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Physiological determinants of fertile floret survival in wheat as affected by earliness *per se* genes under field conditions

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ABSTRACT

Variations in wheat yield are largely explained by changes in grain number per m^2 which is linked to the number of fertile florets at anthesis. This, in turn is the outcome of developmental processes which control floret initiation and mortality. Earliness '*per se*' (*Eps*) genes are involved in fine-tuning time to anthesis in wheat (and other cereals) but their effect on development prior to anthesis is less well studied. We aimed to determine effects of *Eps* genes on spike fertility, quantifying aspects of floret developmental which influence this trait. Field experiments were carried out to record floret primordia generation/degeneration dynamics in near isogenic lines (NILs) with contrasting *Eps* alleles (late flowering *vs* early flowering alleles; *Eps*-late and *Eps*-early, respectively) derived from the Avalon x Cadenza (AxC) cross with the *Eps* gene on either chromosome 1D or 3 A and from the Spark x Rialto (SxR) cross with the *Eps* gene on chromosome 1D. *Eps* NILs varied in spike fertility: *Eps*-late alleles increased fertility. Although the effect was in general slight, the magnitude was affected by the particular alleles and the cross used to produce the NILs. Differences in the number of fertile florets were explained by differences in the dynamics of floret development. NILs with *Eps*-late alleles improved the development of a small number of labile florets allowing them to complete their development to become fertile florets instead of dying, as in lines carrying early alleles. Thus, these alleles improved floret fertility mainly through reducing the rate of floret mortality with no influence on the dynamics of floret primordia initiation or in maximum number of floret primordia. Therefore, *Eps* genes could be exploited in wheat breeding not only to fine-tune time to anthesis but also to improve spike fertility.

1. Introduction

Increased wheat yields are an urgent priority for global food security (Reynolds et al., 2012). Yield is determined by the number of grains per unit land area and their average weight. Grain size is a minor source of variation for yield improvements (Slafer et al., 2014). It has been widely reported that wheat yield depends far more on the number than on the weight of the grains (Peltonen-Sainio et al., 2007; Sadras, 2007), and that grain number is the main factor limiting wheat yield potential (e.g. Fischer, 2011). Consequently, the improvement of agronomic performance of wheat (and other crops) the number of grains, of which spike fertility is a critical player, must be increased (Slafer et al., 2014). The number of grains is defined from sowing to anthesis with a critical period from the emergence of the penultimate leaf (20–30 days before anthesis) until *c.* 7–10 days post anthesis (Fischer, 1985; Slafer and Savin, 1991), while the average seed weight is defined from a few days before anthesis until maturity (Calderini et al., 2001; Ugarte et al., 2007). Understanding the mechanisms involved in defining the grain number would allow the identification of traits which might be critical for efforts to increase grain yield. Although some fertile florets may not set grains, grain set under field conditions usually exceeds 70% (Ferrante et al., 2013; Savin and Slafer, 1991), which is expected in a cleistogamous species. Therefore, grain number is largely defined by the number of fertile florets, which in turn depends on the dynamics of floret generation/degeneration. This process begins with floret primordia development but, as development proceeds, involves varying levels of floret mortality/survival during the late reproductive phase. Thus, studying genotypic differences in the dynamics of floret generation/degeneration could be critical for the further improvement of yield.

Earliness '*per se*' (*Eps*) genes are important for fine-tuning the adaptation of wheat (Herndl et al., 2008) and therefore are common in

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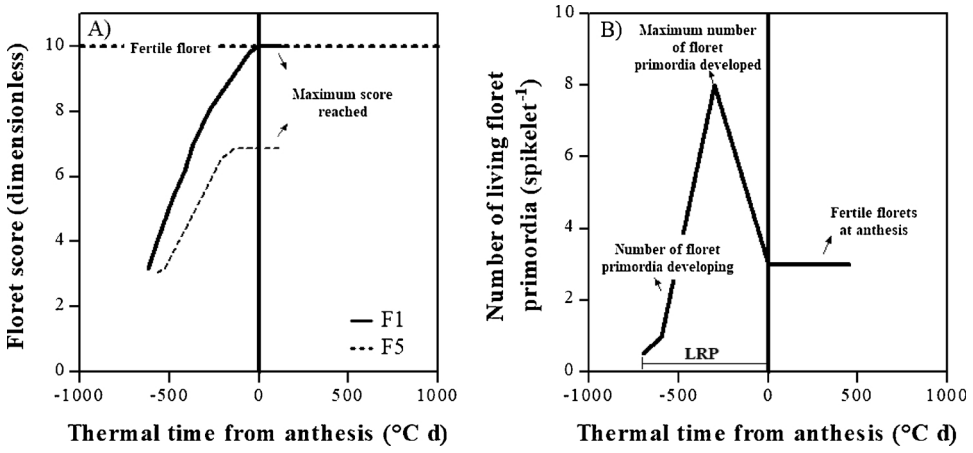

Fig. 1. Schematic diagrams of the dynamics of (A) floret development and (B) number of living florets through thermal time from anthesis (negative values represent the period before anthesis). In panel A we exemplified the expected dynamics of developmental progress for two contrasting floret positions, e.g. the floret most proximal to the rachis (i.e. F1) and the fifth floret (i.e. F5). In all cases these dynamics are strongly bi-linear with a growing phase in which the floret primordium develops and a plateau indicating the maximum score of development reached by that primordium (that can be a fertile floret if it reached 10, or the maximum stage it reached before starting its degeneration). Panel B shows the typical dynamics in which during the late reproductive phase (LRP) the number of living floret primordia increases until a maximum number is reached followed by a floret mortality phase and finishing with the final number of floret primordia that reached the fertile floret stage at anthesis.

germplasm adapted to different regions (e.g. Appendino et al., 2003; Worland et al., 1994). As the magnitude of their effects is frequently minor (Griffiths et al., 2009) it has been unusual to find studies reporting on their likely impact on number of organs developed. An exception is the earliness *per se* locus *Eps-A^{m}1*of diploid wheat *Triticum monococcum* L. (Valárik et al., 2006), with a rather large effect on phenology, that also affected spike fertility (Lewis et al., 2008). Although, to the best of our knowledge, this was never studied before, the effect on spike fertility might indicate an impact of these genes on the dynamics of floret development. If *Eps* genes in hexaploid wheat could affect these dynamics they could directly affect spike fertility, and breeding programs might exploit *Eps*-late alleles to increasing floret fertility, in addition to any use they may have for fine-tuning time to anthesis.

To the best of our knowledge there have been no studies reporting the effects of *Eps* genes in hexaploid wheat on the dynamics of floret development and in setting a particular level of the spike fertility. In the companion paper (Ochagavía et al., 2018) we reported the effects of *Eps* genes on the duration of different phases as well as on the dynamics of leaf and spikelet initiation. These *Eps* genes were those identified in Griffiths et al. (2009) and characterised for the effects on phenology by Zikhali et al. (2014) and Farré et al. (2016). In this paper, we studied their effect under field conditions on the dynamics of floret development. We also showed the consequences of these effects on spike fertility by quantifying changes in the number of fertile florets at anthesis.

2. Materials and methods

2.1. General description, treatments and design

Details of these experiments are available in the companion paper (Ochagavía et al., 2018). To recap briefly, fields experiments were carried out from 2012-13 to 2014-15 in Lleida, NE Spain. Sowing dates and rates were always within the optimum and the experiments were irrigated to avoid water stress.

Treatments consisted of wheat NILs, developed at John Innes Centre (Farré et al., 2016; Zikhali et al., 2014), differing in earliness *per se* alleles that resulted from the crosses of Avalon x Cadenza (AxC) or Spark x Rialto (SxR), each pair of NILs carrying either the early or the late allele in Chromosome 1D (in both AxC and SxR) or in 3 A (AxC). NILs, within each of the three groups (*Eps-D1* of AxC, *Eps-D1* of SxR, and *Eps3 A* of AxC) were arranged in a completely randomised design with eighteen (for NILs from AxC) and twelve (NILs coming from SxR) replications. Even though the lines are derived from UK material, they are well adapted to our environment. Due to the relatively strong winter of Lleida, wheat crops sown in fall are exposed to a sufficiently long period of vernalising temperatures (in these experiments more than 10 weeks of vernalising temperatures; Ochagavía et al., 2018) and consequently they flowered in spring, like cultivars released by local breeding programs do.

2.2. Measurements and analyses

As explained in the companion paper (Ochagavía et al., 2018), the stages of terminal spikelet and anthesis were determined allowing estimation of the duration of the late reproductive phase, which is the stage when florets develop. Thermal time was calculated using the mean air temperature and a base temperature of 0 °C.

Floret development dynamics were determined as in Prieto et al. (2018). Briefly, from terminal spikelet onwards, one representative plant per plot (i.e. 18 plants of each NIL of the AxC cross and 12 plants of each NIL of the SxR cross) was randomly sampled and taken to the lab two or three times a week, the main spikes dissected under a microscope (Leica MZ 7.5, Leica Microscopy System Ltd., Heerbrugg, Switzerland), and each individual floret primordium from the central spikelets was characterised for stage of development following the scale described by Waddington et al. (1983). Pistil development progress was observed and recorded in each floret primordium from the early stages until W10, when florets were considered fertile or, in the case of floret primordium that did not reached W10, until the maximum 'Waddington stage' was reached. Florets were numbered from 1 to n, from the closest to the most distal positions respect to the rachis, respectively. The score used for describing the pistil development was plotted against the thermal time from anthesis (Fig. 1A).

The number of living floret primordia within spikelets was counted at each sampling time in the central spikelets allowing representation of the dynamics of floret primordia generation/degeneration. Floret generation phase lasts until the maximum number of floret primordia is reached. From then on, floret mortality or a degeneration phase takes place until the surviving primordia constitute the number of fertile florets, which is set near to anthesis. Plotting these dynamics facilitates the comparison, not only of differences in the maximum and final number of florets, but also the length of each phase among genotypes (Fig. 1B). To start counting a floret primordium as a living floret it should have reached the stage of development of W 3.5; i.e. the first stage when an individual floret primordium can be identified (Waddington et al., 1983).

Samples of aboveground biomass were taken at anthesis from each replicate of each genotype from a sample area of 0.5 m long of a central row, which had been labelled shortly after seedling emergence. Thus ensuring that the plant density and uniformity was that expected and that the interplant variability within the sample was minimised. From

this sample, four plants were selected at random and the number of fertile florets (style and stigmatic branches spreading and green or yellow anthers visible) was counted in the main-shoot and tiller spikes, within each spikelet from one side of the entire spike.

To determine when differences between particular treatments were significant we subjected the data to analysis of variance (ANOVA) and to *a posteriori* contrasts LSMeans Differences Student's *t*-test using JMP® Pro version 12.0 (SAS Institute Inc., Cary, NC, USA). Linear regression analyses were performed to determine the degree of relationships between variables.

3. Results

3.1. Spike fertility

When there was an effect of the *Eps*-late alleles considered in this study, it increased the number of fertile florets relative to the NILs with the *Eps*-early allele along the spikes borne by main-shoots and tillers.

In the case of the *Eps* gene in chromosome 1D of AxC the number of fertile florets along the main-shoot and tiller spikes were not significantly different between the early and late NILs, although the *Eps*-late NILs tended to present (a non-significantly) higher number of fertile florets than the *Eps*-early (Fig. 2, top panels).

The *Eps*-late allele in chromosome 3 A of AxC showed more clear results than that seen between the 1D AxC NILs: the late allele induced a significantly higher fertility in the spike than the early allele (Fig. 2, middle panels). Fertility significantly improved in almost all of the spikelets, in both the main-shoot and tiller spikes, though the magnitude of the effect was larger in the main-shoot spikes (Fig. 2, middle panels).

When NILs from the SxR cross with contrasting alleles of the *Eps* gene on chromosome 1D were compared, again the lines carrying the *Eps*-early exhibited spikes with reduced fertility: the number of fertile florets per spike was significantly higher in lines with the late allele than in those with the early allele in both the main-shoot and tiller spikes (Fig. 2, bottom panels).

Whenever a difference between NILs in spike fertility was detected, it was representative of most spikelets but clearer in the most prolific spikelets which are those in the central part of the spikes. Thus, a detailed analysis of floret development in these spikelets could shed light on the origin of the differences in spike fertility.

3.2. Floret development and living floret primordia dynamics in the central spikelets

A more detailed analysis was carried out to try to find out the likely origin of the overall increase in number of fertile florets due to the introgression of *Eps*-late alleles. Dynamics of development of each individual floret primordium as well as that of all living floret primordia were studied for each of the NILs. Florets 1, 3, 4 and 6 were chosen to illustrate the main results because they represent extreme cases of floret development: while floret 1 always reaches the fertile stage, floret 6 never develops that much (under field conditions) and florets 3 and 4 are those most commonly labile. So, determining their developmental dynamics provides a very direct view of whether or not treatments affect floret fertility (all other floret positions are shown in Appendix A).

There were no clear differences in the dynamics of development of the three most proximal floret primordia between NILs carrying the late and early *Eps* alleles in chromosome 1D in the AxC cross, all reached the stage of fertile floret (score 10) (Fig. 3A Florets 1 and 3; Fig. S1 in Appendix A Floret 2). On the other hand, floret 4 reached that stage only in the *c.* 60% of the NILs plants carrying either the *Eps*-early or late allele (Fig. 3A) without any differences between them, whilst the remaining 40% of the NILs reached a stage of *c*. W9. Florets in more distal positions never developed to stages even close to fertile florets in any of the NILs (Fig. 3A Floret 6; Fig. S1 in Appendix A Florets 5, 7, and 8) and did not exhibit any difference in development between NILs. Consequently, there were no differences between the *Eps*-late and *Eps*-early NILs either in the dynamics of generation and degeneration of floret primordia nor in the final number of fertile florets when considering the *Eps-D1* in the genetic backgrounds of Avalon and Cadenza (Fig. 3B).

Similarly to what was described for the NILs varying in *Eps-D1* from AxC, when the effects of the *Eps*-late vs -early allele in chromosome 3 A, there were no clear differences in the dynamics of florets 1, 2 and 3, which always reached the fertile floret stage simultaneously (Fig. 4A, Fig. S2 in Appendix A). However, floret 4 reached the fertile stage in *c.* 80% of the plants carrying the *Eps*-late allele (the other 20% reached a stage of *c*. W8.5), while it did so only in *c.* 40% of the plants carrying the early allele (Fig. 4A). This difference could be reflected in the final number of fertile florets at anthesis (Fig. 4B), despite the fact that both NILs initiated a similar number of floret primordia, the plants carrying the *Eps*-late allele showed a slightly slower rate of death towards the end of the floret mortality period resulting in a higher number of fertile florets at anthesis.

Finally, dynamics of floret 1,2 and 3 of the NILs carrying either the *Eps-D1*-early or -late allele from SxR did not show any clear differences (Fig. 5A, Fig. S3 in Appendix A), while floret 4 was fertile in all plants of the NILs carrying the *Eps*-late variant but only in several (though not all) plants carrying the *Eps*-early (Fig. 5A). More distal florets (5–8/9) (Fig. 5A; Fig. S3 in Appendix A) never reached the stage of fertile florets at anthesis in NILs with *Eps*-early alleles while in the lines with the *Eps*-late 30% of the plants produced a fifth floret primordium through to fertile floret classification at anthesis (Fig. S3 in Appendix A). Therefore, the slight differences in the final number of fertile floret was related to a reduced floret mortality due to an improved development of labile florets when the *Eps* allele was the late form (Fig. 5B) even though both NILs variants initiated the same maximum floret primordia (Fig. 5B).

3.3. Relevance of the duration of late reproductive phase

As shown in the companion paper (Ochagavía et al., 2018), NILs carrying the *Eps-D1*-early alleles had a shorter duration of the late reproductive phase than their counterparts carrying the *Eps-D1*-late alleles (in both the AxC and the SxR NIL groups but more so in the former) whilst the *Eps* alleles on 3 A had no effect.

The differences in late reproductive phase, although slight between NILs in most cases, exerted a clear positive effect on spike fertility (Fig.6A) and the relationship was even stronger if the actual duration of floret initiation (from when F1 reached the stage 3.5 to anthesis) is considered instead (Fig. 6B). In addition, excluding the cases of chromosome 3 A (squares in Fig. 6) due to the lack of effect of the *Eps* alleles on the length of the LRP, the coefficients of determination increased to $R^2 = 0.78$ and $R^2 = 0.91$*, respectively. The relationship was not only due to differences in genetic backgrounds (with SxR having longer LRP and more fertile florets per spike than AxC) but also genuinely due to the action of *Eps*-late alleles in chromosome 1D, in general it seems that within genetic backgrounds (AxC or SxR) the NILs with *Eps*-late alleles tended to exhibit a longer duration of the phases when floret development takes place and increased the spike fertility as well (Fig. 6).

4. Discussion

Although exceptions can be found (Slafer and Rawson, 1994), particularly in diploid wheat under controlled conditions (Appendino and Slafer, 2003; Bullrich et al., 2002; Lewis et al., 2008), the *Eps* genes normally have relatively small effects on developmental attributes and are useful to fine-tuning adaptation. The number of grains per m^2 depends on the number of spikes and spike fertility. The former component is normally critical for coarse regulations (large changes in grain number due to genetic or environmental factors) whilst spike fertility is

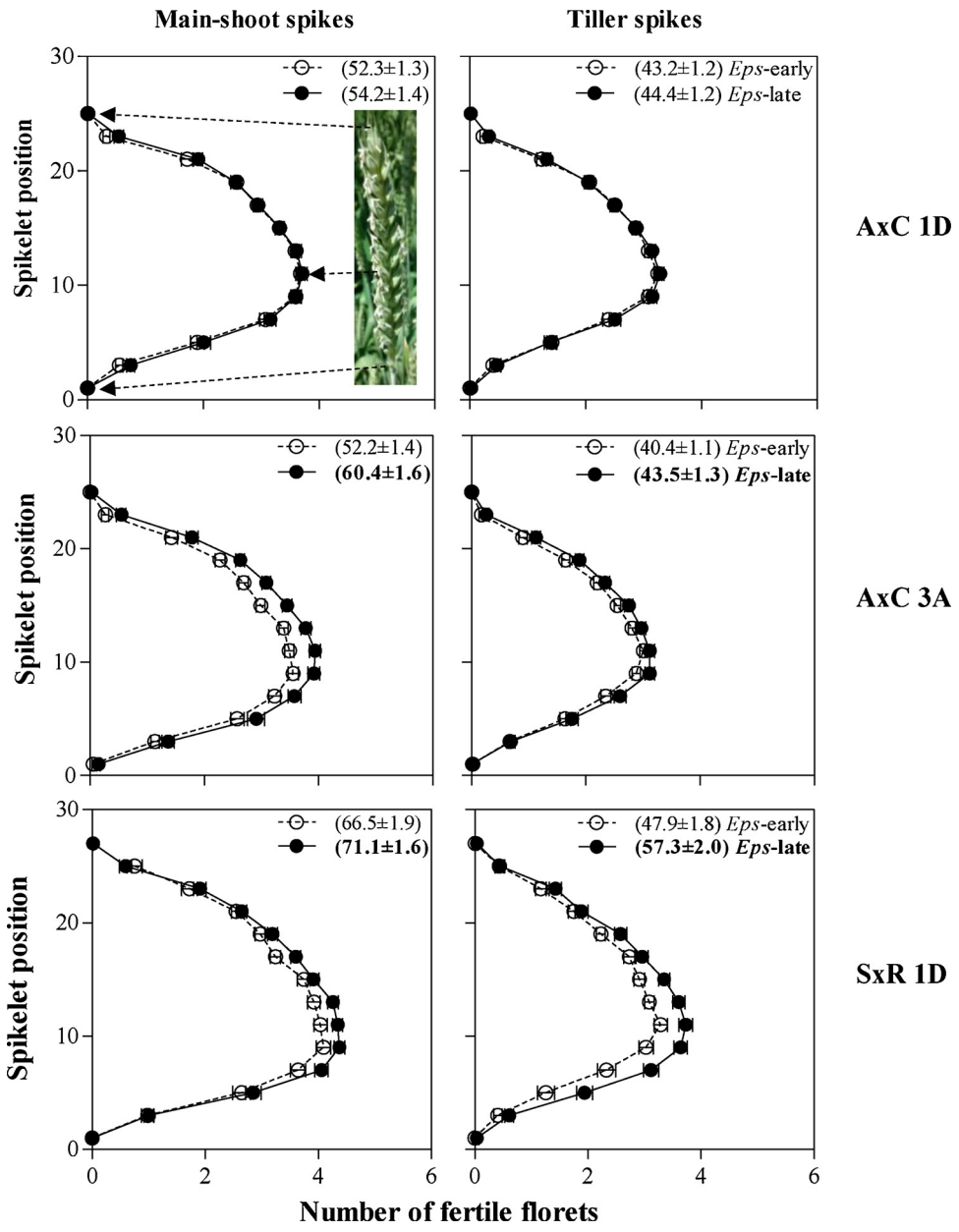

Fig. 2. Mapping of fertile florets on the main-shoot (left panels) or tiller spikes (right panels) for the *Eps* NILs AxC 1D (top panels), AxC 3 A (middle panels) and SxR 1D (bottom panels) carrying the *Eps*-early allele (open symbols, dashed lines) or the *Eps*-late allele (closed symbols, solid lines). Spikelet position is counted from the base to the tip of the spike (i.e. spikelet 1 is the basal spikelet closest to the peduncle) to the terminal spikelet. Each data-point is the average of all replicates across two growing season and within each replicate the value was the average of 4 plants. The segment in each data-point stands for the standard error of the means (not visible when smaller than the symbol). The average of the number of fertile florets per spike and the standard error of the means are indicated in brackets inset each panel, and when differences between *Eps*-early and -late NILs were statistically significant ($P < 0.05$) the figure corresponding to the latter was typed in bold.

the component of grain number more likely responding as a fine regulator to affect more subtle changes in grain number (Slafer et al., 2014). As *Eps* effects on developmental processes are relatively small, expectedly they did not include major changes in dynamics of tillering and tiller mortality (Ochagavía et al., 2018). Thus, effects on the number of fertile florets had to operate, if existing, on the number of fertile florets per spike, through effects on floret development.

Concordant with most of the literature (Bullrich et al., 2002; Lewis

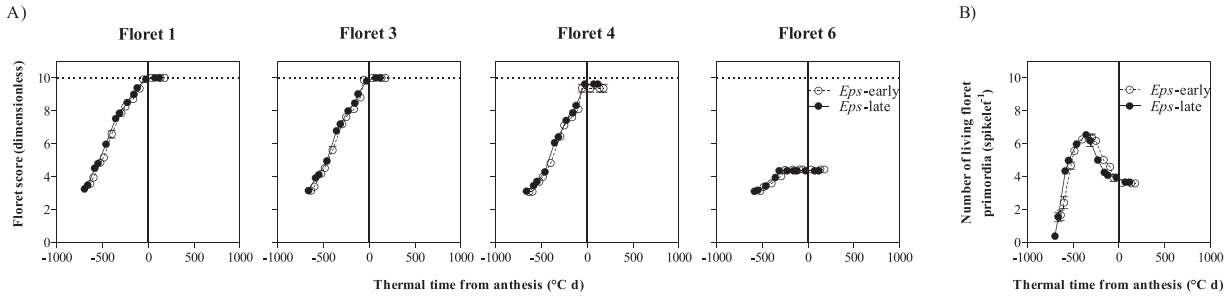

Fig. 3. Dynamics of the floret development of F1, F3, F4 and F6 in central spikelets of the main-shoot (A) and the number of living floret primordia (B) through thermal time from anthesis in the *Eps* NILs carrying either the late (closed circles, solid lines) or the early allele (open symbols, dashed lines) from the AxC in 1D. Each data-point is the average of all replicates across two growing season and within each replicate the value was the average of 3 plants, bars represent the standard error of the means.

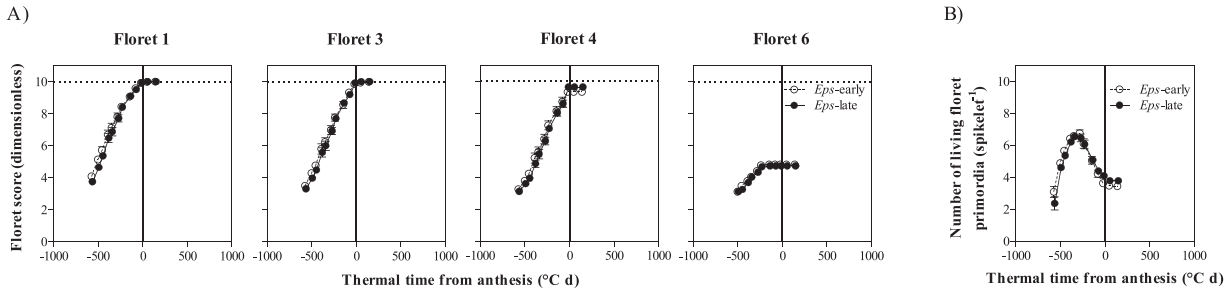

Fig. 4. Dynamics of the floret development of F1, F3, F4 and F6 in central spikelets of the main-shoot (A) and the number of living floret primordia (B) through thermal time from anthesis in the *Eps* NILs carrying either the late (closed circles, plain lines) or the early allele (open symbols, dashed lines) from the AxC in 3 A. Each data-point is the average of all replicates across two growing season and within each replicate the value was the average of 3 plants, bars represent the standard error of the means.

et al., 2008) the *Eps* alleles had only relatively minor effects on time to anthesis, NILs with *Eps-D1*-early alleles from Spark and Cadenza flowered slightly (though significantly) earlier than in those with the corresponding -late alleles from Avalon and Rialto (Ochagavía et al., 2018). Most of the literature on the effects of these alleles has focused exclusively on the duration of the whole cycle; assuming most commonly that any difference seen in time to heading would be reflecting differences in either the duration of vegetative phases (with others unaffected) or in all pre-anthesis phases equally (Slafer, 1996; Lewis et al., 2008; Faure et al., 2012). In a previous study it was shown that there is genotypic variation in *Eps* (comparing cultivars after vernalizing the seedlings and under long days) that were not restricted to particular phases (Slafer and Rawson, 1995). However, Ejaz and von Korff (2017) showed *Eps* alleles affected the phase of inflorescence development in barley when it was subjected to high temperatures. The results in the companion paper showed that *Eps* alleles on chromosome 1D mainly influenced the late reproductive phase, while those in chromosome 3 A did not (Ochagavía et al., 2018). This proves that, by quantifying in detail the phenological differences between NILs of particular *Eps* genes, we might provide the knowledge necessary for breeders to achieve fine-tuning not only of the time to anthesis, but also the partitioning of time among pre-anthesis phases with positive consequences for spike fertility.

In general, there was a subtle effect of *Eps* genes on the number of fertile florets at anthesis: the NILs with the *Eps*-late alleles produced more fertile florets per spike in comparison with *Eps*-early, and the increased spike fertility was apparent in most spikelets, likely through reducing the rate of floret mortality (the main determinant of floret survival) immediately before flowering. This is in line with previous studies in that whenever a treatment increases spike fertility it does so most frequently through increased floret survival (e.g. Ferrante et al., 2013; González et al., 2011; Guo et al., 2016; Miralles et al., 1998; Prieto et al., 2018; Siddique et al., 1989). There are evolutionary reasons for this to be so, based on an 'optimistic strategy' of generating more reproductive primordia than can survive as the required investment involved in initiating primordia seems negligible (Sadras and Slafer, 2012). The mechanism by which late alleles seemed to decrease floret mortality was by allowing a slightly longer period for the development of labile florets: whilst these florets (e.g. Floret 4 in central spikelets) developed insufficiently to reach the stage of fertile florets and were induced to a late mortality in NILs with the *Eps*-early allele, when the late allele was introgressed into the same genetic background that late mortality was prevented. A similar situation is observed for *Ppd-1* alleles for photoperiod insensitivity, which also lengthen the duration of the late reproductive phase (González et al., 2005; Prieto et al., 2018). In many cases the same effect, of lengthening the developmental phase of particular floret primordia was also seen in more distal positions which would never have otherwise reached the stage of fertile florets, suggesting that the effect of floret development is generic (not linked to particular florets).

Thus, as the effects of the *Eps* genes on the length of developmental phases are relatively weak (Bullrich et al., 2002; Slafer et al., 2015) our findings were not unexpected in that we find correspondingly slight differences in spike fertility in this study. However, within any one mega-environment major genes such as *Ppd-1* and *Vrn-1* are usually fixed, to produce the desired combination of growth habit and photoperiod sensitivity. So the only way variation in phenology can be used to increase spike fertility is via more subtle allelic variation at those

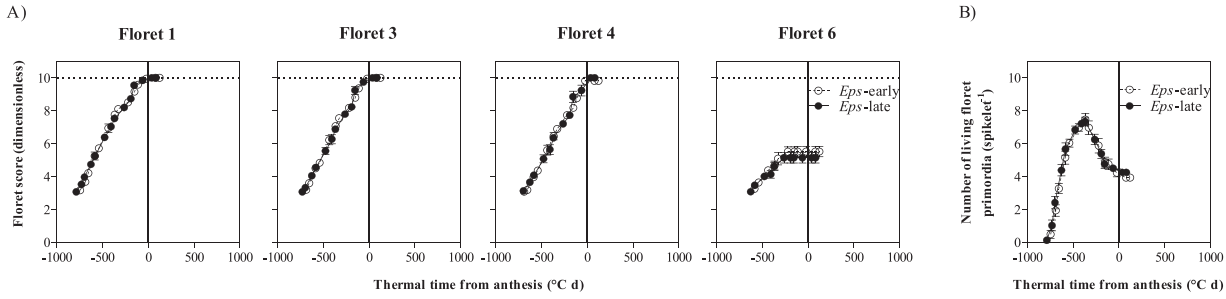

Fig. 5. Dynamics of the floret development of F1, F3, F4 and F6 in central spikelets of the main-shoot (A) and the number of living floret primordia (B) through thermal time from anthesis in the *Eps* NILs carrying either the late (closed circles, plain lines) or the early allele (open symbols, dashed lines) from the SxR in 1D. Each data-point is the average of all replicates across two growing season and within each replicate the value was the average of 3 plants, bars represent the standard error of the means.

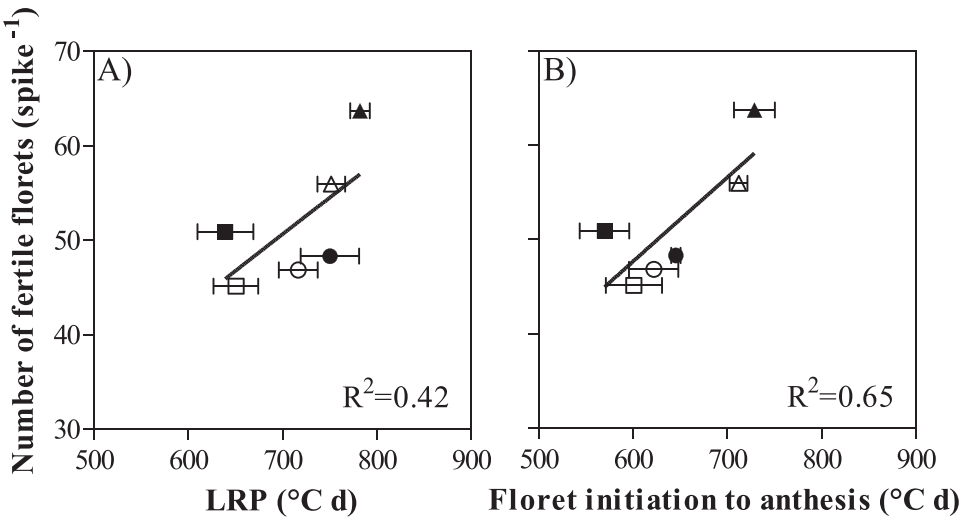

Fig. 6. Relationship between the number of fertile florets per spike and either (A) the duration of the late reproductive phase (LRP), or (B) the duration of the period from initiation of F1 to anthesis for the *Eps* NILs carrying either the early (open symbols) or late (closed symbols) alleles from the AxC 1D (circles), AxC 3 A (squares) and SxR 1D (triangles). Data resulted from the average across two growing seasons. Segments on each symbol represent the standard errors of the means. The coefficient of determination (R^2) and the level of significance (p-value) for linear regression are shown.

genes or *Eps*. We concluded that *Eps* genes considered in this study affected floret development differently, through modifying the rate of floret mortality. *Eps*-late alleles providing the developmental space for a few labile floret primordia to continue to develop, with the outcome that some of them reached the stage of fertile floret instead of degenerating and dying as in the NILs with *Eps*-early alleles. This extended period of development reduced the rate of floret mortality by increasing the survival of labile florets with positive consequences for spike fertility in NILs with *Eps*-late alleles. The magnitude of the effect was expectedly small (as all effects of *Eps* genes are rather minor) and variable depending on the particular allele and in the case of *Eps-D1* depending on the particular cross used to create the NILs (only a non-significant trend in *Eps-D1* of AxC whilst a significant a more clear effect on spike fertility in *Eps-D1* of SxR). Consequently, the manipulation of particular *Eps* genes may be useful not only to fine-tuning time to anthesis but also to influence on spike fertility. Together with the discovery that the gene underlying *Eps-D1* is the wheat homologue of *ELF-3* (Zikhali et al., 2016) the spike fertility effects described here provide a strong motivation to search for new alleles (and homoeoallelic combinations) of *Eps-1* and test their effects on this important trait.

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Appendix A

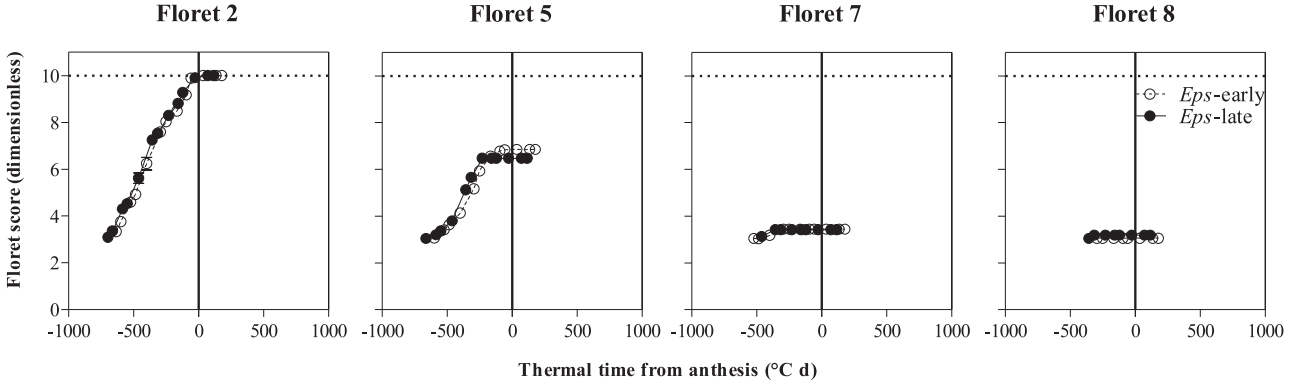

Fig. S1. Dynamics of the floret development of F2, F5, F7 and F8 in central spikelets of the main-shoot through thermal time from anthesis in the *Eps* NILs carrying either the late (closed circles, plain lines) or the early allele (open symbols, dashed lines) from the AxC in 1D. Each data-point is the average of all replicates across two growing season and within each replicate the value was the average of 3 plants, bars represent the standard error of the means.

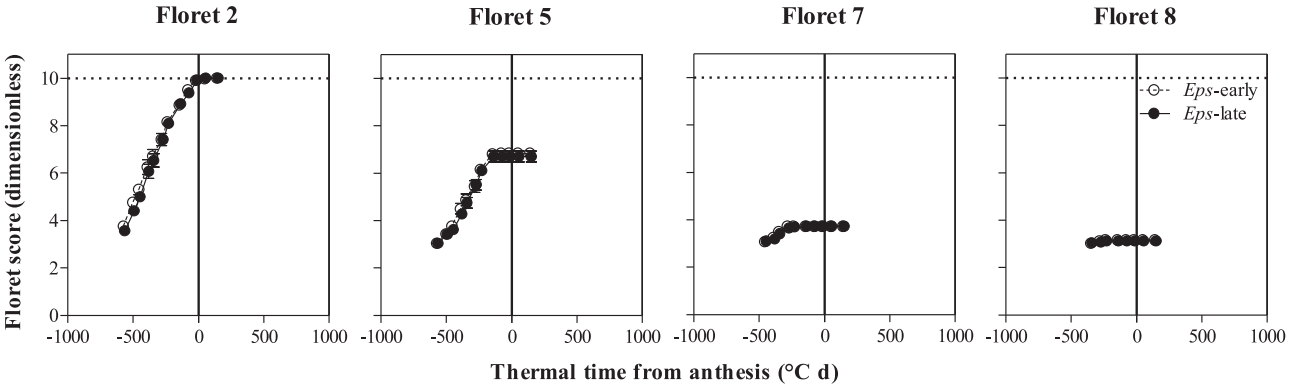

Fig. S2. Dynamics of the floret development of F2, F5, F7 and F8 in central spikelets of the main-shoot through thermal time from anthesis in the *Eps* NILs carrying either the late (closed circles, plain lines) or the early allele (open symbols, dashed lines) from the AxC in 3 A. Each data-point is the average of all replicates across two growing season and within each replicate the value was the average of 3 plants, bars represent the standard error of the means.

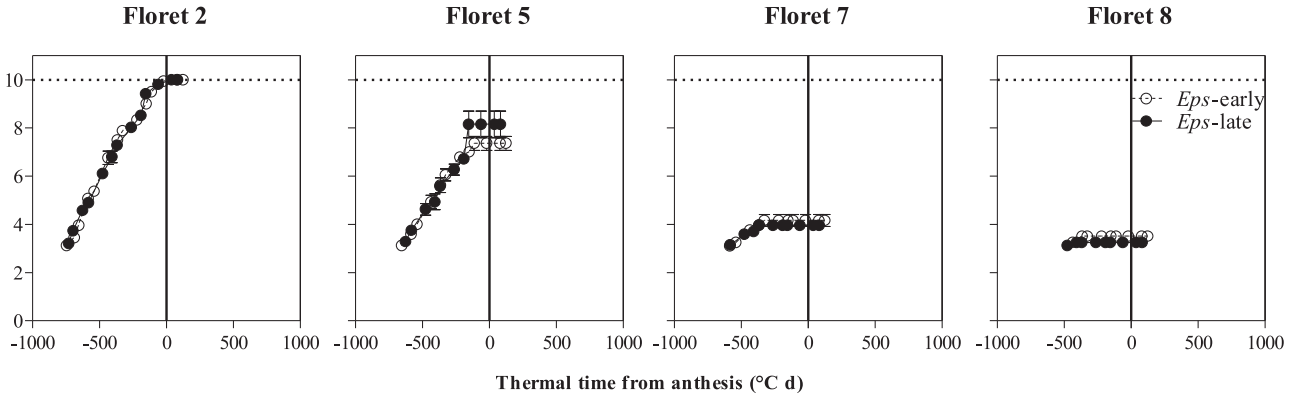

Fig. S3. Dynamics of the floret development of F2, F5, F7 and F8 in central spikelets of the main-shoot through thermal time from anthesis in the *Eps* NILs carrying either the late (closed circles, plain lines) or the early allele (open symbols, dashed lines) from the SxR in 1D. Each data-point is the average of all replicates across two growing season and within each replicate the value was the average of 3 plants, bars represent the standard error of the means.

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