + Eps-2B-late |
| Paragon x Baj 104      | early (Baj)         | Eps-7D-early + Eps-2B-early |
| Paragon x Baj 147      | early (Baj)         | Eps-7D-early + Eps-2B-early |

**Table 2.** Lines selected for this study, possessing contrasting alleles of both Eps genes (Eps-7D and Eps-2B). For comparing the effects of these alleles we averaged the results across lines possessing the same alleles of these two genes (see scheme for contrasts on the right and footnote). That offered four contrasting combinations of the two alleles (late or early) of the two Eps genes; and depending on the particular comparisons made the direct and interactive effects of these genes were studied. 1 and 2: direct effects of Eps-7D and Eps-2B genes, respectively. 3 and 4: effects of Eps-7D gene when the background has the late and early allele of Eps-2B gene, respectively. 5 and 6: effects of Eps-2B gene when the background has the late and early allele of Eps-7D gene, respectively.

### Table 3

| Source of variation | Degrees of freedom | Mean squares (°C day) | F-ratio | Significance (P-value) |
|---------------------|--------------------|-----------------------|---------|------------------------|
| Season              | 1                  | 422.73                | 1.71    | 0.212                  |
| Eps-7D              | 1                  | 28,698.98             | 116.38  | < 0.001                |
| Eps-2B              | 1                  | 478.60                | 1.94    | 0.185                  |
| Eps-7D × Eps-2B     | 1                  | 2721.61               | 11.04   | 0.005                  |
| Season × Eps-7D     | 1                  | 88.65                 | 0.36    | 0.558                  |
| Season × Eps-2B     | 1                  | 489.38                | 1.98    | 0.181                  |
| Season × Eps-7D × Eps-2B | 1                 | 614.34               | 2.49    | 0.137                  |
| Blocks              | 2                  | 858.61                | 3.48    | 0.059                  |
| Error               | 14                 | 246.61                |         |                        |

**Table 3.** ANOVA for time to anthesis. Factors that significantly affected this trait are in bold.

**Dynamics of leaf appearance and final leaf number.** There was no significant effect of the Eps-7D gene on the final leaf number (FLN), lines with the Eps-7D-late and -early alleles produced 9.55 and 9.57 leaves (averaged across the two Eps-2B alleles in the background and seasons), respectively (Fig. 3a,b,e,f). On the contrary, presence of the allele Eps-2B-early tended to reduce the FLN (Fig. 3c,d,g,h). But this effect exhibited an interaction with the allelic form of the Eps-7D gene in the background. Lines with Eps-2B-early showed c. 0.7
leaves less than Eps-2B-late lines (averaged across seasons) when the background was the Eps-7D-late gene (Fig. 3c,g), while the difference was less clear (c. 0.4 leaves), and statistically not significant, when in the background the Eps-7D gene had the early allele (Fig. 3d,h).

Although in all cases the relationship between the number of appeared leaves and thermal time had a strong linear component (Fig. 3), the relationships were actually bi-linear with early leaves appearing significantly faster (and having therefore a shorter phyllochron) than later leaves (Table 4). Opposite to the differences between Eps genes on FLN, the Eps-7D gene affected phyllochron (in general both that of the early and late leaves and regardless of the Eps-2B allele in the background) and in line with the effects of this gene on time to anthesis the effect was smaller and less clear in the second growing season (Table 4, top half). The effects of Eps-2B on phyllochron was not clear, as differences between lines with the Eps-2B-early and Eps-2B-late were small and inconsistent (Table 4, bottom half).

**Dynamics of leaf and spikelet primordia development.** The dynamics of leaf and spikelet primordia initiation showed that, in general, both Eps-7D and Eps-2B effects increased the number of primordia initiated when lines carrying the late allele were compared with those with the early allele, particularly when the other Eps gene in the background was the late allele (Fig. 4). As differences in FLN were small or negligible (see above), these differences in number of primordia reflected those in spikelets initiated per spike. Overall, none of the two Eps genes affected the rate of primordia initiation in any of the two cropping seasons (Fig. 4). Thus, the differences in the total number of primordia initiated between lines carrying the late and early Eps alleles were mostly due to the differences in duration of primordia initiation (Fig. 4).
Figure 2. Duration of the two pre-anthesis phases considered: time from sowing to terminal spikelet (TS) and from then to anthesis, the late reproductive phase as affected by Eps-7D (left panels) and Eps-2B genes (right panels) on backgrounds contrasting in the allelic form of the other Eps gene (top and bottom panels for the late and early alleles of the other Eps gene). Data are shown for each of the two cropping seasons (CS1 and CS2). Segments in each bar stand for the SEM. Significance level shown for the differences between alleles within each season: *p < 0.05; **p < 0.01; NS non-significant.

Figure 3. Relationship between number of leaves appeared on the main shoot and thermal time from sowing as affected by Eps-7D (left panels: a,b,e,f) and Eps-2B genes (right panels: c,d,g,h) on backgrounds contrasting in the allelic form of the other Eps gene (left and right panels within each Eps gene) in cropping seasons 1 (top panels: a–d) and 2 (bottom panels: e–h). Inside each of the panels are the final leaf number (FLN) with their SEM.
Table 4. Rates of leaf appearance (leaves [100 °C day]⁻¹; ± SE) corresponding to the early- and late-appearing leaves (RLA-I and RLA-II, respectively) as affected by Eps-7D and Eps-2B genes (top and bottom parts of the table, respectively) on backgrounds contrasting in the allelic form of the other Eps gene in the two cropping seasons (CS1 and CS2), and coefficients of determination of the segmented linear regression (in all cases P < 0.001). The corresponding phyllochron values (°C d leaf⁻¹) are included between square brackets.

| Eps-7D effect | Eps-7D-early | Eps-7D-late |
|---------------|--------------|-------------|
| Eps-7D-early  | 1.12 ± 0.02 [89.3] | 0.89 ± 0.04 [112.4] | 0.997 |
| Eps-7D-late   | 0.81 ± 0.03 [122.6] | 0.69 ± 0.04 [142.9] | 0.997 |

| Eps-7D effect | Eps-7D-early | Eps-7D-late |
|---------------|--------------|-------------|
| Eps-7D-early  | 0.95 ± 0.01 [105.6] | 0.88 ± 0.03 [114.2] | 0.998 |
| Eps-7D-late   | 0.99 ± 0.01 [100.4] | 0.81 ± 0.03 [122.6] | 0.998 |

| Eps-2B effect | Eps-2B-early | Eps-2B-late |
|---------------|--------------|-------------|
| Eps-2B-early  | 1.22 ± 0.02 [89.3] | 0.89 ± 0.04 [112.4] | 0.997 |
| Eps-2B-late   | 0.81 ± 0.02 [122.6] | 0.69 ± 0.04 [142.9] | 0.997 |

| Eps-2B effect | Eps-2B-early | Eps-2B-late |
|---------------|--------------|-------------|
| Eps-2B-early  | 0.95 ± 0.01 [105.6] | 0.88 ± 0.03 [114.2] | 0.998 |
| Eps-2B-late   | 0.99 ± 0.01 [100.4] | 0.81 ± 0.03 [122.6] | 0.998 |

Figure 4. Relationship between number of primordia initiated on the main shoot apex and thermal time from sowing as affected by Eps-7D (left panels: a, b, c, d) and Eps-2B genes (right panels: e, f, g, h) on backgrounds contrasting in the allelic form of the other Eps gene (left and right panels within each gene) in cropping seasons 1 (top panels: a–d) and 2 (bottom panels: e–h). Inside each of the panels are the total number of primordia with their SEM.

Dynamics of floret development. Detailed study of developmental dynamics of each individual floret initiated in 3 spikelet positions of the spike (apical, central and basal spikelets) was carried out to understand the basis of Eps-7D and Eps-2B spike fertility effects. In order to focus on aspects of floret dynamics which directly influences grain number we only presented the dynamics of florets that reached at least the stage W4.5 (carpel primordia visible); i.e. up to the fifth floret from the rachis. Outside of this range seeds were hardly ever produced. At the other end of the floret survival scale the two florets most proximal to the rachis (F1 and F2) were very similar and the likelihood of survival was always 100% for these florets, hence we focused here on the developmental progress of the F2, F3, F4 and F5 florets to reflect any effects of Eps genes on floret fertility.

For the same reason, as results were consistent across growing seasons, we only included data for the first season in the main text and the equivalent figures for the second season are presented as supplementary figures. Overall the effect of Eps-7D on development of florets showed evidence of its interaction with Eps-2B, and this...
interaction effect was also seen when comparing the differences between lines with Eps-7D-early and late which depended on the early or late allelic backgrounds of Eps-2B. When the background had the Eps-2B-late development of individual floret primordia was in general faster in lines with the Eps-7D-early allele and this resulted in a different likelihood of some labile florets to become fertile at anthesis (Fig. 5 and Supplementary Fig. S2). There was no difference in final fertility of the F2 floret among any of the lines in any of the three spikelet positions in both the seasons: F2 always reached the fertile floret stage (Fig. 5a,e,i and Supplementary Fig. S2a,e,i). However, even when not producing a difference in fertility for this particular proximal floret, the rate of development seemed consistently faster for this F2 for lines with Eps-7D-early allele (Fig. 5a,e,i and Supplementary Fig. S2a,e,i). The remaining distal florets (F3, F4 and F5) also showed the same trend of faster rate of floret development in lines carrying Eps-7D-early than those carrying Eps-7D-late, affecting the likelihood of these florets becoming fertile depending on the particular floret and spikelet positions. Despite the different rates of floret development, F3 reached similar final levels of fertility in central spikelets in lines with any of the alleles of Eps-7D; but in the basal and apical spikelets the lower rate of floret development in lines with Eps-7D-late allele determined that F3 was fertile in only c. 33% of the plants measured whilst it was fertile in almost all plants of the lines with the Eps-7D-early allele (Fig. 5b,j and Supplementary Fig. S2b,j). When considering F4, these lines differed in the time of initiation along with rate of development and plants, with Eps-7D-early had earlier initiation and faster rate of development of F4 than those with Eps-7D-late. When the Eps-7D was the early type c. 60% of F4 reached W10 in apical and basal position, while they were never fertile in lines with the Eps-7D-late allele (Fig. 5c,k and Supplementary Fig. S2c,k). Floret F5 reached W4.5 in almost all the lines but was fertile only in the central spikelet of most, but not all, plants with the Eps-7D-early (Fig. 5h and Supplementary Fig. S2h). When we compared the rates of developments and fate of floret primordia of lines with the Eps-7D-early and -late alleles but with the Eps-2B-early allele in the background, there were no noticeable differences in any of the spikelets and floret positions (Fig. 6 and Supplementary Fig. S3). That is, the improved rates of development in the lines with the Eps-7D-early allele evidenced consistently across floret and spikelet positions and over the two growing seasons when the background was the Eps-2B-late were not evident anymore. Regarding the effect of Eps-2B
on development of florets, the effects were more clear when in the background the Eps-7D was the early allele and generally subtler than those of Eps-7D (Figs. 7, 8 and Supplementary Figs. S4, S5). When the background had the Eps-7D-late development of individual floret primordia was rather similar in all lines regardless of the Eps-2B allele (Supplementary Fig. S4). On the other hand, when the Eps-7D in the background was the early allele, there were no clear differences in rates of development for most proximal florets but for the labile floret primordia (i.e. those reaching W10 or dying depending on the conditions) lines with the Eps-2B-late allele had maintained a faster rate of development than in lines with the Eps-2B-early allele, allowing these labile florets reaching the W10 stage in the former, (as well as attaining higher floret score in florets not being fertile in any of the lines; Fig. 8 and Supplementary Fig. S5).

Floret primordia initiation and death. Detailed analysis was carried out to calculate the living floret primordia (again considering only floret primordia that reached a score ≥ W4.5, and therefore the maximum number of primordia was “only” 6–7). The patterns of floret initiation and mortality for each genotype were relatively similar for the three different spikelet positions considered (Supplementary Figs. S6 and S7) and therefore we reported here an average of these three spikelets (Fig. 9). The largest effect was that produced by the Eps-7D gene when in the background the allele of the other Eps gene was Eps-2B-late. In this case the lines carrying the Eps-7D-early produced more floret primordia (reaching W4.5 or higher; if all florets visible microscopically were taken into account there would be no difference in maximum number of floret primordia) and increased the rate of floret survival, both contributing to an improved number of fertile florets compared with line carrying the Eps-7D-late (Fig. 9a). These Eps-7D-early lines initiated floret development earlier and therefore the duration of the process of floret initiation was not reduced (Fig. 9a), although the duration of the LRP tended to be shorter (Fig. 2b). When the Eps-2B in the background was the early allele the Eps-7D showed no clear effect on the dynamics of floret initiation-mortality (Fig. 9b). Considering the effect of Eps-2B, there was no clear trends in the dynamics of floret initiation/mortality when the Eps-7D in the background was the late allele (Fig. 9c). However, when the background had the Eps-7D-early the Eps-2B-late slightly increased spike fertility with respect to

Figure 6. Dynamics of floret development (floret score) in F2, F3, F4 and F5 florets at apical (top panels: a–d), central (middle panels: e–h) and basal (bottom panels: i–l) positions of the spike with thermal time from sowing in lines with Eps-7D-late (open symbol) and early (closed symbol) allele with Eps-2B-early allele in the background in the first cropping season.
**Eps-2B**-early mainly through improving floret primordia survival, as the maximum number of floret primordia initiated (and reaching at least W4.5) was similar (Fig. 9d).

**Fertile florets at anthesis.** There was a clear interaction between Eps-7D and Eps-2B on the number of fertile florets per spike. Lines carrying the Eps-7D-early allele had higher spike fertility than those with the Eps-7D-late alleles when the other gene in the background was Eps-2B-late allele (Fig. 10a,e), but not when the Eps-7D-late allele was in the background (Fig. 10b,f). Reflecting the interaction between the two Eps genes, the Eps-2B also affected significantly spike fertility when the Eps-7D in the background was the early type (Fig. 10d,h), and the magnitude of the difference was reduced when the Eps-7D-late was in the background (Fig. 10c,g). Differences in fertile florets per spike between lines with contrasting forms of the Eps-7D were concentrated in central spikelets of the spike and the advantage was substantial enough to override the fact that the lines with Eps-7D-late produced more spikelets per spike (Fig. 10a,e). On the other hand, the effect of Eps-2B on fertile florets was subtle and not significant yet very consistent compared to that of Eps-7D, and differences in floret fertility were relatively minor but consistent across most spikelets (Fig. 10d,h).

**Discussion**

The two newly identified Eps QTLs, Eps-7D and 2B affected the whole cycle from sowing to anthesis but the effects were influenced by the allele of the other Eps in the background. The overall effect of these two Eps genes was relatively small, although Eps-7D was stronger than Eps-2B. The effect of Eps-7D on time to anthesis was as strong as it has been reported before for other Eps genes (e.g. Refs. 21,24,26,27,35,37,38) but effect of Eps-2B was relatively very small again such smaller effects were also noted for other Eps35. Therefore, either of them, but particularly the Eps-7D due to its more consistent effect, might be exploited to fine-tune time to anthesis in elite germplasm.

It is true that the changes in the whole phase from sowing to anthesis can be brought about by various combination of changes in its component pre-anthesis phases (with more or less independency) and its importance...
lies in the fact that each of these phases determine initiation and survival of various organs that may end up affecting yield components\(^3\). Eps-7D affected both time to TS and LRP while the subtle effect of Eps-2B on whole phase was only evident in time to TS\(^2\). Our study provides the first time proof for the interaction between two Eps in particular which was only speculated before\(^3\). Our observations in addition to that of other researcher\(^3\) suggest that different Eps genes may differ in their effects on individual developmental phases, even if their effect on the overall time to anthesis were similar.

It was important to note that the delay due to late allele of one Eps was enhanced in presence of the late allele of the other Eps (the line carrying late allele at both Eps-7D and 2B had the longest duration from sowing to anthesis), pointing towards probable additive effects for the duration of developmental phases. And discrepancies about the effect of same Eps in the literature (under different temperatures or background) also suggests the epistatic interaction of Eps with other unknown flowering genes and interaction with temperature. Therefore, it cannot be overemphasized how important it is to study individual Eps from a particular genetic background with the known flowering gene complex (other major and minor flowering genes involved) acting in the background in order to decode the functions of Eps (certainly beyond time to anthesis) for exploiting them in breeding. As Eps, unlike Ppd and Vrn, are present in almost all of the chromosomes, their abundance provide an enormous opportunity to work with, although as most are likely to be minor genes with a probable additive effect, their abundance also poses difficulty in clear understanding of their function (and pathway).

The QTLs also differed in their effect on FLN, while Eps-7D did not alter the FLN Eps-2B-early had significantly (though naturally only slightly) lower FLN than the respective late allele when the background was Eps-7D-late. Effect of Eps-2B on FLN support the study conducted by Hoogendoorn (1985)\(^4\) observing variations in FLN in their vernalized photoperiod insensitive lines. This differential effect of Eps-7D and Eps-2B on FLN when having similar effects on the duration of early phases of development shows another differential mechanisms of action of these genes in early developmental traits when seen in more detail. While both affected similarly the rate of development of the early phases (and might be presumed acted in that phase similarly), it seemed that
**Figure 9.** Number of living floret primordia per spikelet (average across apical, central and basal spikelets) and thermal time from sowing as affected by *Eps-7D* (left panels: a,b,e,f) and *Eps-2B* genes (right panels: c,d,g,h) on backgrounds contrasting in the allelic form of the other *Eps* gene (left and right panels within each *Eps* gene) in first (top panels: a–d) and second (bottom panels: e–h) cropping seasons. *Eps-7D*-late and -early (open and closed symbols, respectively) and *Eps-2B*-late and -early allele (triangle and circles, respectively). Bars are the standard errors among spikelet position.

**Figure 10.** Mapping of fertile florets at anthesis per each spikelet in the spike as affected by *Eps-7D* (left panels: a,b,e,f) and *Eps-2B* genes (right panels: c,d,g,h) on backgrounds contrasting in the allelic form of the other *Eps* gene (left and right panels within each *Eps* gene) in first (top panels: a–d) and second (bottom panels: e–h) cropping seasons. Inside each of the panels are the fertile florets per spike with SEMs.
the Eps-7D affected the rate of leaf initiation in parallel. Consequently, the lines with the Eps-7D-early allele had the same FLN as the lines with Eps-7D-late did; while Eps-2B seemed to have not affected the rate of leaf initiation and therefore the effects on duration of an early developmental phase is reflected on FLN. Furthermore, the Eps-7D gene affected the rate of leaf appearance as the main mechanism for changing time to anthesis, as also found by Ochagavia et al.35 for another Eps gene. These opposite effects on phyllochron, are commensurate with the fact that Eps-7D did also affect the duration of the LRP while Eps-2B restricted its effect to the earlier phases.

Further, both Eps-7D and 2B affected the number of primordia initiated and the differences in the primordia was mainly contributed from spikelet primordia as the differences in FLN between late and early alleles of both the QTLs were rather small. And the differences in the primordia were mainly due to the differences in the duration of primordia initiation as there was no alteration in the plastochron. Such marginal effect of Eps on spike number per spike mainly due to the Eps effect on phenophases without affecting the rate of primordia initiated have been observed in other studies involving "major"37 and "minor"35,36,40 Eps genes. Among the few studies reporting Eps effects beyond time to anthesis the studies by Lewis et al. (2008)37 in diploid wheat (under field condition) and Alvarez et al. (2016)25 in tetraploid wheat (under controlled conditions) were exceptions showing the effect of Eps-Am 1 bringing about larger changes in spikelet number per spike. This exception was justified by the unusual larger effect of that Eps on time to anthesis. Like other studies in the literature have reported, Eps QTLs bringing about significant (moderate to major) variation in developmental patterns have only brought about subtle variations in the components related to yield (spike number42–45, spike fertility25). Further, our results indicate that both the Eps QTLs alter developmental phase resulting in modification of anthesis time have contrasting pattern of their function on the dynamics of organs initiated.

Eps-7D and Eps-2B had a contrasting effect on spike fertility presented as floret development and fertile floret per spike at anthesis, again the effect of both Eps on spike fertility was influenced by the presence of the early or late allele of the early Eps in the background. Unexpectedly, lines with the Eps-7D-early allele had a higher spike fertility compared to late allele despite the shorter LRP, at least when the background allele for Eps-2B was late. A general hypothesis is having a longer LRP would improve spike fertility which has a straight-forward explanation that longer duration allows distal florets to become fertile along with positive effect from improved spike dry weight (SDW)13. However, this is true only when the genetics involved in altering the duration do not affect the rate of development and the differences in the duration were big enough to cause variations in SDW. In this particular case, the situation was different as the period of floret development was similar between lines with contrasting alleles of Eps-7D, despite their differences in the LRP. The Eps-7D-early had faster rate of development of each individual floret and similar duration of floral development (due to an early onset of floret development) and that determined the improved rate of survival of distal florets to become fertile. On the other hand, the Eps-7D-late delayed the initiation of distal florets which then developed at a slower rate, consequently diminishing the likelihood of survival of many of the labile florets primordia, reaching anthesis with less fertile distal florets, overriding the effect of bearing slightly higher spikelet number per spike on the overall number of fertile florets per spike. The effect of Eps-2B on spike fertility was in alignment with the general hypothesis mentioned above in that the Eps-2B-late allele improved spike fertility (when the allele of Eps-7D in the background was late) due to a slightly longer duration of floret development resulting in lower floret mortality as evidenced with other Eps genes25, and through extending the LRP manipulating the photoperiod34,42. Our results (when spike fertility was higher) further provide proof to the concept that variations in the floret fertility are mainly due to differences in the survivability (low mortality) of produced florets (due to variations in duration, rate of development or resource availability) rather than improved maximum number of florets initiated41–43.

As for the possible identity of genes underlying the two QTL described we are constrained by the genetic resolution offered by a population of 169 RILs. However, the QTL peak marker of the 7D effect is located at 58,868,234 bp (IWGSC RefSeq v1.0). TaFT-D1 (TraesCS7D02G1116001, Vrn-D3) is just 10 Mb distal to this marker at 68,416,466 and is an excellent candidate gene. In the spring wheat background described here, with saturating vernalisation, Vrn-D3 would exhibit the eps profile described here and elsewhere41. The 2B QTL peak marker, AX-94940971, is at 33,839,683 bp which is close to the predicted position of Ppd-B1 although this gene is not contained within the current 2B assembly of Chinese Spring. However, there is some evidence that this QTL is not a photoperiod insensitive allele of Ppd-B1. No QTL was identified at 2B when the same population was grown in Mexico (data not shown) although the 2D and 7D effects were just as strong. If the earliness effect described here was due to photoperiod insensitivity the additive effect and significance associated with this QTL would increase. It is also noticeable that the QTL effect is weaker than would be expected of Ppd-B1 and that there are two LOD peaks for this QTL leading us to suspect that the actual location is more proximal than Ppd-B1.

Materials and methods

Plant material, field conditions and management. A population of 169 recombinant inbred lines was developed to generation F4 from a cross of variety ‘Paragon’ and ‘Baj’ following usual procedures40,41. The population was grown in unreplicated 1 m² plots in Norwich, UK (Lat: 52°37’ N, 1°10’ E) in the 2015–2016 season (sowing date: 26 October 2015) under standard growing conditions and phenotyped for time of heading. Further, two more field experiments were carried out with eight RILs selected from the population (Table 2) in two consecutive cropping seasons, 2016–17 (CS1) and 2017–18 (CS2) under similar environmental conditions near Bell-lloc d’Urgell (Lat. 41°38’ N, 0°44’ E in CS1 and Lat. 41°37’ N, 0°47’ E in CS2), Lleida, North-East Spain. Both experiments were sown within optimum sowing dates (16 November 2016 and 17 November 2017). The seeds were sown at a density of 125 kg ha⁻¹ with the aim of attaining a uniform plant density of 250 plants per m². The experiments were maintained under stress-free conditions (i.e. weeds, insects, and diseases were controlled or prevented, and plots were irrigated and fertilized as required).
Weather data were collected daily from Meteocat (agro-meteorological network of Catalonia, en.meteocat. gencat.cat) from a station located near the experimental site. The average temperatures from sowing to maturity (growing period) were 12.6 and 11.8 °C (Fig. 11a) and accumulated precipitations for the whole growing period were 263.6 and 286.9 mm for CS1 and CS2, respectively (Fig. 11b). With respect to average temperature, the two growing seasons were slightly colder in the early phase (sowing to 2 leaf stage) but very similar during early and late reproductive phases until maturity when compared to the average of the last five years (2010–2016).

Treatments and experimental design. Treatments consisted of eight lines (Table 2) derived from the cross Paragon × Baj where Paragon and Baj carry late and early allele of both Eps-7D and Eps-2B, respectively. Although not NILs, lines mostly differed for these two Eps QTLs (7D and 2B) while having similar genetic composition (mixture of Paragon and Baj) for the other major genes influencing anthesis time. The heading date QTL identified in Paragon × Baj are shown in Table 1 and QTL plots in supplementary Fig. S1. RILs chosen for detailed study were fixed for Ppd-D1 and Rht-B1, which are both segregating in this population. Combining the different lines for their alleles of these two Eps genes, we end up with four contrasts to test the direct effects of these alleles as well as the interactions between them (i.e. to what degree the allelic form of one of these genes may condition, qualitatively or quantitatively, the effects of the other one).

Genotypes were arranged in a randomized complete block design with three blocks. Plot size was six rows (0.20 m apart) wide and 4 m long. Three plants in each plot (9 plants per treatment) were marked after the seedling emergence stage. These marked plants were visually representative of the whole plot as well as had uniform plant density around them and were randomly chosen from any of the 4 central rows. In addition, we marked 1 linear meter in each experimental unit which showed uniform emergence of seedlings and optimum plant-to-plant density to be sampled for measurements at anthesis.

Measurements and data analysis

Genetic mapping and QTL analysis. The Paragon × Baj RILs were genotyped using the 35 k Axiom Wheat Breeders’ array (Affymetrix46) and a genetic map was constructed using MSTmap online (http://mstmap.org/) with default setting and a grouping threshold of LOD = 10. Linkage groups were separated into different chromosomes manually and, as the length of linkage groups were inflated, the total length of the map was adjusted to 2100 cM. Full genotype files and genetic maps are given as supplementary material can be found at. Employing the genetic map, QTL detection was performed on heading date measurements from the UK trial, using package “qtl” (vs. 1.44–947) in two steps, the first scan determining co-factors and the second scan identifying robust QTL, taking the co-factors into account45.

Leaf number and dynamics of leaf appearance. The number of leaves emerging from the main shoot was recorded in the three marked plants once or twice a week depending on temperature. Measurements began from 1 leaf stage and continued until the flag leaf was fully emerged, using the scale described by Haun (1973)48. Leaf appearance dynamics were analyzed by regressing the number of leaves emerged against the calculated thermal time (using daily average temperature and assuming a base temperature of 0 °C) from sowing. Despite the fact that the linear regressions in all cases were highly significant, the distribution of residuals indicated that a bi-linear trend was required (to have a random distribution of residuals). Therefore, we fitted a segmented linear regression forcing a breakpoint in rate of leaf appearance at leaf 7, because it has been shown that (1) this change in phyllochron coincides with Haun stages between 6 and 812,49–51 and (2) using that threshold for the change in slope of the bi-linear regression produces excellent outputs in a wide range of environmental (e.g. Ref.14) and

Figure 11. Monthly minimum (Tmin), maximum (Tmax) for first (CS1) and second (CS2) cropping season and mean of the monthly average temperature (Tavg) of CS1 and CS2 as well as monthly Tavg of cropping seasons from the past 5 years (2010–2016, a); monthly accumulated precipitation and global radiation for CS1 and CS2 (b).
genotypic contexts (e.g. Refs. 51,53). Phyllochron is the time between the appearance of two successive leaves. It was calculated as the reciprocal of the rate of leaf appearance. In each case two values are generated, corresponding to the early (leaves 1–7, phyllochron I) and late appearing leaves (leaf 7–flag leaf, phyllochron II).

Shoot apex dissection and dynamics of primordia initiation. To determine the apical stage and number of primordia in the main shoot apex, one representative plant per experimental unit (three plants per treatment) was sampled randomly from the central rows. This was performed once or twice a week, depending on temperature, from the 2-leaf stage to terminal spikelet stage. The plants were taken to the laboratory for dissection under a binocular microscope (Leica MZ 8.0, Leica Microsystems, Heerbrugg, Switzerland). Apical stages were determined using the scale described by Kirby and Appleyard14. Time taken to reach apical stages including double ridge and terminal spikelet (TS) were recorded for all the lines in each block. Along with the apical stages, the number of leaf and spikelet primordia initiated at each sampling were counted.

After TS stage, one plant from each experimental unit was sampled two or three times a week, again depending on temperature, until anthesis. This was to count the number of florets initiated and determine the stage of development for each floret primordium following the scale developed by Waddington et al. (1983)35. Wheat exhibits asynchronous development and growth of spikelets within the spike and florets within the spikelets is different. For that reason, we dissected 3 spikelets per spike, one from apical (3rd or 4th from the top) and one from central and one from the basal (3rd or 4th from the bottom) position of the spike. The florets within each spikelet position were numbered from F1 to Fn according to their position with respect to rachis, where F1 being the floret that was most proximal to the rachis and Fn the most distal floret. Floret development was observed from an early floret primordia stage (< W3.5) to until W10 stage (stage when floret is already fertile) or highest stage attained by florets that was aborted. The time taken for the F1 to reach W10 stage from sowing was considered as the anthesis time and late reproductive phase (LRP) was calculated as period between TS and anthesis time.

Living floret primordia and fertile floret number. The number of living floret primordia was derived from the individual floret (F1 to Fn) development curve. The sum of all the florets that continued to develop was calculated for each sampling date, and then plotted against thermal time. This shows the maximum number of florets that reached W4.5 (stage when stamen, pistil and carpel primordia are present) were considered for calculating living floret primordia. While F1 and F2 florets invariably (in all genotypes and spikelet positions) reached W10, fate of F3 to F6 florets was depended on genotype, spikelet position and field conditions but these florets at least reached W4.5 in all cases. Florets F7–F10 were initiated in most cases, but did not reach the threshold of W4.5 stage in most spikelets.

Fertile floret mapping at anthesis. Three plants were randomly selected from the marked 1 m of uniform form in each experimental unit (9 plants per treatment) to “map” the number of fertile florets per spike, through counting them at each spikelet in the main-shoot spike (i.e. the number of fertile florets was mapped for every spikelet position from basal to terminal spikelet; S1 to Sn). Florets were considered fertile if they were at least at the green anther stage (> W8.5), considering the florets within and across spikelets have asynchronous development and that floret death is highly unlikely when florets have progressed in development to very close to the stage of fertile floret.

Analysis. The data of the variables studied here was subjected to analysis of variance using the statistical software JMP Pro Version 14.0 (SAS Institute Inc. Cary, NC, USA). The significance level for the differences between Eps allele were calculated using LSmMean contrast. The two-way interaction between two Eps QTLs and three-way interaction between Eps QTLs and season were analyzed using full factorial model in JMP Pro version 14.0.

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Author contributions
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Competing interests
The authors declare no competing interests.

Additional information
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