Understanding developmental processes responsible for adaptation- and yield- related traits in elite wheat germplasm

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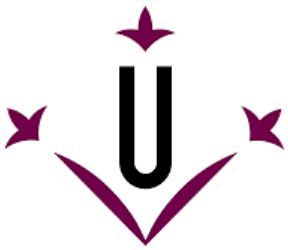

Universitat de Lleida

TESI DOCTORAL

Understanding developmental processes responsible for adaptation- and yield- related traits in elite wheat germplasm

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ನನ್ನ ಮೇಲೆ ಅತ್ಯಂತ ಕನಿಷ್ಠ ಆಕಾಂಕ್ಷೆಗಳನ್ನು ಇಟ್ಟು ಕೊಂಡ ನನ್ನ ಅಮ್ಮಾ ಮತ್ತು ಅಪ್ಪಾ ಜಿಗೆ,

ನಾನು ಅವರು ಬಯಸಿದಂತ ಮೊಮ್ಮಗಳಾಗಿಲ್ಲದಿದ್ದರು ನನ್ನ ಮೇಲೆ ಅತೀ ಹೆಮ್ಮೆ ಪಡುವ ನನ್ನ ಅಜ್ಜಿ ಮತ್ತು ಅಜ್ಜನಿಗೆ ಹಾಗೂ ನನಗೆ ತುಂಬಾ ಪ್ರಿಯವಾದ ನನ್ನ ತಮ್ಮಂದಿರಿಗೆ.

I dedicate this thesis to lovely people mentioned below who by the way are never going to read this-

To my parents, Yashodha and Anant, who had minimum hopes on me after I did not get into Engineering and Medicine universities (for which I am extremely thankful). My grandparents, Sharamma and Tirakappa, who are extremely proud of me although I haven't been the most ideal grand-daughter they wished for. And to my annoyingly smart and well-behaved brothers, Vinnu and Pawan who still think their sister is cool.

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Abstract

Increases in wheat yield are essential to meet the growing demand under a complex situation of impossibilities to further expand the arable lands, climate change, and the challenge to produce grains under environmentally friendly techniques. In the past, genetic gains were brought about by optimised plant height and time to anthesis which along with improving lodging resistance and wheat adaptation resulted in an important increase in grain yield. Further genetic gains will mostly depend on other traits, like the duration of particular sub-phases of wheat development or the combination of final leaf number and phyllochron and the effects on floret fertility (a major determinant of the number of grains, and hence grain yield). Therefore, this Thesis was focused on identifying traits underpinning the grain yield and discussing some important trade-offs between physiological components of grain yield as well as to understand the effect of newly identified *Eps* QTLs on wheat development.

The main aim of the present Thesis was to improve the understanding of the physiological traits underlying grain yield such as distribution of pre-anthesis phases, spike fertility and their influence on grain number. Particularly to identify (i) traits and trait combinations that affect grain yield, evaluating possible genetic variability in pre-anthesis phases in elite lines with similar time to anthesis; and plant height; (ii) the functions of a newly identified *Eps* QTLs *Eps-7D* and *Eps-2B* beyond their known effect on time to anthesis such as their effect on the duration of individual pre-anthesis phases and spike fertility; and (iii) the interaction of the *Eps-7D* with temperature and photoperiod under controlled conditions. To accomplish these objectives, four experiments under field and one experiment under control conditions were performed.

A large set of bi-parental population derived from elite parents were evaluated under field conditions and sub-set of lines with similar plant height and time to anthesis were selected to identify traits driving the grain yield variability in them. The selected lines carried large variability for grain yield (c. 500-1000 g m^{-2}) which was explained better by spike dry weight at anthesis and fruiting efficiency, determined during late reproductive phase (traits that are components of grain number). Improving grain number did not reduce grain weight as two third of the lines presented high grain number (high grain yield) also had higher grain weight compared to those with low grain number.

Evaluation of eight lines differing in *Eps-7D* and *-2B* under field conditions revealed an epistatic interaction between the two QTLs which affected the dynamics of leaf appearance, spikelet and floret primordia development in addition to the duration of pre-anthesis phases. Evaluation of *Eps-7D* under

controlled conditions disclosed the interaction between *Eps-7D* × temperature on pre-anthesis phases and dynamics of organ development. Overall, the effect of *Eps-7D* was stronger than *Eps-2B* and the effects of *Eps-7D* depended on allelic status of *Eps-2B*. The allelic forms of *Eps-7D*, *Eps-7D-late* and *–early,* had different degree of sensitivity to temperature and the differences in their effect was clearer at 9 ºC under short day.

The work reported in this Thesis may be useful in further improving grain yield in well adapted wheat regions, as the variability in individual pre-anthesis phases in the studied population with similar time to anthesis carried reasonable variability for grain yield. In addition, the evaluation of allelic combinations of two newly identified *Eps* and their epistatic interactions help in tailoring allelic combination to produce a desired phenotype with advantageous distribution of time to important phenophases. The interaction between two particular *Eps* is reported for the first time here which was only speculated before.

Resumen

Los aumentos en el rendimiento del cultivo de trigo son fundamentales para satisfacer la creciente demanda en una situación compleja, con imposibilidades de expandir aún más las tierras cultivables, el cambio climático y el desafío de producir granos mediante prácticas agronómicas sostenibles. En el pasado, las ganancias genéticas obtenidas mediante la optimización de la altura de la planta y el tiempo hasta antesis, conjuntamente con la mejora de la resistencia al acame y la adaptación del trigo, resultaron en incrementos importantes del rendimiento del grano. Futuras ganancias genéticas dependerán de otros atributos como la duración de alguna sub-fase de desarrollo particular del trigo o la combinación del número final de hojas y filocronos y la fertilidad de las flores (uno de los determinantes más importantes del número de granos y, por ende, del rendimiento). Es por ello, que esta Tesis se centró en identificar los atributos en que se fundamenta el rendimiento del grano y en discutir las compensaciones entre los diferentes componentes fisiológicos del rendimiento del grano, así como comprender el efecto de los QTLs *Eps* recientemente identificados en el desarrollo del trigo.

El principal objetivo de esta Tesis fue mejorar la comprensión de los atributos responsables del rendimiento del grano como la distribución de las subfases durante prefloración, la fertilidad de la espiga y su influencia en el número de granos. Particularmente identificar (i) los atributos y las combinaciones de estos que afectan al rendimiento del grano, evaluando la posible variabilidad genética en las fases prefloración de poblaciones élite que poseen similar duración hasta floración y en altura de la planta; (ii) las funciones de los nuevos QTLs identificados, *Eps-7D* y *Eps-2B* en sub-fases particulares de la duración tiempo preantesis y la fertilidad de la espiga; (iii) la interacción de *Eps-7D* con la temperatura y el fotoperíodo bajo condiciones controladas. Para cumplir estos objetivos, se llevaron a cabo cuatro ensayos de campo y uno bajo condiciones controladas.

Un gran número de poblaciones bi-parentales derivadas de progenitores élite fueron evaluadas en condiciones de campo y se seleccionó una muestra de líneas con altura de planta y tiempo hasta antesis similares para identificar los atributos que determinan la variabilidad en el rendimiento. Las líneas seleccionadas presentaban una gran variabilidad en rendimiento (c. 500-1000 g m^{-2}) que se pudieron explicar por un mayor peso de la espiga en antesis y mayor eficiencia de fructificación (ambos componentes del número de granos) determinadas en la fase reproductiva tardía. El aumento en el número de granos no redujo el peso de los granos, ya que dos tercios de las líneas presentaron un

número de granos alto (alto rendimiento) también tuvieron un peso de grano más alto en comparación con aquellas con un número de granos bajo.

La evaluación de ocho líneas que se diferenciaron en los *Eps-7D* y *-2B* en campo reveló una interacción epistática entre ambos, que afectaba la dinámica de aparición de las hojas, y el desarrollo de las espiguillas y los primordios florales. La evaluación de *Eps-7D* en condiciones controladas reveló los efectos de la interacción entre este y la temperatura sobre las fases preantesis y las dinámicas de desarrollo de órganos. La magnitud del efecto de *Eps-7D* fue mayor que la de *Eps*-2B y los efectos de *Eps-7D* dependían del estado alélico de *Eps-2B*. Las formas alélicas *Eps-7D*, *Eps-7D-late* y *Eps-7D-early* mostraron diferentes grados de sensibilidad a la temperatura y las diferencias en sus efectos fueron claramente demostradas en condiciones de días cortos y a 9 ºC.

El trabajo realizado en esta Tesis contribuye a entender y mejorar aún más el rendimiento de grano en de trigo, ya que la variabilidad en algunas subfases de desarrollo en pre-antesis en la población estudiada (con un tiempo similar hasta antesis) presentó una variabilidad razonable para el rendimiento de grano. Además, la evaluación de combinaciones alélicas de dos *Eps* recientemente identificados y sus interacciones epistáticas ayudan a adaptar la combinación alélica para producir un fenotipo deseado con una distribución ventajosa del tiempo para fenofases importantes. La interacción entre dos *Eps* particulares es informado por primera vez en esta Tesis.

Resum

Increments en el rendiment de blat són necessaris per tal de fer front a la demanda creixent sota situacions complexes com la impossibilitat de expandir les terres cultivables, el canvi climàtic i el repte de produir grans mitjançant pràctiques agronòmiques sostenibles. En el passat, els guanys genètics s'han aconseguit optimitzant l'altura de la planta i el temps fins a antesi i, conjuntament amb la millora de la resistència al allitament i l'adaptació del blat, resultaven en increments importants del rendiment del gra. La millora genètica més enllà dependrà d'altres trets com la duració de cadascuna de les sub-fases del desenvolupament del blat o la combinació del nombre final de fulles i filocrons i els efectes en la fertilitat de les flors (un dels determinants més importants del nombre de grans i per extensió del rendiment). És per això, que aquesta Tesi es va centrar en la identificació dels trets en què es fonamenta el rendiment del gra i en debatre el compromís entre els diferents components fisiològics del rendiment del gra així com entendre l'efecte dels recentment identificats QTLs (locus de caràcters quantitatius) *Eps* en el desenvolupament del blat.

El principal objectiu d'aquesta Tesi era millorar la comprensió dels trets responsables del rendiment del gra: com la distribució de les fases abans de floració, la fertilitat de l'espiga i la seva influència en el nombre de grans. Més particularment, identificar (i) els trets i les combinacions d'aquests que afecten al rendiment del gra, avaluant la possible variabilitat genètica en les fases abans de floració de les poblacions elit similars en temps fins a floració i en altura de planta; (ii) les funcions dels nous QTLs identificats, *Eps-7D* i *Eps-2B* en sub-fases particulars del temps abans d'antesi i fertilitat de l'espiga; (iii) la interacció de *Eps-7D* amb la temperatura i el fotoperíode sota condicions controlades. Per acomplir aquests objectius, es van dur a terme quatre assaigs de camp i un sota condicions controlades.

Un gran nombre de poblacions bi-parentals derivades de progenitors elit van ésser avaluades en camp i es va seleccionar una mostra de línies amb altura de planta i temps fins a antesi similars per tal d'identificar els trets que en dirigeixen la variabilitat en rendiment. Les línies seleccionades presentaven una gran variabilitat en rendiment (c. 500-1000 g m^{-2}) que es basaven en una millor fertilitat de l'espiga. Com que la major part de línies seleccionades amb un alt rendiment mostraven un nombre i un pes de grans elevat, podem concloure que la subsegüent millora del rendiment pot venir de l'augment en el nombre de grans sense implicar una reducció en el pes.

L'avaluació de vuit línies que es diferencien en els *Eps-7D* i *-2B* en camp va revelar una interacció epistàtica entre ambdós, que afectava la dinàmica d'aparició de fulles, i el desenvolupament de les

espiguilles i els primordis florals a més a més de les fases abans d'antesi. L'avaluació de *Eps-7D* en condicions controlades va revelar els efectes de la interacció entre aquest i la temperatura sobre les fases abans d'antesi i les dinàmiques de desenvolupament d'òrgans. La magnitud de l'efecte de *Eps-7D* era major que la de *Eps-2B* i els efectes de *Eps-7D* depenien de l'estat al·lèlic de *Eps-2B*. Les formes al·lèliques *Eps-7D*, *Eps-7D-late* i *Eps-7D-early* mostraven diferents graus de sensibilitat a la temperatura i les diferències en els seus efectes eren aparents en dies curts i a 9 ºC.

Les conclusions d'aquesta Tesi van ser que el rendiment de gra pot millorar-se sense canviar el temps fins a antesi (optimitzant les fases d'abans d'antesi). Els *Eps* estudiats poden ser una eina per ajustar les fases d'abans d'antesi amb poques alteracions del temps fins a antesis. Aquí es demostra per primer cop la interacció entre dos *Eps* la qual només havia estat suggerida.

Chapter 1: General Introduction

1. Chapter I: General introduction

1.1. A brief description of the general background

Wheat is one of the important cereals for it is a staple food cultivated worldwide. Large portion of the population in developing countries not only derive calories, but also their nutrition from wheat (Shewry and Hey, 2015). Thanks to its adaptability, it is grown in very diverse environments around the world from sea level to more than 3000 m above sea level, from temperate to tropical regions and from drought prone area to regions with 1700 mm average rainfall (Curtis, 2002). Such wide adaptability requires large trait variations that was possible by efforts of breeding to put together adaptive traits suiting diverse regions (Braun et al., 1996). Wheat yield improvements are critical to meet the growing demand under an un-expandable agricultural land with the uncertain changes imposed by the climate (Reynolds et al., 2016).

Although there have been large improvements in wheat grain yield, particularly during the green revolution, the present rate of improvement is not promising: (i) the genetic gain in yield has been much slower over the last decades than in the previous 3-4 decades (see many examples in Foulkes and Reynolds, 2015), and (ii) it does not keep up with the growing demand (increasing population and changing food habits; Reynolds et al., 2009a). Agronomic practices have been optimized according to changing soil and climate scenarios to maintain grain yield (at least in wheat regions of developed countries), it is inevitable that the major improvements from now will mostly depend on breeding. Genetic gains in wheat over a century have been achieved either by cautious selection of high yielding lines or because of favouring a phenotype which had pleiotropic effects on yield (e.g. Austin et al., 1980; Calderini et al., 1995). Over the time, there has been shift in the selection pressure on traits from selecting directly for yield *per se* itself (especially in high-input agriculture) to towards the improvement in the knowledge on physiological mechanism underlying yield (Reynolds et al., 2012, 2009b).

1.2. Grain yield generation: components and their physiological determinants as affected by phenology

1.2.1. Wheat growth and development

Wheat development is a continuous process from sowing to physiological maturity yet encompasses distinguishable phases delimited by morphological stages viz., seedling emergence (DC10), initiation

of tillering, onset of stem elongation (OSE, DC30) or terminal spikelet (TS), flag leaf emergence (DC39), heading (DC59), anthesis (DC65) and physiological maturity (DC95), denoted by decimal code proposed by (Zadoks et al., 1974) . These developmental stages can be grouped into four important phases based on differentiation of organs, such as, vegetative phase (sowing to floral initiation), early reproductive phase (floral initiation to TS/OSE), late reproductive phase (TS to anthesis, LRP) and grain filling phase (anthesis to physiological maturity). The length of each of these phases influences growth and developmental rate of various organs (tillers, spikelets and florets) determining final grain yield (Slafer et al., 2015; Slafer and Rawson, 1994a).

1.2.2. Grain yield components and their trade-off

Average grain weight (AGW) and grain number per m^2 (GN) are two major components that define grain yield in all field crops as wheat. These two grain yield components are often negatively related to each other (Miralles and Slafer, 1995a; Siddique et al., 1989b; Slafer et al., 1996, 2014) posing a complex situation as improving one would negate the other. Heritability of GN is lower than that of AGW, but the former has reasonable genetic variability and much higher plasticity (Sadras and Slafer, 2012). Then, as a result, larger yield improvements are attributed to GN (Acreche et al., 2008; Calderini et al., 1995a; Shearman et al., 2005) than to AGW. Although grain yield is the end result of growth and development occurring during the whole growth cycle, the length of LRP is relatively more critical for determining GN (e.g. Fischer, 1985; Kirby, 1988; Savin and Slafer, 1991; Siddique et al., 1989a), component that best explains the grain yield. Grain yield is mostly limited by GN (Fischer, 2008; Reynolds et al., 2009a) as cultivars have an excess of available assimilates per every grain that is set indicating sink limitation during grain filling phase (Acreche et al., 2009; Borrás et al., 2004; González-Navarro et al., 2015; Slafer and Savin, 1994). Larger variation in GN is attained by spike dry weight at anthesis (SDWa) (Slafer et al., 2014) which is influenced by time before and after TS (Fischer, 1985; González et al., 2011; Thorne and Wood, 1987). GN is set close to anthesis time (flag leaf to anthesis) when the spike growth is at its peak and during which the fate of florets that initiated since terminal spikelet stage is determined (Fischer, 1985; González et al., 2003a; Miralles et al., 2000) defining spike fertility. SDWa and grains per gram of spike at anthesis (fruiting efficiency, FE) are physiological determinants of GN, the former is responsible for larger increments while the latter brings minor improvements in GN (Slafer et al., 2015).

1.2.3. Past yield improvement strategies

The past yield improvements have been successful from two major traits viz., plant height and time to anthesis that brought improvements in larger magnitudes (Fig. 1.1). Grain yield improvements were achieved by selecting for a plant ideotype that was shorter in height compared to its predecessors (Brooking and Kirby, 1981; Fischer and Stockman, 1986; Reynolds et al., 1996) which was mainly preferred for lodging resistance. Reducing height directly shrunk the yield losses due to lodging and indirectly improved the harvest index by better biomass partitioning to the growing spike with reduced competition from the growing stem (Siddique et al., 1989b; Slafer and Andrade, 1993). Reducing plant height was achieved by introgressing *Rht* genes which lead to higher GY without the need to bring major changes in the total biomass produced (Calderini et al., 1995a).

Tuning the time to anthesis improved wheat adaptation to much wider environments and improved yield by optimising the time devoted for certain growth stages during which critical organ development occur. Also, in regions where wheat was already adapted, adjusting time to anthesis to avoid stresses like frost and high temperatures brought about yield improvements (Richards, 1991).

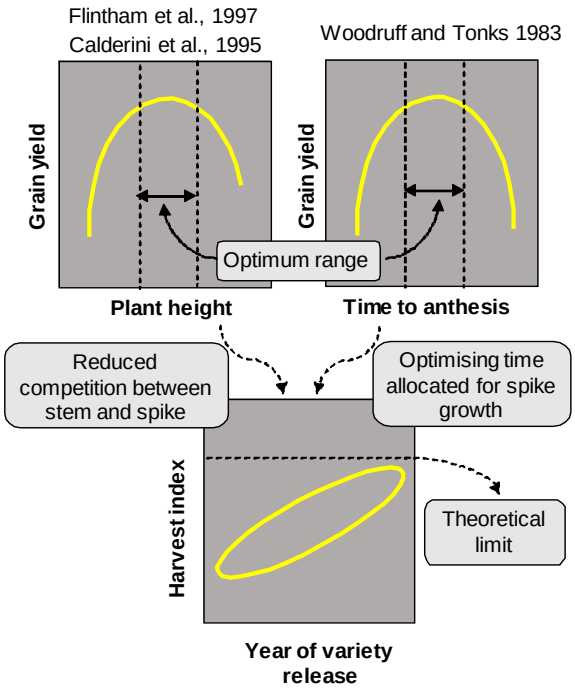

Fig. 1.1. Scheme illustrating the past yield improvements through plant height and time to anthesis that have brought harvest index close to threshold. Present day cultivars have optimum plant height and time to anthesis limiting further alterations in these traits prompting the need to identify new traits directly or indirectly bringing yield improvements.

Present cultivars have the optimum plant height (Richards, 1992) and time to anthesis (Woodruff and Tonks, 1983; Fig. 1.1) and further sizeable alterations may not be beneficial (Flintham et al., 1997). While further reductions in plant height can cause yield penalties due to low biomass (Miralles and Slafer, 1995a) as a result of low radiation use efficiency, taller plants have poor biomass partitioning and high risk of lodging; major alterations in time to anthesis are not sensible as this critical stage has been carefully placed in a safer time frame in many wheat growing areas to avoid late frost and terminal drought (Whitechurch and Slafer, 2001). Future GY improvements might still depend on GN and so it is critical to improve our understanding on GN determinants and cause of negative relationships with AGW.

1.3. Wheat phenology and its genetic control

A greater flexibility of time to anthesis in wheat is the major reason for its adaptation which makes it the crop that is grown worldwide (Snape et al., 2001; Worland, 1996). And this flexibility in time to anthesis in wheat is possible because of the presence of genetic factors that respond to environmental stimuli like cold temperature (vernalization), photoperiod and temperature. Although temperature has universal effect (qualitative) on whole growth and development of wheat (e.g. crops: Johnson and Thornley, 1985; wheat: Rahman and Wilson, 1978; Slafer, 1996; in general metabolism of all living organisms: Clarke, 2004; Gillooly et al., 2002), the duration of individual developmental phases may have different degree of sensitivity for temperature (Slafer, 1996).

1.3.1. Vernalization (Vrn)

Requirement of prolonged exposure to cold temperature in wheat is termed as vernalization sensitivity and the genetic factors responsible for this sensitivity are called *Vrn* genes. Wheat is classified as winter and spring type based on the vernalization sensitivity, the former type is sown in autumn and has strict requirements of cold temperature to accelerate flowering (i.e. developmental progress is thus slowed down until the spring) while the latter is sown in spring and flowers without vernalization treatment. Earlier studies have shown that *Vrn* mostly affects vegetative phase i.e., until floral initiation or double ridge stage (Flood and Halloran, 1986a) but some recent studies working on lines with different *Vrn* allelic combination and vernalization treatment have shown the effect of *Vrn* beyond vegetative phase

(e.g. Slafer et al., 2015; Steinfort et al., 2017).

1.3.2. Photoperiod (Ppd)

Wheat is sensitive to length of the day and the response is mainly quantitative. The photoperiod sensitivity is controlled by *Ppd* genes. Almost all the phases viz., sowing to floral initiation, floral initiation to TS and TS to anthesis (LRP) are known to be affected by *Ppd* (González et al., 2005a; Whitechurch and Slafer, 2002). Photoperiod effect and *Ppd* genes influence GY indirectly by either lengthening time to anthesis or by optimising the length of the phase before anthesis. Responses of the *Ppd* genes are known to be influenced by the presence of *Vrn* allele (Steinfort et al., 2017) and vice versa interaction has been also recorded (Davidson et al., 1985).

1.3.3. Earliness per se (Eps)

The genotypes with insensitive *Vrn* and *Ppd* genes or sensitive genotypes after the vernalization and photoperiod requirements have been satisfied still display certain degree of differences in earliness which has been termed as earliness *per se*. The genetic factors responsible for these differences are called *Eps* genes and have been identified on almost all chromosomes (Sukumaran et al., 2016). *Vrn* and *Ppd* effects are quite obvious as the presence of their insensitive allele completely knock out the requirement of cold temperature and long day respectively. As *Eps* effects are normally rather subtle (Griffiths et al., 2009), both in relative and absolute terms, *Eps* genes are mostly identified in QTLs (Zikhali et al., 2014a). Recent studies have shown *Eps* effect on not only phenology but also spikelet number (Alvarez et al., 2016; Lewis et al., 2008) and spike fertility (Prieto et al., 2018a). It can be speculated that the indirect effect of *Eps* on grain yield attributes could be the probable reason for their indirect selection in the past (Alvarez et al., 2016). There have been fewer studies on detailed effect of *Eps* on various pre-anthesis phases (Lewis et al., 2008; Ochagavía et al., 2018a). And so far the study by Prieto et al. (2018a) is the only study in hexaploid wheat to understand the effect of *Eps* on spike fertility. Further, there have been speculations about interaction of *Eps* with temperature (Appendino and Slafer, 2003a; Bullrich et al., 2002) which needs to be understood better and study by Ochagavía et al., (2019) gave the first evidence confirming that interaction.

Wheat development is controlled by complex network of these three group of genes namely *Ppd*, *Vrn* and *Eps* (Slafer et al., 2015; Worland, 1996). *Vrn* and *Ppd* genes have played an important role in wheat adaptation and have helped to manipulate time to anthesis to suit the local climate and improve grain yield. The magnitude of effect of these genes on different developmental phases is different depending on the allele itself and background on which the genes is introgressed. *Vrn* and *Ppd* genes have been extensively studied to understand their precise effect on individual phases occurring before anthesis (González et al., 2005a; Miralles and Richards, 2000; Scarth et al., 1985; Steinfort et al., 2017; Whitechurch and Snape, 2003). There have been studies that have shown *Vrn* and *Ppd* effects on time to floral initiation (Rawson and Richards, 1993; Whitechurch and Snape, 2003) or terminal spikelet (Foulkes et al., 2004; Rawson and Richards, 1993; Scarth et al., 1985) or length of LRP (González et al., 2005a, 2003a; Miralles et al., 2000; Scarth et al., 1985; Whitechurch et al., 2007) or effect was found on all the pre-anthesis phases (González et al., 2005a).Considering major changes in time to anthesis may not be always desirable the *Eps* genes with minor effect serve as better tool compared to *Vrn* and *Ppd* in optimizing time distribution among pre-anthesis phases.

1.4. Future improvements in genetic gain and grain yield potential

Wheat genetic gains needs to be increased again to achieve yields matching expected rise in demand and this time in an ever changing and uncertain climate (Lobell et al., 2015; Reynolds, 2010). It is required to improve yield potential while keeping up with the reduction in gap between actual and potential yield in wheat. Understanding better the physiological mechanism underlying grain yield would assist in addressing the limitations imposed on genetic gain (Foulkes and Reynolds, 2015). Major traits such as plant height and time to anthesis that have once brought dramatic grain yield improvements are not that sensible anymore (reason explained above), and only fine-tuning them might be required. Therefore, to improve yield more significantly it is critical now to identify other relevant traits. Nonetheless, the yield improvements, however gradual, can be achieved through improving either biomass and its partitioning or/and time allocated for the individual developmental phases to maximize the grain yield attributes.

As discussed above the future grain yield improvements will mainly depend on further increasing GN than AGW (Slafer et al., 2014); and identifying traits that influence GN will eventually be instrumental in genetically increasing grain yield, although care must be taken to understand cause and consequence of the trade-offs between its components. Selecting for higher spike fertility and high grain yield might

also reduce the likely compensation caused by GN and AGW trade-off (Alonso et al., 2018).

Owing to the criticality of time to anthesis most wheat growing regions already have a narrow window of time that is most favorable for growth and development. But grain yield variability for a particular anthesis time is defined by the potential growth around time to anthesis (Abbate et al., 1997; Woodruff and Tonks, 1983) when the spike growth is very responsive eventually determining fertile floret number and so GN (Stockman et al., 1983). In the past *Vrn* and *Ppd* genes were extensively used to coarse tune anthesis time (González et al., 2005a; Miralles et al., 2000), which may not be an option anymore in many wheat growing regions where only fine tuning time to anthesis may be favorable (although it could still be sensible in improving or maintaining yield under the climate change scenarios; Chairi et al., 2018). In addition to minor changes in time to anthesis, altering the LRP at the expense of earlier stages would bring about yield improvements (e.g. Acuña et al., 2019). In this context, *Eps* genes are far more crucial than the other major genes (Griffiths et al., 2009) when the concern is to enhance regional adaptation (fine tune, Fig. 1.2). At least some *Eps* genes have been shown to affect time to anthesis by altering mainly LRP (Ochagavía et al., 2018a) suggesting they might affect floret development, which needs to be extensively studied.

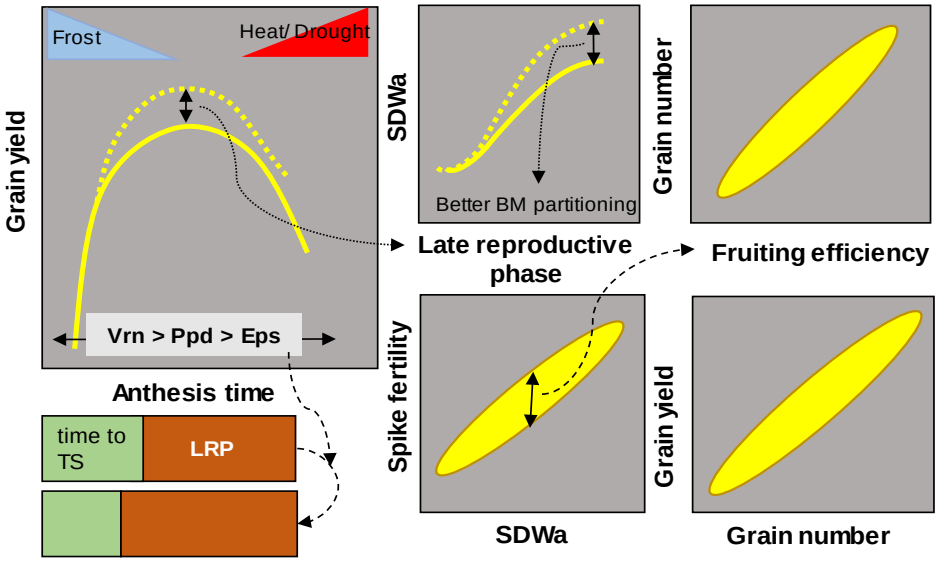

Fig. 1.2. Major alterations in time to anthesis may not be sensible but to improve spike dry weight at anthesis (SDWa) and spike fertility, late reproductive phase (LRP) can be lengthened with little changes in time to anthesis or at the expense of time from sowing to terminal spikelet (TS). For fine tuning anthesis time or re-adjusting pre-anthesis phases the importance of adaptation genes go in reverse order of magnitude of their effect on phenology (*Vrn* < *Ppd* < *Eps*).

Studies by Slafer et al. (2015) and Garcia et al. (2019) have shown genetic variability present in FE in modern cultivars and among various cereal species respectively, suggesting the scope to utilize this trait for real breeding program for improving yield potential. Although the importance of spike fertility or FE has been discussed recently current cultivars have reasonable genetic variability (Abbate et al., 1998). FE should be considered as a complementary trait to SDWa, and once SDWa of the cultivars has been maximized the further GY improvements will depend on FE (Garcia et al., 2019). Further, simultaneously the length of LRP should be increased (Fig. 1.2) which poses limitation in maximum attainable FE for a particular SDWa (Gonzalez-Navarro et al., 2016).

1.5. Material importance- where to identify new traits and variability?

As breeders are generally reluctant to use wild or exotic germplasm when pyramiding genes to improve yield (Moore, 2015; Singh et al., 2018) and normally prefer to keep crossings within their elite germplasm (Cowling, 2013), it is relevant to identify useful traits and their yield correlations within elite material where most of the favourable genes (disease resistance, phenology genes, plant ideotype and grain yield QTLs) have been put together through several decades of breeding efforts (Slafer, 2003). Elite material have relatively less variability for most of the traits compared to respective wild relatives or populations derived from wide crosses, but variability identified within this elite germplasm is most accessible source of genetic variability for real breeding programmes (when aiming to improve complex traits). Breeders tend to use elite lines in designing strategic crosses in order to attain transgressive segregation hence quantifying the magnitude of genetic variability for new traits and trait combinations in such material is effective for improving yield potential (Foulkes et al., 2011; Reynolds et al., 2012).

1.6. Objectives

The present thesis aims at improved understanding on the physiological traits underlying grain yield such as distribution of pre-anthesis phases, spike fertility and their influence on grain number. Also, within this aim to discuss the probable trade-off between physiological determinants and their causes as well as consequences. These general aims will be dealt in three distinctive objectives to evaluate:

- Elite lines with similar time to anthesis and plant height to identify traits and trait

combinations that affect grain yield. Evaluating possible genetic variability in phases occurring before and after terminal spikelet with similar time to anthesis (Chapter III).

- The functions of a newly identified *Eps* QTL - 7D beyond its known effect on time to anthesis. Duration of individual pre-anthesis phases and spike fertility as affected by this *Eps* QTL-7D (Chapter IV).
- The effect of *Eps* QTL-7D on different phenophases and floret development under contrasting *Ppd*-D1 background under long and short day. To study temperature interaction of the *Eps* QTL-7D under three temperature regimes in controlled conditions (Chapter V).

1.7. Outline of the Thesis

The present Thesis is divided into six chapters. These chapters include a general introduction and the main objectives (this Chapter I), followed by general procedures with the methodology used in most experiments throughout the Thesis (Chapter II). Then, three experimental chapters (III, IV and V), and a general discussion and conclusion of the entire thesis (Chapter VI). This last chapter highlights key results and conclusions of each chapter. Each experimental chapter is based on papers which have been submitted or prepared to be submitted to international journals. This approach has the advantage that each chapter can be read independently of the others, but the counterpart is that there are unavoidable repetitions, especially in their introduction and material and methods sections.

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Chapter 11: General procedures

2. Chapter II: General procedures

2.1. Briefing about experiments

This chapter includes the general procedures which were standard for most of the experimental chapters in the present Thesis that were standard in all the experiments. The thesis consists of three independent studies conducted under field condition (Chapters III and IV) and under controlled conditions (Chapter V). The detailed descriptions of a particular experiment related to each chapter will be dealt in the Materials and methods sections of respective chapter.

2.2. Growing conditions under field experiments

The field experiments were carried out avoiding water and nutrients stresses: experiments were always sown within the optimal dates of the region to maximize yield and fully fertilized. The experiments were fertilized with urea (46% N) at the initiation of tillering stage (DC21, Zadoks et al., 1974) at the rate of 150 kg N ha^{-1}. The experiments were irrigated to supplement the precipitation when found necessary. Weeds, pests and diseases were prevented or controlled using chemicals (following the doses prescribed by the manufacturer). The particular experimental design, size of the plots and density of plants (per m^2) is described in each respective chapter.

2.3. Measurements and calculation

2.3.1. Thermal time calculation

Thermal time was calculated considering "0 ºC" base temperature and minimum temperature (Tmin) was not below the base temperature and the maximum temperature (Tmax) was within the optimum temperature for the growing duration. The daily weather data was collected from Meteorological station of Meteocat (Servei Meteorologic de Catalunya) close to the experimental fields and Tmin and Tmax were used to calculate the average daily temperature. Thermal time is summation of daily average temperature (above the base temperature) for that phase or interval of time (Monteith, 1984).

2.3.2. Developmental stages

Developmental stages like seedling emergence (DC10), onset of tillering (DC21), onset of stem elongation (DC30), flag leaf emergence (DC39), heading (DC59), time to anthesis (DC65) and physiological maturity (DC95) was recorded in each experimental unit (plot) following the scale proposed by (Zadoks et al., 1974). Each experimental unit was visually observed and time when at least 50% of the plot showed that stage was recorded. Onset of stem elongation (OSE) was recorded by touching the main stem at the base to detect the first node (felt as a hard lump) which was repeated on several plants within the plot (http://www.cerealsdb.uk.net/cerealgenomics/cgi-bin/grain3.pl?topic=Stem%20elongation accessed on 08.09.2020). Data from apex dissection was used to correct for OSE using terminal spikelet (TS) stage as reference.

2.3.3. Above ground biomass, partitioning and yield

In all the field experiments plants in 1 linear meter were sampled at anthesis (DC65) and physiological maturity (DC95) to measure above ground biomass and yield components. Plants in sampling area were manually pulled out to recover the whole above ground biomass and taken to the laboratory where they were processed to record number of plants, shoots, and productive shoots (shoots bearing spikes) and plant height (stem length from the soil level to the base of the spikes). Leaves (leaf blades), stem (shoot with leaf sheath) and spikes were separated and dried in a hot-air oven at 65 ºC for 72 h after which dry weights were recorded. At physiological maturity, the spikes were threshed after measuring the dry weight to collect grains, later these grains were counted, and dry weight was measured to obtain grain number and average grain weight.

2.3.4. Leaf appearance dynamics

The leaf appearance was recorded in the studies discussed in Chapters IV and V. Three plants in each experimental unit were selected randomly from the central rows (field experiments) or pots (controlled conditions) and representing the stage of the crop in that whole plot and these plants were marked. The marked plants were identified and measurements of leaf number on main shoot using Haun scale (Haun, 1973) were made once or twice a week depending on the temperature and rate of advancement or treatment combinations in control conditions. Leaf appearance dynamics is shown by plotting the number of leaves emerged against the calculated thermal time (Fig. 2.1) or days. The statistically

significant slope determines the rate of leaf emergence at particular thermal time. Phyllochron, time interval between appearances of two consecutive leaf, is equal to inverse of the slope calculated from the trend (Wilhelm and McMaster, 1995).

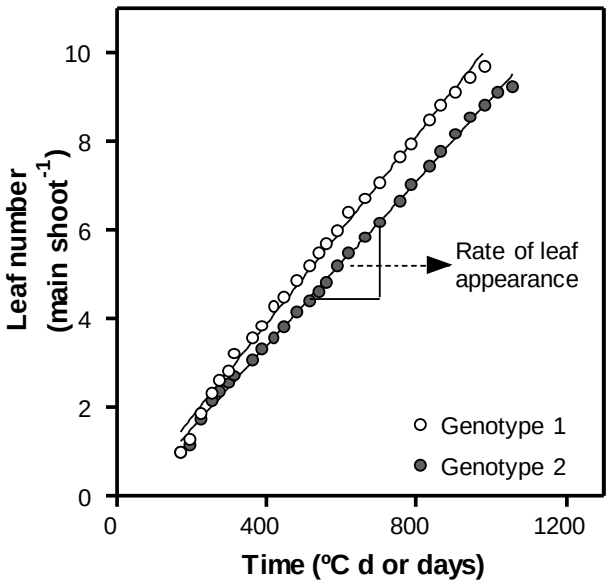

Fig. 2.1. Example of number of leaves plotted against time expressed either in ºC d or days for two hypothetical genotypes (Genotype 1 and 2). Slope of the trend was calculated for each genotype to derive phyllochron. Each data point is average of measurements taken in nine plants and standard error bar depicting standard error of mean.

2.3.5. Dissection of shoot apex and spike

2.3.5.1. Shoot apex to measure number of leaf and spikelet primordia

One plant per experimental plot was chosen randomly that represents the whole plot (avoiding border rows) and sampled at an interval of 5-7 days (depending on temperature) to study the stage of main shoot apex. The main shoot was dissected under binocular microscope (Leica MZ 8.0, Leica Microsystems, Heerbrugg, Switzerland) to evaluate number of primordia and stage of the apex (Fig. 2.2) following the scale proposed by Kirby and Appleyard (1981). Time taken to reach important apical stage like 2.5 (double ridge) and 3.5 (terminal spikelet) were plotted to compare between genotypes. Total primordia (leaf and spikelet) recorded at every sampling was plotted against thermal

time and the plastochron (time interval between two successive primordia) for the trend was calculated as the inverse of the slope.

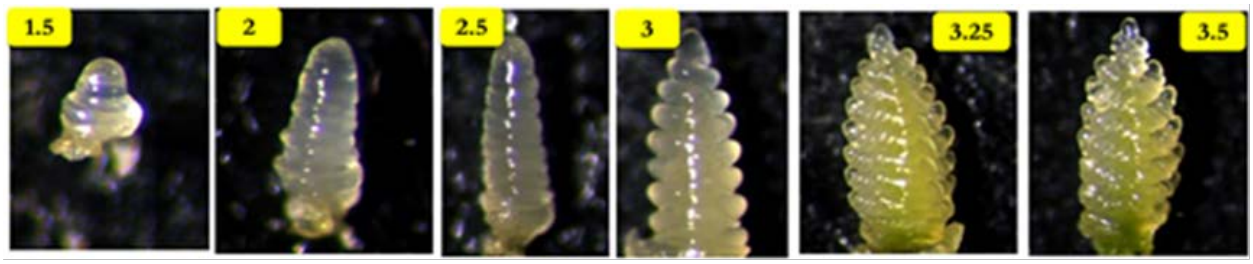

Fig. 2.2. Pictures depicting various apex stages denoted in the corner of each picture. The scale used to identify apex stage was proposed by Kirby and Appleyard (1981). Apex stage 1.5 and 2 are vegetative apex and 2.5 (double ridge) is reproductive stage and apex stage 3.5 is terminal spikelet stage.

2.3.5.2. Spike dissection to record floret development

From the terminal spikelet stage onwards one plant per each experimental unit was randomly selected avoiding border effect, although the selection of plant was random it was made sure that it was representative of the whole experimental unit. Plants were sampled at an interval of two days (twice or thrice a week) and spike was dissected under the microscope (Leica MZ 8.0, Leica Microsystems, Heerbrugg, Switzerland) to study floret development in three spikelets each from apical, central and basal position of the spike. Stage of the floret primordia was identified using the scale proposed by Waddington et al. (1983). Florets within a spikelet were numbered from 1 (F1) to n (Fn) and all the florets were studied from W3.5 (when primordia have differentiated as floret primordia) until W10 (floret is fertilized) or maximum stage reached (for the aborted florets).

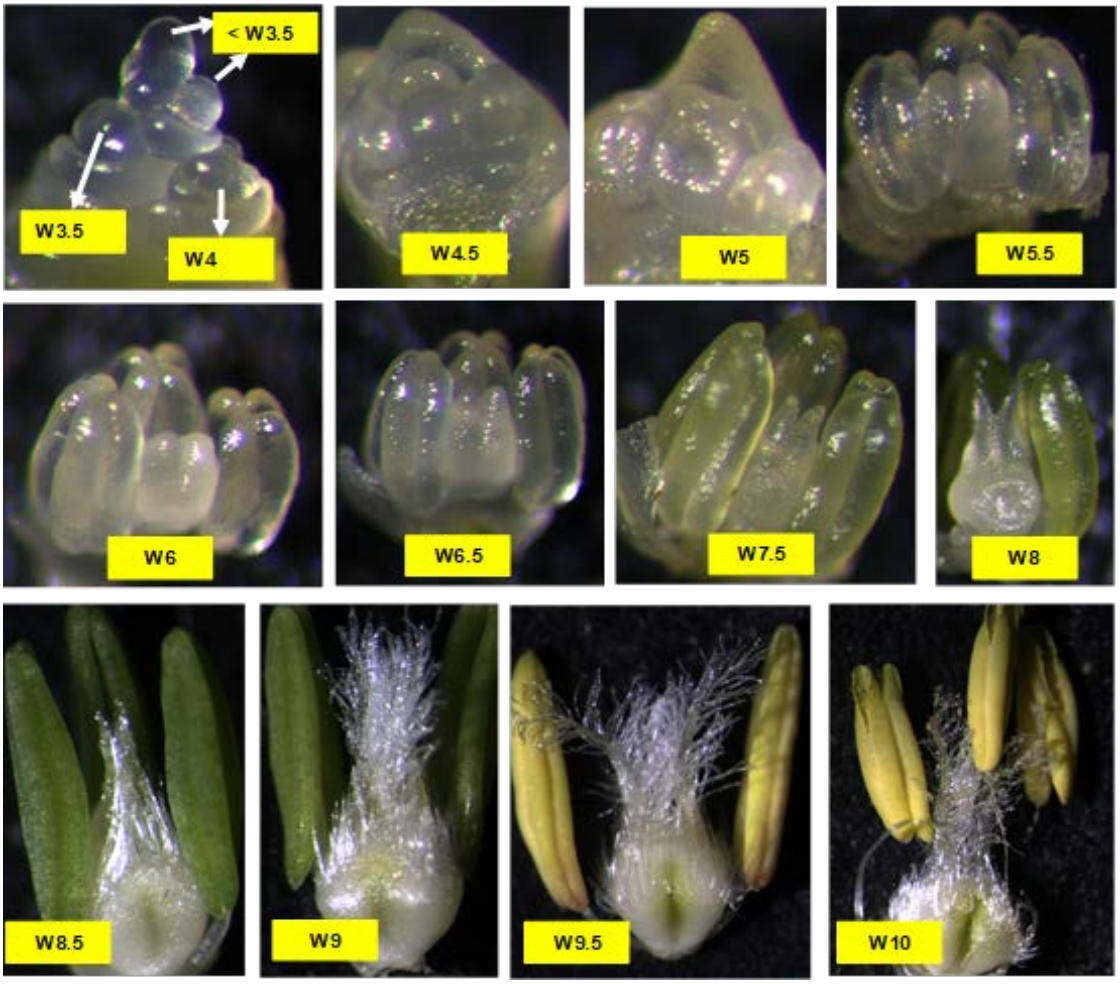

Fig. 2.3. Photo description of floret primordium with the floret score following the Waddington scale (Waddington et al., 1983). The pictures were captured during dissection using the camera attached to Leica MZ 8.0.

2.3.5.3. Floret development dynamics

Data from the spike dissection carried out from terminal spikelet stage to anthesis is generated to present floret score (from 3.5 to 10 or highest attained stage) in Waddington scale, a scale that is qualitative and dimensionless. For the purpose of presenting results that are meaningful in the final effect on the number of fertile florets the dynamics of florets is shown for those florets that reached at least the stage of development W4.5 (carpel primordium visible), which included up to the F6 (higher order florets would virtually never develop enough to produce fertile florets; indeed, a sixth floret primordium was only seldom fertile in central spikelets and never a sixth fertile floret was fertile in any of the spikelets analysed in detail). Average floret score for each floret (F1 to Fn) was calculated

considering three replicates (blocks) in each spikelet position and plotted against the time with the error bars depicting standard error of mean (Fig. 2.4). In some cases, the time to anthesis was extrapolated from averaging thermal time when all the F1 from three spikelet position reached W10 (case specified in the chapter).

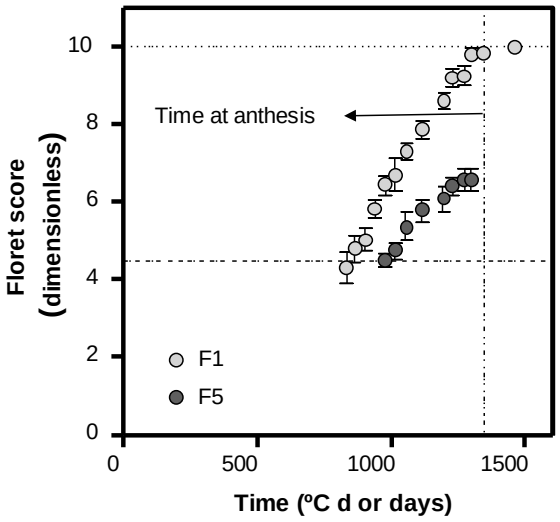

Fig. 2.4. Example of floret stage for F1 and F5 depicted against time expressed either as ºC d or days, floret score starting from W4.5 to W10 (F1) or highest attained stage in case of aborted floret (F5). Thermal time at F1 reaching W10 was considered as anthesis time.

2.3.6. Living floret primordia curve

Living floret primordia was derived from the individual floret (F1 to Fn) development curve. Then the sum of all the florets that continued to develop was calculated and plotted against thermal time which shows the maximum number of florets initiated and final number of florets (florets that reached stage W10) at anthesis (Fig. 2.5). Only florets that at least reached W4.5 (stage when stamen, pistil and carpel primordia are present, see fig. 2.3) were considered for calculating living floret primordia. While F1 and F2 florets invariably (genotype, spikelet position and growing conditions) reached W10, fate of F3 to F6 florets was depended on genotype, spikelet position, controlled or field condition but these florets at least reached W4.5. Florets F7-F10 in most cases did not even reach the threshold of W4.5 stage.

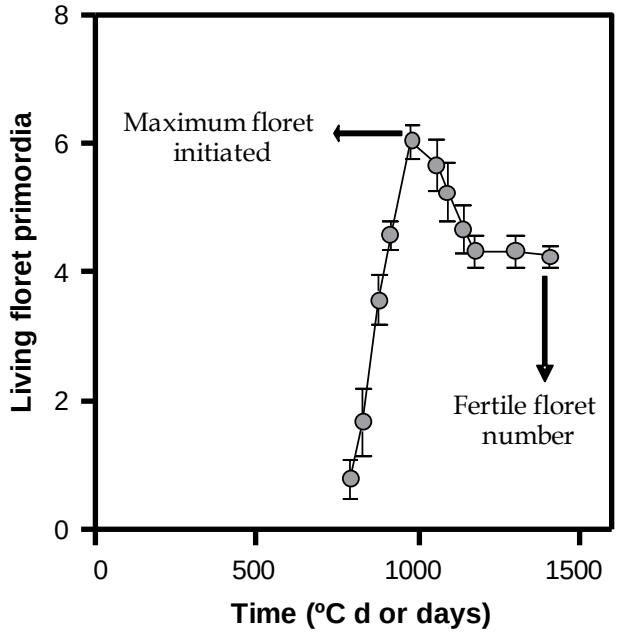

Fig. 2.5. Example of living floret primordia considering a threshold of W4.5 stage calculated by summation of averaged floret score plotted against time expressed either as ºC d or days indicating maximum florets initiated (florets at least reaching W4.5) and fertile florets after floret abortion.

2.3.7. Fertile floret mapping at anthesis

Three representative plants of the sampled meter were randomly selected and fertile floret per spikelet in all the spikelets in a main shoot spike was recorded. The floret that was at least at the green anther stage (>W8.5) was counted as fertile floret considering the florets within spikelet have asynchronous development. Then the number of fertile florets is mapped for every spikelet position from basal to terminal spikelet (S1 to Sn) like shown in Fig. 2.6.

2.3.8. Grain number mapping at physiological maturity

Three plants per each experimental plot from the sampled meter were randomly chosen to record the grain number per spikelet. The grains per spikelet was plotted against spikelets from basal to terminal spikelet (Fig. 2.6).

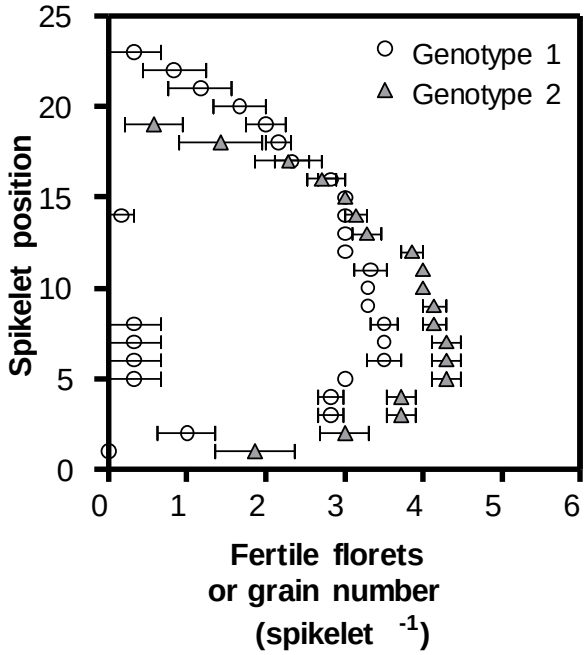

Fig. 2.6. Example for fertile floret or grain number mapped per spikelet at each spikelet position from basal to terminal spikelet for two hypothetical genotypes (Genotype 1 and 2) for comparison. Each data point is average from nine plants and error bar depicts the standard error of mean.

2.4. References

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Chapter III. Genotypic differences in wheat yield determinants within a NAM population based on elite parents

3. Chapter III. Genotypic differences in wheat yield determinants within a NAM population based on elite parents

3.1. Abstract

Future grain yield (GY) improvements require the identification of beneficial traits within the context of high yield potential and not just based on the pleiotropic effect of traits such as crop height and heading date. I evaluated 1937 lines from Nested Association Mapping (NAM) population derived from 13 bi-parental varietal crosses under field conditions. I selected 493 lines with similar time to anthesis to that of the two checks used in the study (across and within each family) which reduced the range of plant height in the selected lines. Yield components were measured in these 493 lines from which 231 lines were selected by excluding lines with lowest number of grains so excluded low yielding lines. Later the subset of 231 lines were evaluated in two field experiments (2016-17, CS1 and 2017-18, CS2). Numerical and physiological components of grain yield were measured. The two-step selection maximised GY within an acceptable range of variation for height and anthesis. GY in 231 lines showed very high G×E interaction. Taking both seasons together, the lines from upper and lower quartile GY groups to identify stable beneficial trait combinations for improved GY were selecetd. Differences in GY were explained by grain number driven by increased spike dry weight at anthesis (SDWa) and fruiting efficiency (FE). Increased GY was accompanied by sink limitation. The data points towards increases in grain number as the route towards future GY increases in wheat breeding.

3.2. Introduction

Present rates of genetic gains in wheat grain yield (GY) are insufficient to satisfy future demands (Reynolds et al., 2012) which is estimated to increase 50% by 2050 from current level of demand (https://www.cimmyt.org/work/wheat-research/; accessed on 08.09.2020). In recent decades the rate of genetic gain has decreased (e.g. Aisawi et al., 2015; Flohr et al., 2018; Maeoka et al., 2020), in many cases to a standstill (e.g. Acreche et al., 2008; Chairi et al., 2018; de Oliveira Silva et al., 2020; Lo Valvo et al., 2018). To address this problem, there is need to improve our understanding of physiological attributes likely to underpin future GY gains as well as to identify variation available within elite germplasm for these traits. Grain number per m^2 (GN) and average grain weight (AGW)

are the two most important GY components (Slafer et al., 2014). Owing to larger plasticity it is GN that has delivered most GY improvements (Abbate et al., 1995; Calderini and Slafer, 1999; Fischer, 1985; Reynolds et al., 2009; Serrago et al., 2013; Siddique et al., 1989a; Slafer et al., 1990; Slafer and Andrade, 1989), even though it has much lower heritability than AGW (Sadras and Slafer, 2012).

Past improvements in wheat GN and GY came through the gradual accumulation of beneficial quantitative variation as well as a limited set of step changes such as the widespread deployment of semi-dwarf genes, chiefly Rht-1 (e.g. Calderini and Slafer, 1999; Flintham et al., 1997) and improving adaptation by changing time to anthesis to be more adequate for a specific region (e.g. Araus et al., 2002) particularly through changes in photoperiod and vernalisation sensitivity (González et al., 2005b; Griffiths et al., 2009; Shaw et al., 2012; Whitechurch and Snape, 2003). Reductions in plant height mediated by *R*ht-1 enhanced biomass partitioning to the juvenile spikes prior to anthesis (Brooking and Kirby, 1981; Fischer and Stockman, 1986; Miralles et al., 1998) which in turn allowed for an improved development of florets resulting in higher GN (Ferrante et al., 2013; Fischer and Stockman, 1986; Miralles et al., 1998; Siddique et al., 1989a). Introgression of Rht-1 alleles and homoeoalleles increased harvest index (HI) through increased GN and improved GY without major changes in biomass and a reduction in AGW, that naturally did not compensate the GN benefits (Bingham and Wellington., 1981; Calderini et al., 1995; Flintham et al., 1997; Miralles and Slafer, 1995; Shearman et al., 2005; Siddique et al., 1989b). Adjustments in time to anthesis have been critical to improve GY through improving adaptation mainly when the life cycle of the original genotypes exploited in a region did not allow maximum use of available resources or for stress avoidance (Araus et al., 2002). These two traits, that have been critical to improve yields in the past, would be of limited importance in the future as they have already been optimised in major wheat growing regions (e.g. Acreche et al., 2008; Maeoka et al., 2020; Slafer et al., 2005).

Future gains in GN will provide the increased sink strength which many studies have pointed to as required to increase GY, because of the frequent sink limitation for grain filling in wheat (Borrás et al., 2004; Borrill et al., 2015; Reynolds et al., 2005; Serrago et al., 2013 and references quoted there in). Final GN is a highly integrative trait (highly plastic and with low heritability; Sadras and Slafer, 2012) with many of the development processes that lead to contributing to the final number. So, the identification of major genes or QTL directly and consistently controlling it is unlikely. For these reasons it is important to understand which traits are responsible for differences in GN within elite

material and to show how they could be deployed by breeders aiming to improve GY within elite × elite pedigrees by reducing sink limitation in their finished varieties.

While time to anthesis is tightly controlled in breeders selections around a local optimum, the partitioning of the cycle into different duration of phases occurring before and after terminal spikelet (TS) might still be improved (Slafer et al., 2001). Components of GN are formed from sowing to a few days after anthesis (Slafer and Rawson, 1994a) but the most sensitive phase is demarcated by TS and anthesis, the late reproductive phase or LRP (Slafer, 2003; Fischer, 2011). Thus, it has been hypothesised that lengthening the duration of the LRP, when floret development takes place, would improve GN (Miralles and Slafer, 2007).

By the time anthesis is reached the stage is set for the realisation of GN, in fact the spike dry weight at anthesis (SDWa) has been shown to be highly predictive of GN in a number of experiments (Fischer, 2011; Ferrante et al., 2013). The physiological support for this mechanistic relationship is that floret primordia survival is closely linked to SDWa and in wheat, being a cleistogamous plant, most fertile florets become grains after anthesis. The number of fertile florets at anthesis depends mainly on the balance between the initiation and mortality of floret primordia during the LRP (Kirby, 1988; Prieto et al., 2018b). Both floret mortality (Ferrante et al., 2013; González et al., 2011) and survival (Ferrante et al., 2013, 2012; González et al., 2005a; Siddique et al., 1989a) seems to depend on the availability of resources for spike growth from flag leaf appearance to anthesis. The physiological dissection of this point in development has been taken further by Slafer et al. (2015) using the concept of fruiting efficiency (FE, number of grains produced per unit SDWa) and showing that FE can be useful towards genetically improving wheat GY (see also empirical proofs in (Acreche et al., 2008; Flohr et al., 2018; Lo Valvo et al., 2018b; Zhang et al., 2019).

For a proper identification of traits or trait combinations that are likely to be important and useful in modern wheat breeding programmes (i.e. beyond traits like plant height which are already optimised), it is important to study trait relationships within the context of elite germplasm. Although the analyses restricted to elite genotypes will naturally reduce substantially the degree of variation that could be expected from unselected lines of wider crosses (and would consequently yield less clear relationships). The advantage is that the materials used would resemble better what realistic breeding does (crosses of elite × elite) when aiming to improve yield, and therefore results and conclusions would be more likely truly applicable in actual breeding programmes. Therefore, in the present study a very large population of elite lines (1937 lines of a Nested Association Mapping, NAM, population produced by

crossing elite parents) were grown in the field at Ciudad Obregón, Mexico (Cd. Obregón) and from these initial results I further selected a relatively small, yet rather large, sub-set of 231 lines that were considered best performing (within germplasm that was already elite) to study them more in detail in field experiments carried out in Bell-lloc d'Urgell, Spain (Bell-lloc) over two cropping seasons.

3.3. Materials and Methods

3.3.1. Experimental field conditions

The first field evaluation of the whole NAM population was carried out in the 2015-16 cropping season at CIMMYT's experimental station (within the Norman E. Borlaug Experimental Field, CENEB) in Cd. Obregón, Sonora, North-West Mexico (lat. 27°23' N, 109°55'W). The experiment was sown on 10 December 2015 in small plots ("hills", 80 cm between hills, 30 cm long) at a density equivalent to 5 plants per plot.

In the following two seasons (2016-17, CS1 and 2017-18, CS2), field experiments were carried out near Bell-lloc d'Urgell, Lleida, North-East Spain (Lat. 41°38' N, 0°44' E in CS1 and Lat. 41°37' N, 0°47' E in CS2). Experiments were sown on 16 November 2016 and on 17 November 2017, both at the rate of 125 kg ha^{-1} aiming to attain an effective plant density of 250 plants per m^2. The three experiments were carried out avoiding stresses: plots were always sown within the optimal dates to maximize yield, fully fertilized, irrigated, Weeds, pests and diseases were prevented or controlled. Nitrogen content of the soil from 0-90 cm layer was analysed in CS1 and CS2 experiments in the beginning of the experiments, analysis was carried out in eight samples that were randomly chosen and the average N content of the experimental area was 133.0 ± 9.3 Kg/ha and 115.3 ± 8.7 Kg/ha in CS1 and CS2 respectively. This soil nitrogen was supplemented with Urea which was uniformly applied to each plot at the rate of 150 Kg/ha at onset of tillering stage.

Meteorological data for the cropping periods were recorded from the Meteorological station located near CENEB for the first experiment and from the Meteorological station of Meteocat (Servei Meteorologic de Catalunya) close to the experimental fields in the last two experiments (Table 3.1).

In Bell-lloc (where the more detailed experiments were carried out), the average temperature for the whole cropping duration (November to July) of CS1 was 12.6 ºC whereas CS2 was 11.7 ºC. At the critical stage of anthesis both minimum and maximum temperatures were slightly higher during CS1 than CS2. In general, temperatures, both minimum and maximum were within the ranges normally

occurring in the region during past 5 years. As mentioned above, the experimental fields were irrigated as needed: in Bell-lloc both experiments were irrigated around anthesis but in CS2 an additional irrigation was given at seedling emergence stage as late fall – early winter of 2017 was unusually dry (Table 3.1). Thus, there was only one irrigation in CS1 (on 19 April 2017) and two in CS2 (on 14 December 2017 and 5 May 2018). Each irrigation was equivalent to 80 mm of rainfall.

3.3.2. Genotypes and experimental design

In the experiment at Cd. Obregón the whole NAM population was evaluated while in the two field experiments at Bell-lloc I grew a selection of this population. The NAM population was generated from 13 bi-parental crosses where both parents in each cross were elite spring wheat varieties selected for having particular traits of interest to be included in the crosses. The parents of the crosses used were (i) Paragon, one of the best UK spring wheat cultivars considering yield potential and disease resistance, was the most common parent used; (ii) four CIMCOG (CIMMYT Core Germplasm: Orford et al., 2014) lines viz.: CIMCOG 49, 47, 3 and 32 characterised for their high values of biomass, grains per spike and harvest index; (iii) Weebill, a cultivar well known for having its high yield associated to superior average grain weight; (iv) MISR1, SUPER152, Pfau, Waxwing and Baj, all parents selected for their high yield related to earliness in time to anthesis; and (v) Wyalkatchem a high performing Australian variety. The 13 families were the lines derived of the following crosses: (1) Weebill × CIMCOG3, (2) Weebill × CIMCOG32, (3) Paragon × Pfau, (4) Paragon × Baj, (5) Paragon × Wyalkatchem, (6) Paragon × (Becard × Kachu), (7) Paragon × MISR1, (8) Paragon × Waxwing, (9) Paragon × Garcia, (10) Paragon × Super151, (11) Paragon × Synth type, (12) Paragon × CIMCOG47, and (13) Paragon × CIMCOG49 (please note that the order of the crosses mentioned here from 1 to 13 will be followed in the result section). Detailed descriptions of the populations, including Axiom 35K genotype files and genetic maps can be found at https://data.cimmyt.org/dataset.xhtml?persistentId=hdl:11529/10996, accessed on 08.09.2020. All germplasm is deposited at the CIMMYT genebank.

Table 3.1. Meteorological data for experiments in Ciudad Obregón 2015-16, and in Bell-lloc 2016-17 (CS 1) and 2017-18 (CS 2): monthly average of minimum (T min) and maximum (T max) temperatures (± standard error) as well as monthly cumulative precipitation. In all cases data are provided for the growing season (from the month of sowing to that of harvest).

		Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul
T min (ºC)	**Cd. Obregón 2015-16**		8.08±0.54	5.79±0.31	8.64±0.56	9.84±0.40	11.24±0.39	14.05±0.57	22.12±0.60	
	Bell-lloc 2016-17	2.73±0.57	1.48±0.42	-1.23±0.66	2.70±0.57	3.50±0.44	4.95±0.45	9.60±0.46	15.10±0.51	16.42±0.41
	Bell-lloc 2017-18	-2.42±0.77	-1.67±0.64	1.80±0.67	-0.60±0.66	3.39±0.43	6.56±0.53	10.73±0.63	14.48±0.37	16.90±0.25
T max (ºC)	**Cd. Obregón 2015-16**		26.08±0.75	25.35±0.38	28.45±0.58	27.79±0.51	31.15±0.39	34.46±0.37	37.54±0.35	
	Bell-lloc 2016-17	13.90±0.50	6.96±0.84	8.45±0.81	13.69±0.48	18.49±0.65	21.78±0.67	26.80±0.70	32.09±0.91	32.73±0.63
	Bell-lloc 2017-18	13.28±0.64	8.37±0.61	12.52±0.75	10.86±0.78	15.93±0.52	20.43±0.86	24.02±0.49	29.48±0.70	33.75±0.39
Rainfall (mm)	**Cd. Obregón 2015-16**		0.2	2.1	2.1	8.4	1.0	0.3	1.7	
	Bell-lloc 2016-17	62.1	7.5	14.4	7.2	102.0	22.5	18.1	24.3	5.5
	Bell-lloc 2017-18	0.2	11.7	27.2	44.0	48.5	77.2	53.8	3.0	21.3

In the experiment at Cd. Obregón, the original set of 1937 lines were grown in un-replicated hill-plots together with checks (the parents of the crosses and two well adapted genotypes, Reedling and Sokoll) replicated across the whole experiment. Plots were arranged as different families with embedded checks in an augmented design (all in all considering the lines of the NAM, parents and replicated checks there were 2120 hill plots).

In the two experiments conducted in Bell-lloc, 231 lines were grown which was a sub-set from 1937 lines selected based on their field performances in the initial experiment at Cd. Obregón. Treatments consisted of 231 selected lines grown in un-replicated plots together with replicated check plots across the experiments using augmented design in a regular grid, design which is commonly used to test large lines in early generation where the scarcity of the seeds does not allow replication of lines (Müller et al., 2010). Each plot consisting of 6 rows was 0.2 m apart and 4 m long. Three cultivars viz., Paragon, Garcia and Paledor were the checks used both to quantify the spatial heterogeneity across field and as a reference for performance of well adapted cultivars. The arrangement of these three checks was in a way that each row had two checks (different check cultivar) and each column had at least one check. The experimental design used here dedicated 10% of the experimental plots to check plots which is within the recommended percent (10-20%) in such designs (Martin et al., 2006). Paragon was used, as it was the most common parent of the studied NAM population while Garcia (http://www.genvce.org/variedades/trigo-blando/invierno/garcia/; accessed on 14.01.2020 or http://www.agrusa.com/Semillas.php?_b=&_un=1&_do=18&_tr=19; accessed on 08.09.2020) and Paledor (http://www.genvce.org/variedades/trigo-blando/invierno/paledor/; accessed on 14.01.2020 and http://www.agrusa.com/Semillas.php?_b=&_un=1&_do=18&_tr=22; accessed on 08.09.2020) were chosen to be two of the best performing local cultivars at the time the study was conducted. Paledor was indeed a check in the variety trials at least until the cropping season immediately before the CS1 (https://genvce.org/wp-content/uploads/2019/12/informe-genvce-cereal-de-invierno-2015-2016.pdf, accessed on 27.08.2020). Plots in both experiments were arranged in the field following an augmented randomized complete block design with random allocation of un-replicated 231 genotypes and replicated 3 checks (i.e. there were 26 check plots arranged in order to have two check plots in each of the 13 rows of plots arranged diagonally across rows). The layout of the experiments had 13 rows and 20 columns of plots making it a total of 260 plots, of which 257 corresponded to lines and checks in which traits were measured (the other three plots were sown to complete the rectangular field layout but were not measured). In addition, the whole experiment had

a set of 70 border plots that were not considered for measurements (were sown and maintained to avoid border effects on the plots allocated to rows 1 and 13 and to columns 1 and 20 of the measured plots).

3.3.3. Measurements and determinations

In the field experiment conducted at Cd. Obregón plant height and anthesis date were determined in all the 2120 hill plots. Based on these determinations, 493 lines, which had similar time to anthesis to that of the checks and discarding extremely short lines, were sampled at maturity and yield per hill as well as AGW were determined. Of these 493 lines, the 231 lines that exhibited best field performance were selected to be evaluated in the more detailed study carried out over the following two seasons in Bell-lloc.

In the two field experiments in Bell-lloc, in each plot different stages of development were determined using the decimal code developed by Zadoks et al. (1974): seedling emergence (stage DC10), onset of stem elongation (DC30), flag leaf emergence (DC39), heading (DC59), anthesis (DC65) and physiological maturity (DC95). All the stages were recorded when 50% of the plot showed that stage by monitoring each plot regularly (from once a week to thrice a week, depending on temperature). The onset of stem elongation (OSE) was determined by touching the main shoot at the base just above the ground to detect the first node and was repeated on several plants in each plot to record the stage for that plot. Later, the OSE data from a parallel but smaller experiment conducted in the same field, in which the stage of TS was recorded by periodic dissection of the apex, was used to estimate the timing of TS from the OSE measurements. Length of phenological phases was estimated in thermal time with base temperature of 0 ºC.

Plants were sampled at anthesis (stage DC65) and physiological maturity (DC95) from each individual plot from 1 linear meter which was chosen randomly (from any of the 4 central rows and avoiding the extreme 25 cm of the rows that were left as borders). Plants in that sampling area were manually pulled out to recover the whole above ground biomass and taken to the laboratory where they were processed to record number of plants, shoots, and productive shoots (shoots bearing spikes) and stem length from the soil level to the base of the spikes. Leaves (only leaf laminae), spikes and stems (including leaf sheaths) were separated and dried in a hot-air oven at 65 ºC for 72 h after which dry weights were recorded. At physiological maturity, spikes were dried and weighed and were threshed to obtain grains.

Later, the grains were counted and dried again for at least 24 h to measure the grain weights.

3.3.4. Analyses

For the data from field experiment in Cd. Obregón only descriptive statistics were performed. Data from the field experiments in Bell-lloc were analysed using GENSTAT Pro (Version 19) in the preliminary single environment analysis where checks are considered as extra genotypes that are replicated and effect of spatial heterogeneity on un-replicated lines were accounted for using variation observed in checks which is then used to estimate Best Linear Unbiased Estimates (BLUEs). Relationships between traits were analysed with linear regressions.

3.4. Results

3.4.5. Genetic variation in the whole NAM population and selection of a sub-set

Expectedly, the ranges of variation in phenology and in plant height were rather large when considering the whole NAM population of 1937 lines (Fig. 3.1). Time to anthesis ranged from c. 75 to c. 120 d (Fig. 3.1a), equivalent to a thermal time range from c. 1150 to c. 2000 ºC d with a base temperature of 0 ºC. This large degree of variation was due to a few parents that had a considerably longer time to anthesis than most others in Cd. Obregón as well as a large transgressive segregation particularly for longer periods to anthesis, as the longest times to anthesis in the lines analysed exceeded, by c. 10 d (c. 212 ºC d), the already large range of variation shown by the parents of the population (Fig. 3.1a). This was in part due to the inclusion of cultivars possessing valuable yield-determining traits beyond time to anthesis (such as Paragon) with a strong photoperiod sensitivity conferring late flowering and maladaptation in Cd. Obregón though many of the lines derived from Paragon would (c. three quarter of the parents differed in time to anthesis by less than a week in this growing condition and half of them flowered within the two days of difference shown by the two well adapted genotypes used as checks; Fig. 3.1a). As the study was aimed at identifying traits of value beyond time to anthesis and plant height, the data from the first experiment was used to select against variation in time to anthesis that exceeded that of the best adapted local check varieties. Thus, the range of variation in time to anthesis in the selected 493 lines (which were then sampled at physiological maturity to measure hill-plot yield and AGW) was dramatically reduced (Fig. 3.1a), and

could not be further reduced when selecting the 231 lines for later experiments (Fig. 3.1a).

Plant height in the whole NAM population also varied hugely, from c. 0.25 to almost 2 m (Fig. 3.1b) and in this case mostly due to large transgressive segregation (likely due to segregation of *Rht* alleles resulting in some lines being tall and others double dwarf), as parents of the 13 crosses ranged in height from c. 0.6 to 1.1 m and most parents had a height very similar to that of the two well adapted checks (Fig. 3.1b). The selection of lines that had time to anthesis in the narrow range of best adapted checks reduced the range of variability in height to c. 0.5 to 1.2 m and the final selection of lines to be further tested in later experiments reduced that variation further by discarding the shortest plants (<0.64 m; Fig. 3.1b).

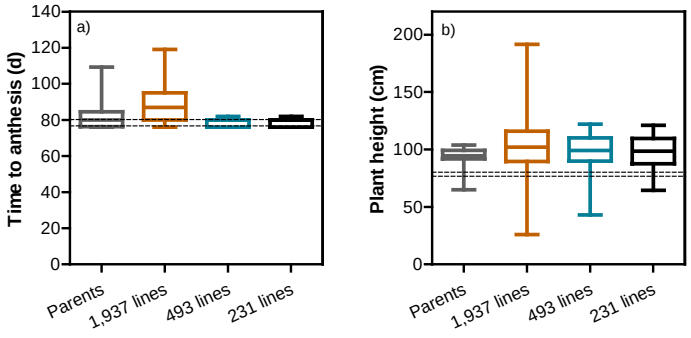

Fig. 3.1. Boxplots for time to anthesis (a) and plant height (b) from the experiment carried out in Cd. Obregón (NW Mexico) in 2015-16 considering the variability within the parents of the 13 crosses, the whole original NAM population of 1,937 lines, the 493 lines that were sampled and for which yield components were determined, and the 231 lines that were finally selected to be further studied in later field experiments carried out in Bell-lloc (NE Spain). Dashed lines show the values corresponding to two well adapted genotypes used as checks in the experiment, viz. Reedling and Sokoll.

To produce the final selection of the subset of 231 lines to be analysed in more detail, the hill-plot yield and yield components of the 493 lines that were sampled in the experiment were considered. The range of GY and its two major components were relatively large (Fig. 3.2), even though these lines displayed virtually no difference in time to anthesis and exhibited a range of plant height that is substantially reduced compared to the whole NAM population. Indeed, variation in time to anthesis or in plant height explained a negligible proportion of the genotypic variation in GY among the 493 lines (0.4% and 6.6%, respectively). As GY was more related to the number than to the weight of grains, and these major components were negatively related to each other (Fig. S3.1), the selection was

made eliminating the lines with lowest number of grains and lowest GY. Consequently, the selected sub-set of 231 lines reduced the variability in GY and its two components shown in the set of 493 lines, through maintaining the lines with highest GY and GN per hill as well as with intermediate values of AGW (Fig. 3.2).

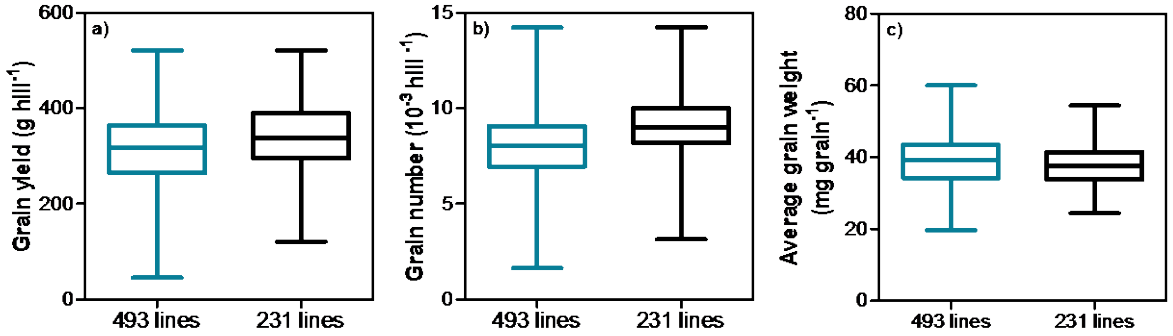

Fig. 3.2. Boxplots of grain yield (a) and its two major components, grain number (b) and their average weight (c) in the experiment carried out in Cd. Obregón (NW Mexico) in 2015-16 considering the variability for the 493 lines that had similar time to anthesis to that of the well adapted checks, and the 231 lines that were further selected to vary less in plant height and which were finally selected to be further studied in later field experiments carried out in Bell-lloc (NE Spain).

3.4.6. Genetic variation in, and relationships between, GY and phenology within the selected lines

When in the next two seasons these selected 231 lines were grown in Bell-lloc, the range of variation in time to anthesis was larger than for the same lines which had been selected in Cd. Obregón with the aim of constraining anthesis data. However, the timeframe of anthesis was much narrower than would be expected from the whole NAM (n=1937) or a random selection of it. Even though the length of cropping cycle is longer in Spain than Mexico, the range in time to anthesis for the selected panel of 231 lines was much narrower than the original population of 1937 lines evaluated in Cd. Obregón (cf. Figs. 1a and 3a and b). Having said that, it is also true that although lines were selected for having similar time to anthesis within the families, there was a noticeable variation, not only considering the whole population (1188-1481 ºC d in CS1 and 1209-1525 ºC d in CS2) but also within most families (Fig. 3.3a and b). There was a reasonable degree of consistency for thermal time to anthesis between the two cropping seasons (Fig. 3.3c).

The same was true for plant height: lines differed in height both across and within families but genotypic differences in height were reasonably consistent across both seasons (Fig. S3.2).

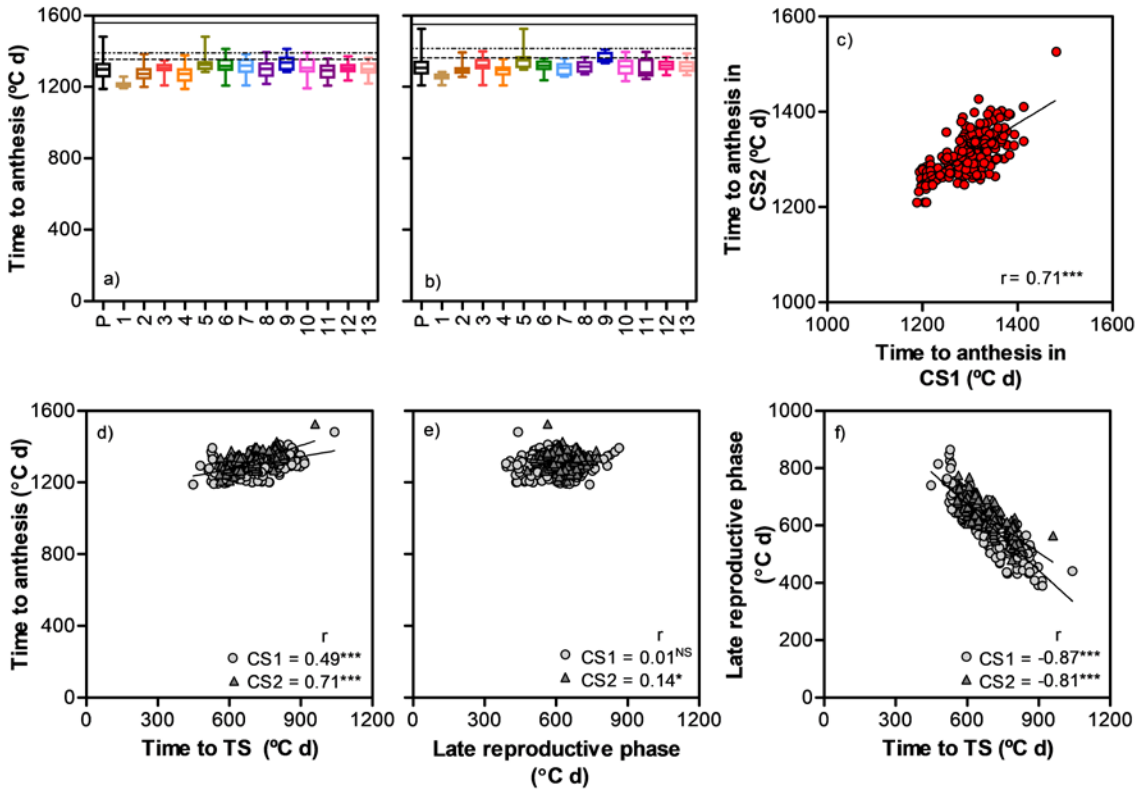

Fig. 3.3. Upper panels: Boxplots showing variability in time from sowing to anthesis within the whole population (P) and within families (13 bi-parental crosses) along with three checks Paragon (solid line), Paledor (dotted line) and Garcia (dashed line) in the first (CS1, a) and second cropping season (CS2, b), and consistency of time to anthesis over the two cropping seasons (c). Bottom panels: Relationships between time to anthesis and its component phases: time from sowing to terminal spikelet (TS, d) and time from then to anthesis, i.e. the late reproductive phase (e); as well as between the two component phases (f) for the 231 lines grown in the first (CS1) and second (CS2) cropping seasons. Coefficients of correlations are shown for each cropping season. Note: The crosses corresponding to serial number 1-13 is given in materials and methods; graphs c, d, e and f do not include checks; origin of the graph c does not begin at 0. Significance level: * $p < 0.05$; *** $p < 0.001$; NS= non-significant.

Genetic variation in thermal time to anthesis was related to variation for each of the two component phases considered: time from sowing to TS (Fig. 3.3d) and time since then to anthesis (Fig. 3.3e), though the correlations were stronger with the initial phase to TS, embracing the vegetative and early

reproductive phases, than with the LRP, suggesting that variation in time to anthesis was mainly controlled by the duration of the first phase in this panel. Furthermore, there was a clear trend for compensation between duration of these two phases both the seasons (Fig. 3.3f), that was naturally only partial (otherwise there would have been no variation in thermal time to anthesis), as revealed by the slopes that were higher (i.e. less negative) than -1 (-0.76 and -0.57 in CS1 and CS2, respectively). This means that in both cropping seasons it was possible to identify lines with the same time to anthesis but differing oppositely in the duration of the phases of leaf and spikelet initiation and of floret development.

Genotypic variation in GY was large (440 to 1181 g m^{-2} and 459 to 1067 g m^{-2} in CS1 and CS2, respectively). And once again, the variation across the subset of the NAM population reflected more the variation within families than differences across families (Fig. 3.4a and b). For most of the families, and therefore for the whole population, there were several lines with greater GY than the local checks, Paledor and Garcia, which were modern commercial high-yielding cultivars. However, unlike with time to anthesis and plant height, there was a very large G×E interaction for GY, evident from the absence of significant relationship between GY of the lines across the two cropping seasons (Fig. 3.4c).

Although the differences in time to anthesis and plant height could potentially interfere with the relationships between GY and traits other than these two, such potential interference would not be critical in this study as there were no clear relationships between GY and either time to anthesis (Fig. 3.4d) or plant height (Fig. 3.4e). Although time to anthesis was significantly related to GY in CS2, it explained only 7% of the GY variation, whilst time to anthesis in CS1 and plant height in both seasons explained less than 1% of the variation in GY (Fig. 3.4d and e).

Even though the total time to anthesis did not explain differences in GY within the whole population, the partitioning of that time into phases occurring before or after TS seemed to have some significance: overall lines and within each of the two seasons GY tended to be negatively related to the duration of the first phase, time from sowing to TS (Fig. S3.3a) and positively related to the length of the following phase, the LRP (Fig. S3.3b). However, even when statistically significant, these relations were weak.

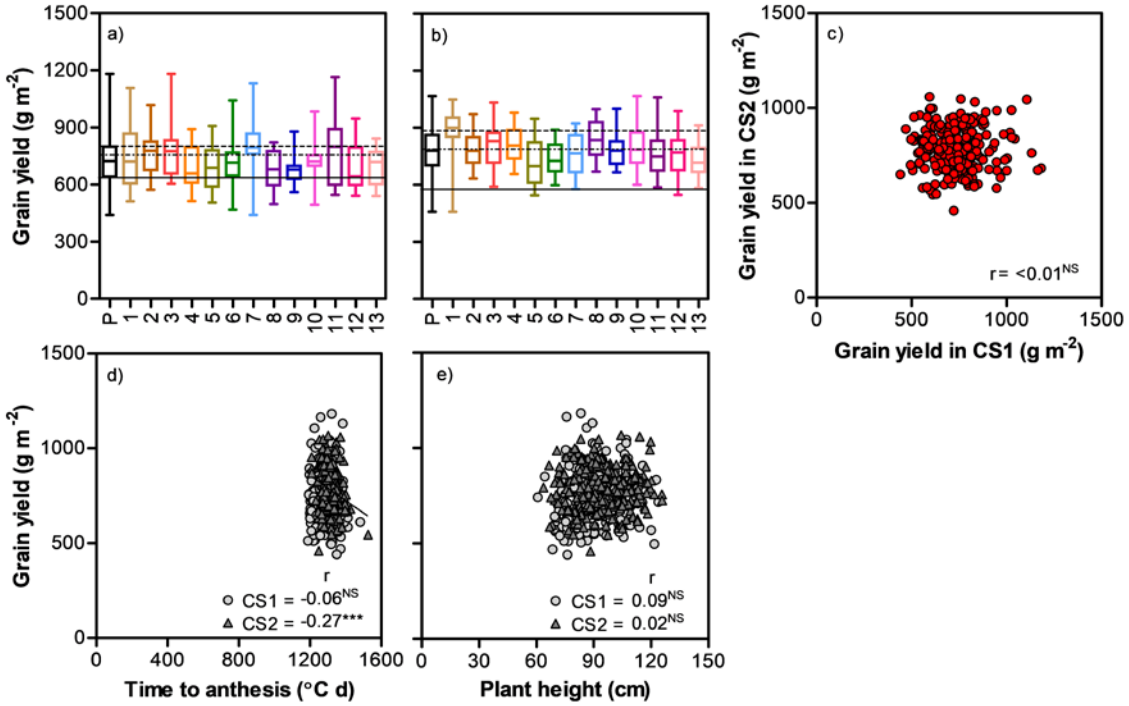

Fig. 3.4. Upper panels: Boxplots showing large variability for grain yield within the whole population (P) and within each family (13 bi-parental crosses) along with three checks Paragon (solid line), Paledor (dotted line) and Garcia (dashed line) in the first (CS1, a) and second cropping season (CS2, b) and inconsistency for grain yield over two seasons (c). Bottom panels: Relationships between grain yield and either total time to anthesis (d) or plant height (e). Coefficients of correlations are shown for each cropping season. Note: The crosses corresponding to serial number 1-13 is given in materials and methods; graph c, d and e do not include checks. Significance level: *** $p < 0.001$; NS= non-significant.

Taking into account the large G×E interaction (reflected by the lack of consistency in GY between CS1 and CS2) and our aim to identify trait relationships that can suggest traits relevant for further raising yield through breeding, I identified sub-groups within the sub-set of 231 lines that consistently expressed the extremes of GY over the two seasons: all lines that were part of the bottom and top quartiles of GY in both seasons, low- and high-GY (GY_L and GY_H, respectively) were selected. Applying this criterion, there were 13 GY_L and 11 GY_H lines.

Naturally these two sub-groups of lines differed in GY highly significantly, with no overlap between them (i.e. the lowest-yielding line of GY_H clearly out yielded the highest-yielding line of GY_L; Fig. 3.5). However, there were also clear genotypic differences in GY within each of these two sub-groups

(Fig. 3.5). By virtue of the data selection made, major genetic variation in GY between the two sub-groups were highly consistent across seasons. The focus was on the analysis of traits determining GY in the selected lines within and across these GY_L and GY_H. But, to extend the understanding on the relationships between traits across the whole sub-set of 231, these relationships will be reported, naturally for each season separately due to the large G×E interaction in GY, in supplementary materials. The mainstream relationships will be shown both for the two sub-groups separately (highlighting whether the considered trait was relevant or not for the genetic variation within GY_L and GY_H lines) and for all of them together (highlighting the contribution of the trait to produce the consistently highest-yielding lines of the population) and the variation levels in these traits between these two sub-groups will be described.

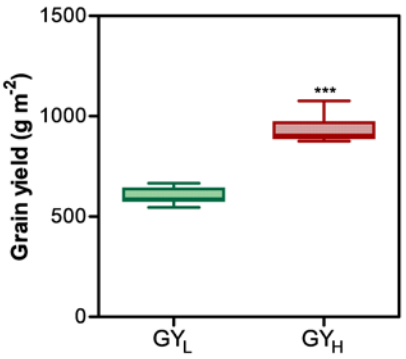

Fig. 3.5. Boxplot depicting variation in grain yield between selected sub-groups of lines being consistently low- and high-yielding in both cropping seasons (GY_L and GY_H, respectively). Significance level: *** $p < 0.001$.

3.4.7. Determinants of grain yield differences in selected lines

GY variation within the GY_L and GY_H lines was totally unrelated to time to anthesis (Fig. 3.6a). Regression of overall lines, i.e. considering both groups, did show a negative relationship (Fig. 3.6a) with the lines from sub-group GY_L slightly later than that of GY_H (Fig. 3.6b). However, the influence of this difference on GY between the two sub-groups would have been negligible for several reasons. Firstly, the overall GY variation explained by time to anthesis variation was relatively small (c. 35%). Secondly, the groups show extensive overlap to the extent that many GY_H lines have the same time to anthesis that many GY_L lines (Fig. 3.6a) still having substantially higher yields (Fig. 3.5). In fact, only few lines in each sub-group account for the significance of the difference in time to anthesis

between the two yielding categories. Finally, and in relation to that distribution, the average time to anthesis between GY_L and GY_H lines was only slightly different (70 ºC d, equivalent to c. 3 d; Fig. 3.6b) and that would hardly explain the large difference in average yield (>300 g m^{-2}; Fig. 3.5).

Regarding plant height, although relatively more variable than time to anthesis, the lack of influence of this trait on GY was even more clear, as the relationships were not significant both within and across yielding sub-groups (Fig. 3.6c), and the range of variation in plant height between the two sub-group contrasting in GY was totally overlapped implying that across them the difference in height was not significant (Fig. 3.6d).

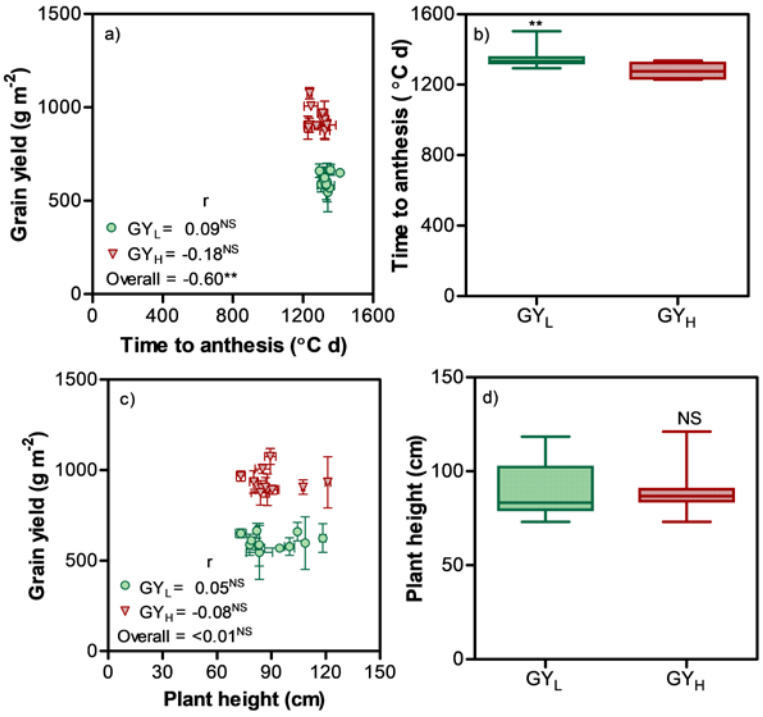

Fig. 3.6. Relationships between yield and either time to anthesis (a) or plant height (c) for the selected sub-groups of low and high yielding lines (GY_L and GY_H, respectively); and boxplots describing the variation in these traits for the two sub-groups (b, d). Coefficients of correlations are shown for each sub-group individually and for the overall data across both sub-groups. Significance level: ** $p < 0.01$; NS= non-significant.

The fact that neither of these two traits had a relevant role is important as the objective was to identify likely traits responsible for differences in GY of elite material other than time to anthesis and plant height. And this lack of relevance was also evident if the analysis were made with the whole sub-set

of 231 lines (see above and Fig. 3.4d and e).

Considering the two major GY components, it was clear that variations in GY were almost solely explained by GN (Fig. 3.7). Considering the variation in GY within sub-groups, it was exclusively explained by those in GN (though the coefficient of correlation was only significant for the GY_L lines; Fig. 3.7a), as GY was completely unrelated to AGW within any of the two sub-groups (Fig. 3.7c); although it is also true that the highest yielding line in the GY_H sub-group had intermediate GN but the highest AGW within that sub-group (Fig. 3.7a and c). But most importantly when trying to determine the reasons why the GY_H sub-group out-yielded the GY_L sub-group, GN was a far more robust determinant of GY than AGW considering all lines across both sub-groups (cf. Fig. 3.7a and c, where it can be seen that more than 80% of the overall variation in GY was related to that in GN, while this percentage was less than 40% when considering AGW).

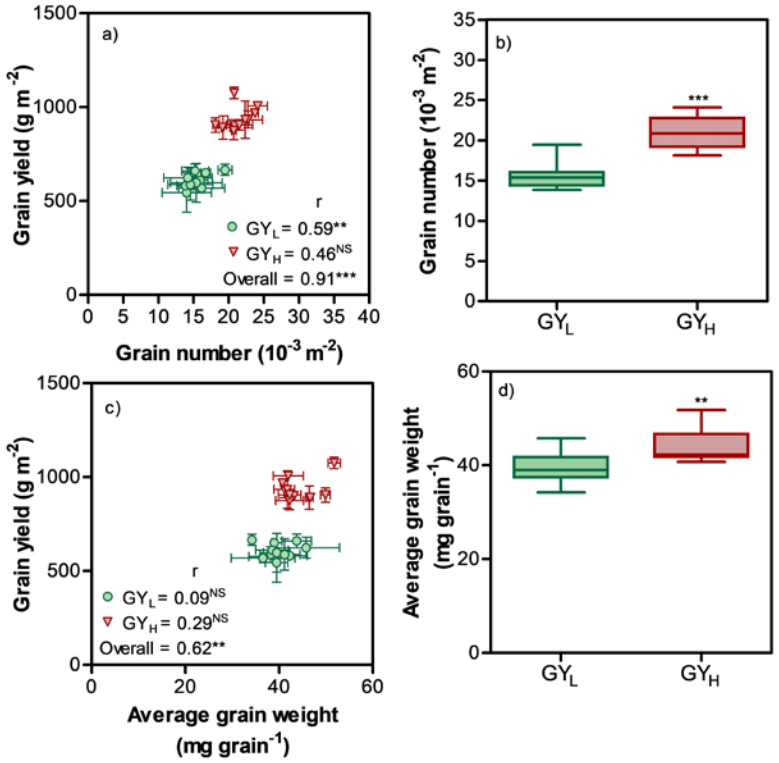

Fig. 3.7. Relationships between grain yield and its two major components: grain number (a) and average grain weight (b) for the selected sub-groups of low and high yielding lines (GY_L and GY_H, respectively); and boxplots describing the variation in these traits for the two sub-groups (b, d). Coefficients of correlations are shown for each sub-group individually and for the overall data across both sub-groups. Significance level: ** $p < 0.01$; *** $p < 0.001$; NS= non-significant.

Finally, whilst there was virtually no overlap between the ranges of GN of GY_L and GY_H lines, which differed as groups significantly (in average GY_H lines had almost 40% more grains m^{-2} than GY_L lines; Fig. 3.7b), AGW of GY_L and GY_H lines display noticeable overlapping (in average GY_H lines had c. 10% heavier grains than GY_L lines; Fig. 3.7d).

The relationship between these GY components were clearly negative within each of the two sub-groups (supplementary Fig. S3.4). However, this did not represent complete compensation as in both sub-groups increasing GN increased GY (Fig. 3.7a). Furthermore, the negative relationship lost significance when all lines were considered together as the GY_L lines did have fewer grains but not so consistently lighter (supplementary Fig. S3.4).

The proposed focus on GN was reinforced by our analysis of the variation within the sub-set of 231 lines in each of the two seasons (supplementary Fig. S3.5). GN was significantly related to GY in both seasons and GY was also significantly related to AGW although only in CS2 and the magnitude of the association was marginal in absolute terms and negligible compared with that of GN (cf. supplementary Fig. S3.5a and b). In both seasons the two major GY components were negatively related across all lines but again this negative relationship did not result in a clear compensation (supplementary Fig. S3.5c).

Thus, to understand the traits responsible for the yield advantage of GY_H over the GY_L lines, it is GN which requires further dissection.

3.4.8. Physiological components of grain number

Physiological components of GN, SDWa and FE, explained part of GN variation (Fig. 3.8). However, their relative relevance seemed to vary with the type of comparison. When comparing the differences across GY_L and GY_H lines it seemed that SDWa was most important as the overall relationship was significant (Fig. 3.8a) and the GY_L lines exhibited significantly lower values than those of GY_H (Fig. 3.8b), whereas this trait was unrelated to GN within each of the two sub-groups (Fig. 3.8a). In general, the contribution of FE to differences in GN was lower than that of SDWa. Although GN in GY_L lines was better explained by FE than SDWa, this was not true for the differences in GN within the GY_H lines (cf. Fig. 3.8a and c). Furthermore, the explanatory capacity of FE decreased noticeably when considering the relationship across all the lines (Fig. 3.8c) and FE was not significantly different between the GY_H and GY_L lines (Fig. 3.8d). Indeed, there was a clear negative relationship between

the two physiological determinants of GN, mainly driven by the different genotypes within each of the two sub-groups (supplementary Fig. S3.6), with the GY_H lines being displaced to the right as a result of their overall higher SDWa (i.e. the increase in SDWa of the GY_H lines compared to the GY_L lines did not bring about any compensation in FE; Figs. 3.8b, d and A3.6).

Again, should the focus of the analysis be in the comparison in sub-set of 231 lines, the outcome would have still been similar. Regardless of the trade-off exhibited by FE and SDWa (supplementary Fig. S3.7c), both similarly influenced GN across all lines in each of the two cropping seasons (supplementary Fig. S3.7a and b).

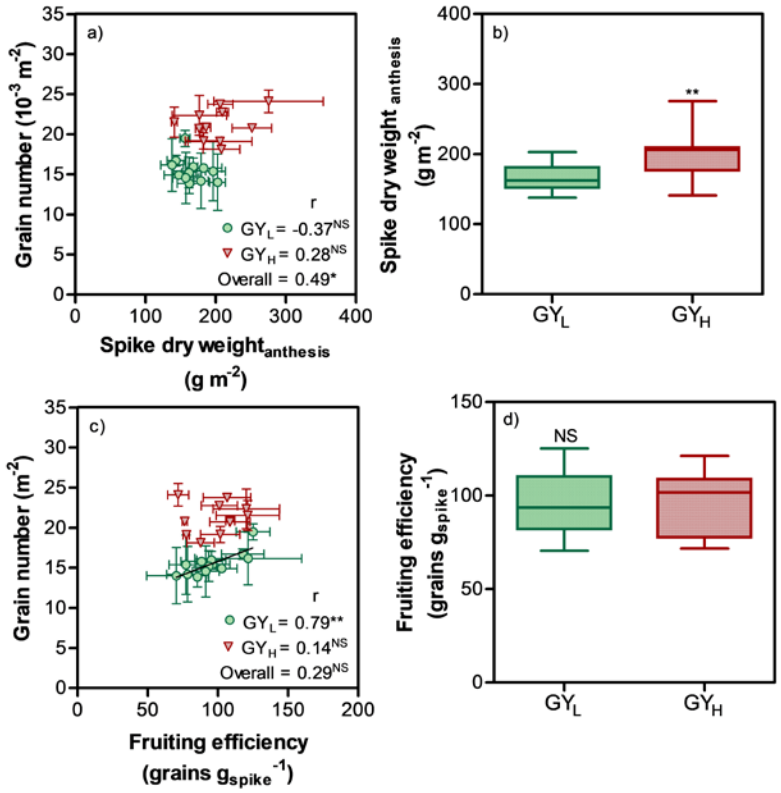

Fig. 3.8. Relationships between grain number and two of its physiological determinants: spike dry weight at anthesis (a) and fruiting efficiency (c) for the selected sub-groups of low and high yielding lines (GY_L and GY_H, respectively). Box plots describing variability for these traits in two sub-groups (b, d). Coefficients of correlations are shown for each sub-group individually and for the overall data across both sub-groups. Significance level: * $p < 0.05$; ** $p < 0.01$; NS= non-significant.

The negative relationship between GN and AGW (supplementary Fig. S3.5c) is mirrored by the negative relationship between AGW and FE. It is the latter which best explains differences in GN

within the GY sub-groups (supplementary Fig. S3.8a). This indirect association with genotypes having higher FE tending to have lower AGW was only significant in GY_L lines but not in GY_H and it was also evident when analysis in sub-set of the 231 lines was considered (supplementary Fig. S3.8b).

3.5. Discussion

The present work aimed at identifying traits responsible for GY differences among lines derived from crosses of elite germplasm, beyond the effects of time to anthesis and plant height. Although not considering time to anthesis, the interest here was in the partitioning of phenological time in the duration of phases occurring before and after TS. In line with previous research (Halloran and Pennell, 1982; Slafer, 2003; Whitechurch et al., 2007) there was a large variation in the two pre-anthesis phases; and there was clear negative relationship between these two phases. This confirms that it would be possible to lengthen the duration of the LRP at the expense of shortening the duration of the phase from sowing to TS (Miralles et al., 2000; Scarth et al., 1985; Slafer et al., 2001). In this context, importance of these observations rests on the hypothesis that a longer LRP would accommodate increased partitioning of biomass to the growing spike (González et al., 2005a; Miralles et al., 2000; Reynolds et al., 2005; Slafer et al., 2005) with the beneficial knock on effect of increased floret survival and final grain number (Ferrante et al., 2013; Sadras and Slafer, 2012), given all other things are constant. However, the relationships between GY and duration of LRP were positive but rather weak, implying that within this experimental material the duration of this phase was not the most critical trait determining yield differences among lines (as also recently seen in Australia; Zhang et al., 2019). GN was the main component explaining variations in GY pointing us towards a prioritization of this trait to explore future genetic gains (Slafer et al., 2014), while not suggesting that maximizing AGW is unimportant in the ultimate high yielding varieties produced by breeders (as illustrated by the fact that within the selected lines used for the more detailed characterization the highest yielding lines had a distinctly higher AGW than the others). To plan for the optimization of both traits it is important to increase understanding of their negative correlation, which was observed here, as in so many previous studies (Miralles and Slafer, 1995a; Siddique et al., 1989a; Slafer et al., 2014). A key question is whether the AGW/GN negative relationship is due to competition for carbohydrates during grain filling. Should there be competition among grains, increasing GN might result in a kind of zero-sum game, with GY not changing significantly. Although, this kind of interpretation of a negative relationship

seems quite intuitive, there is good evidence for the less intuitive scenario in which grains do not compete for resources during grain filling. It follows that the source of the trade-off lies elsewhere and is probably not the consequence of a competitive dynamic (Acreche and Slafer, 2006; Miralles and Slafer, 1995a). This means that further increases in GN are critical in bringing about major improvements in GY (Fischer, 2011; Reynolds et al., 2012; Sanchez-Garcia et al., 2013; Slafer et al., 2014). These extra grains will have access to sufficient resources for filling as supported by studies from source-sink manipulations during grain filling in which grain growth is unresponsive (Abbate et al., 1997; Borrás et al., 2004b; M. P. Reynolds et al., 2005; Serrago et al., 2013 and references quoted there in; Slafer and Savin, 1994) showing that an excess of assimilates are available at this developmental stage (e.g. Bingham et al., 2007; Borrill et al., 2015); although some exceptions can be found and only for particular seasons under extremely high yielding conditions (e.g. Lynch et al., 2017). In other words, that the crop is rather conservative at the time of establishing GN (Sadras and Slafer, 2012), which would be the reason for the differences in plasticity of GN and AGW (being GN determination strongly responsive to source strength and AGW relatively unresponsive). The current study did not involve source-sink interventions like defoliations, shading, de-graining, or thinning the plots and so on, nonetheless a quantitative analysis can be used to estimate whether strong source limitation during the grain filling period was at play. For this purpose, I related AGW to the post-anthesis accumulation of crop growth on a per grain basis (i.e. the ratio between total above ground crop dry weight accumulated from anthesis to physiological maturity and GN). This showed that AGW differences between lines were hardly due to severe source limitations in the low AGW genotypes (Fig. 3.9a). Firstly, there was no clear trend to reduce AGW with reductions in post-anthesis whole crop growth per grain. Secondly, the distributions of the data-points in the figure would be compatible with no source-limitation. Almost all the lines in GY_H were very close to the 1:1 line indicating that the grain growth had more than enough resources: cases in which data points are on the bottom of the 1:1 line would have never being source-limited to the level that even some of the growth produced after anthesis was allocated to other sinks, while cases in which grain weight exceeded the post-anthesis crop growth per grain would still be sink-limited, as post-anthesis growth is only part of the source available to fill the grains (part of the demand of the growing grains can be satisfied by remobilisation of pre-anthesis reserves). This is also true for the data-points from GY_L that fell at the left side of the 1:1 line (Fig. 3.9a). To have a scale that can be more readily compared with the literature I transformed the independent variable into a percentage of AGW (Fig. 3.9b). Calculated as percent difference between AGW and post-anthesis crop growth per grain with respect

to AGW. A negative value means the percentage of GY that was allocated to other organs (i.e. clearly unrealized yield potential due to post-anthesis sink limitation), and positive values represent the percentage of AGW that has been realised thanks to the remobilization of pre-anthesis reserves. The dotted line at 35% indicates a practical limit up to which developing grains can access translocated pre-anthesis reserves derived from Savin and Slafer (1991). This is a rather conservative figure as there have been examples in the literature where up to 50% of the final grain weight was contributed by translocation of reserves accumulated before anthesis (e.g. Borrás et al., 2004; Gent, 1994) which produced an elegant demonstration of the fact that only with a large contribution of pre-anthesis reserves to grain growth the observed AGW would have been possible. In that work it was estimated that, depending on the cultivar and season, up to 55% of the final AGW could be contributed from pre-anthesis reserves and several examples of such large contribution have been observed (see examples in the review by Blum, 1998).

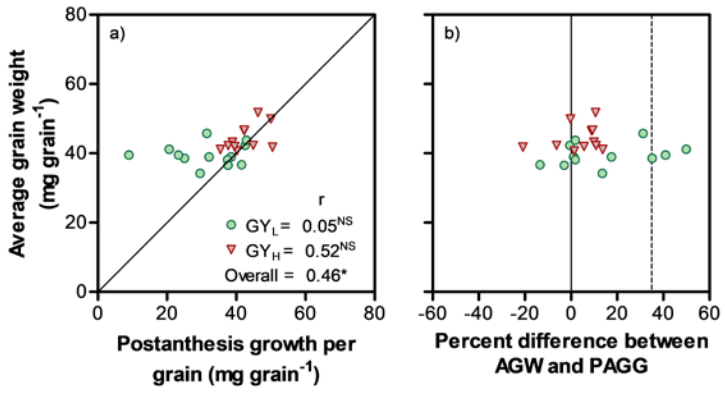

Fig. 3.9. Relationships between average grain weight and either (i) post-anthesis growth per grain (PAGG) in absolute (a), or ii) percent difference between AGW and PAGG with respect to AGW (b) for the selected sub-groups of low and high yielding lines (GY_L and GY_H, respectively). Coefficients of correlations are shown for each sub-group individually and for the overall data across both sub-groups. Plain lines represent the situation when AGW was equal to post-anthesis growth per grain. The dotted line represents a 35% contribution from pre-anthesis reserves to final grain weight, which is more than a highly likely contribution that can be expected (Austin *et al.*, 1980 in barley; Savin and Slafer, 1991 in wheat).

Furthermore, there was a relationship between GY and total dry weight (at physiological maturity) explaining the genotypic GY differences within and across the GY_L and GY_H lines (Fig. 3.10a). The most frequent interpretation of this relationship would be that lines with improved growth capacity produced more grains that, when filled, resulted in a proportionally larger GY. However, this seems

not to be the most likely explanation in this case. Looking at the differences in total accumulated dry weight from sowing to anthesis (TDWa; Fig. 3.10b) and from anthesis to maturity i.e., cumulative growth after anthesis (Fig. 3.10c), it seems that the more common cause-consequence hypothesis can be inverted to reach an interpretation that is at least as likely. Indeed, there was only a marginal difference in TDWa between GY_L and GY_H lines, with a large overlap in this trait between lines of the two sub-groups (Fig. 3.10b), while the difference became relevant in post-anthesis growth (Fig. 3.10c). Thus, it may well be that the physiological basis for the higher GY of the GY_H lines is more efficient translation of pre-anthesis growth into GN. These lines increased the sink strength during grain filling lowering the extent of sink limitation. This, in turn, would reduce the down regulation of post-anthesis canopy photosynthesis (that has been shown to exist due to insufficient sink demand in different conditions; e.g. Serrago et al., 2013) driving the improved crop growth during grain filling. This would be in line with previous studies showing that higher GN would increase post-anthesis growth (Acreche and Slafer, 2009; Reynolds et al., 2005), through its positive effect on canopy photosynthesis.

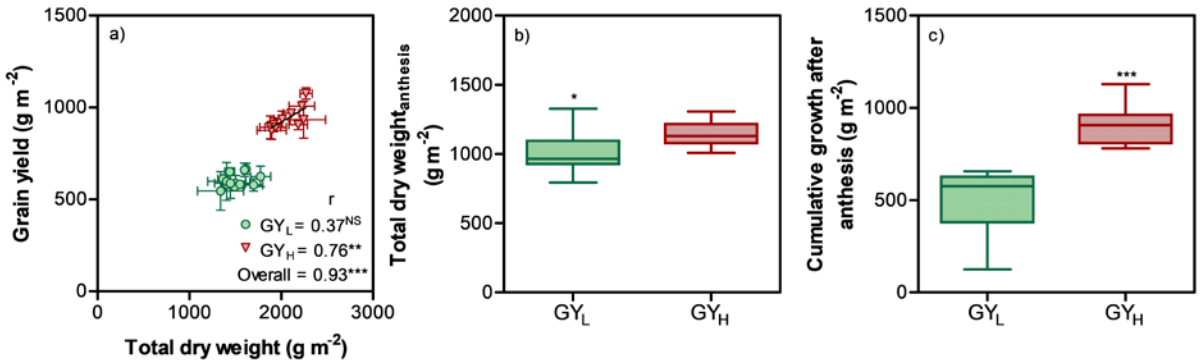

Fig. 3.10. Relation between total dry weight (at maturity) and grain yield (a); box plots showing variations in total dry weight at anthesis (b) and cumulative growth after anthesis (c) for the selected sub-groups of low and high yielding lines (GY_L and GY_H, respectively). Coefficients of correlations are shown for each sub-group individually and for the overall data across both sub-groups. Significance level: * $p < 0.05$; *** $p < 0.001$.

Both physiological components of GN, SDWa and FE, seemed to have been relevant to improve GY, which is in line with recent results from Australia in a study combining many commercial cultivars, elite lines and a MAGIC population (Zhang et al., 2019). As lines did not differ much in TDWa their differences in SDWa implies a better dry matter partitioning to the juvenile spike growing immediately

before anthesis in high GY_H lines. This is critical because wheat GY is clearly source-limited just prior to anthesis (Borrás et al., 2004; Slafer and Savin, 1994) and SDWa is critical in determining post-anthesis sink strength (Fischer, 2011; Slafer, 2003). This is because the development of labile florets in the juvenile spikes immediately before anthesis is highly sensitive to allocation of resources (Ferrante et al., 2013, 2010; Fischer, 1985; González et al., 2005b; Siddique et al., 1989b; Slafer et al., 2015), which in turn is the mechanistic basis for the strong and consistent relationship between GN (or number of fertile florets) and SDWa (Fischer, 1985 and a plethora of papers confirming this relationships in different background conditions, in response to various different treatments). In the past, breeding has improved GY through increasing GN exploiting this mechanism. Specifically, modern semi-dwarf cultivars out yielded their older traditional height (tall) predecessors due to an improved dry matter partitioning to the spike before anthesis (e.g. Brooking and Kirby, 1981; Calderini et al., 1995; Fischer and Stockman, 1986; Flintham et al., 1997; Miralles et al., 1998; Shearman et al., 2005; Siddique et al., 1989a; Slafer and Andrade, 1993). But these gains were achieved through plant height reduction. In the present study I showed that there is opportunity to further improve partitioning of dry matter to the spike beyond reductions in plant height (Foulkes et al., 2011) that would be instrumental to further improve GY through reducing the degree of sink-limitation during the post-anthesis phases of development. A recent paper by Rivera-Amado et al. (2019) clearly illustrates how this further improvement in pre-anthesis partitioning to juvenile spikes would be possible. The other trait that can help in reducing the sink-limitation during grain filling is FE (e.g. (Slafer et al., 2015). In this study FE explained part of the GN differences within the segregants from elite parents. Although I observed trade-off between SDWa and FE that had been also reported before (e.g. Ferrante et al., 2012; Lázaro and Abbate, 2012), it seemed feasible to identify genotypes with best combinations of both traits maximising GN, and therefore crossing parents with high SDWa and high FE could increase the likelihood of transgressive segregation for GN (and GY).

3.6. References

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3.7. Supplementary material

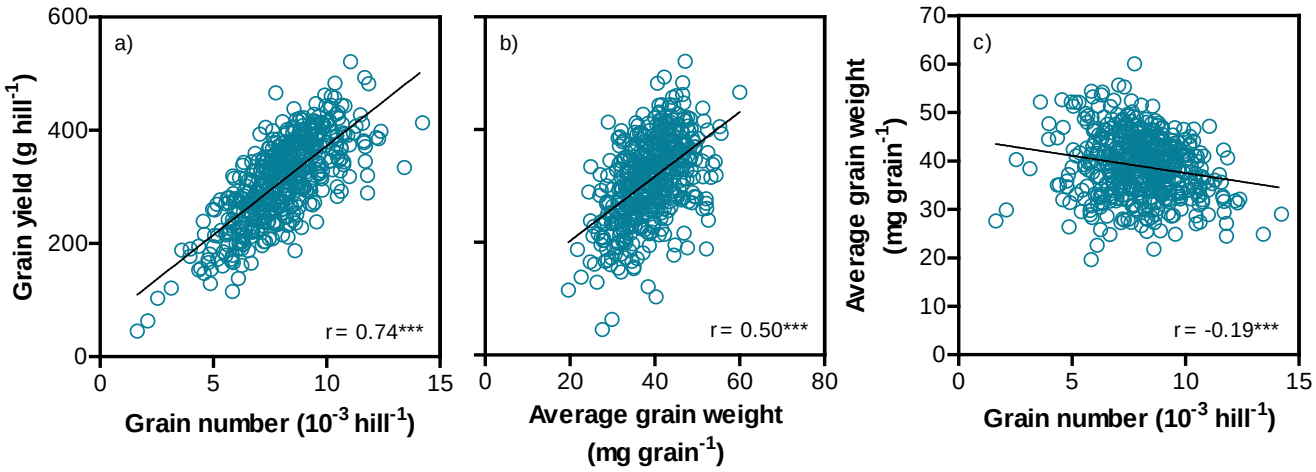


Fig. S3.1. Relationships between grain yield and two component phases of time to anthesis: time from sowing to terminal spikelet (a), and time from then to anthesis, the late reproductive phase (b) in the selected sub-set of 231 lines. Significance level: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

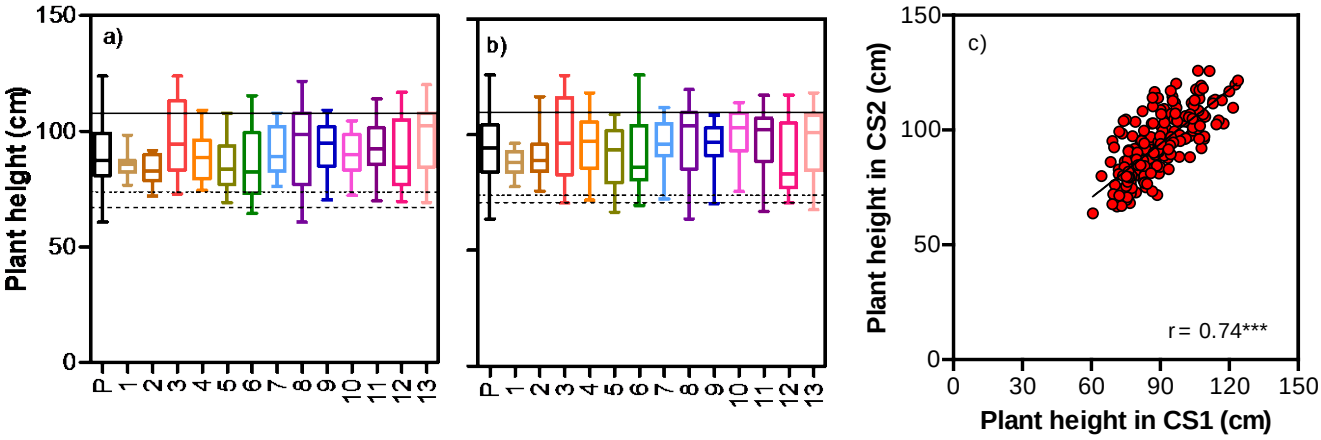


Fig. S3.2. Boxplots showing variability for plant height within the whole population (P_W) and within families (13 bi-parental crosses) along with three checks Paragon (solid line), Paledor (dotted line) and Garcia (dashed line) in the first (CS1, a) and second cropping season (CS2, b), and consistency for plant height over the two cropping seasons (c). Significance level: *** $p < 0.001$.

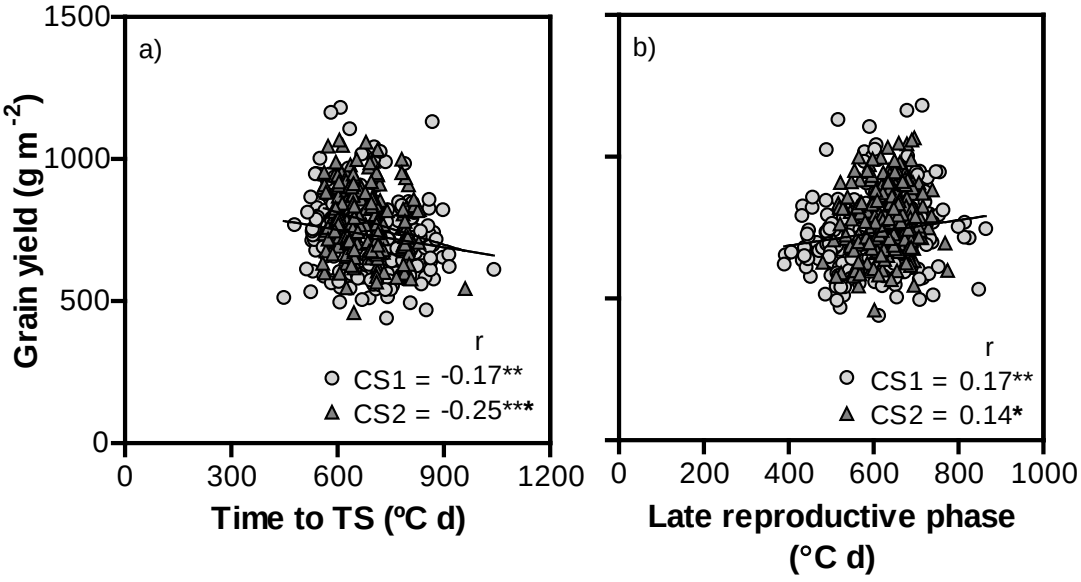


Fig. S3.3. Relations between grain yield and two component phases of time to anthesis: time from sowing to terminal spikelet (a); time from then to anthesis, late reproductive phase (b) in the whole population of 231 lines. Significance level: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

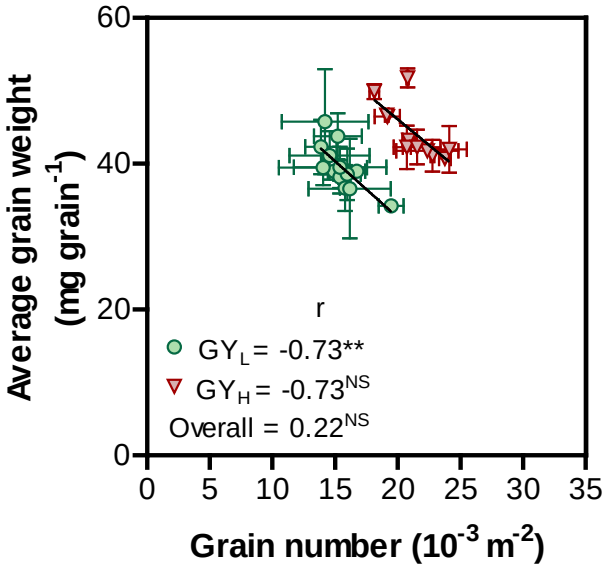


Fig. S3.4. Relationship between the two components of grain yield, average grain weight and grain number, for the two sub-groups of low and high yielding lines (GY_L and GY_H, respectively). Coefficients of correlations are shown for each sub-group individually and for the overall data across both sub-groups. Significance level: ** $p < 0.01$; NS= non-significant.

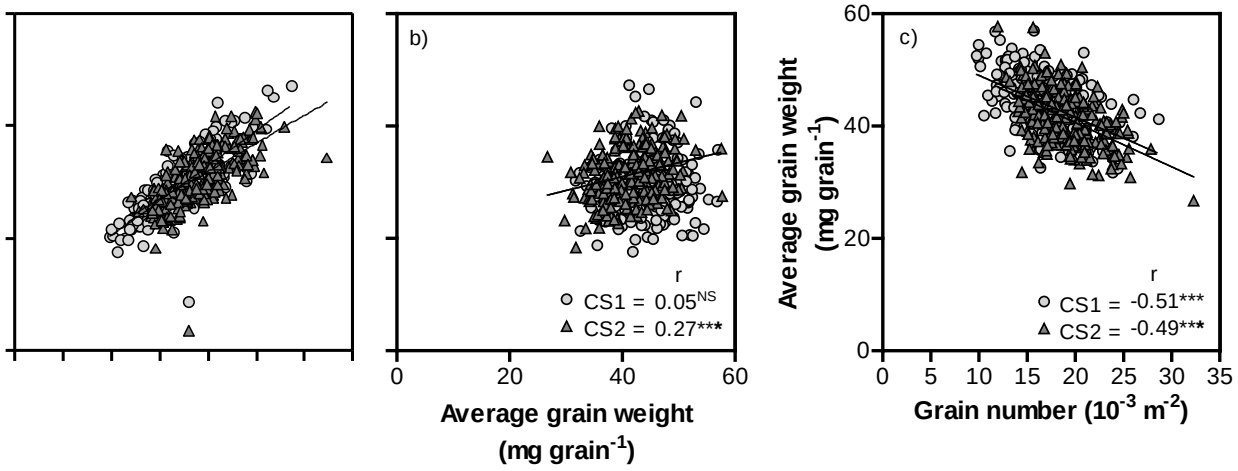


Fig. S3.5. Relationships between grain yield and its components: grain number (a) and average grain weight (b); and between them (c) in the sub-set of 231 lines. Significance level: *** $p < 0.001$; NS= non-significant.

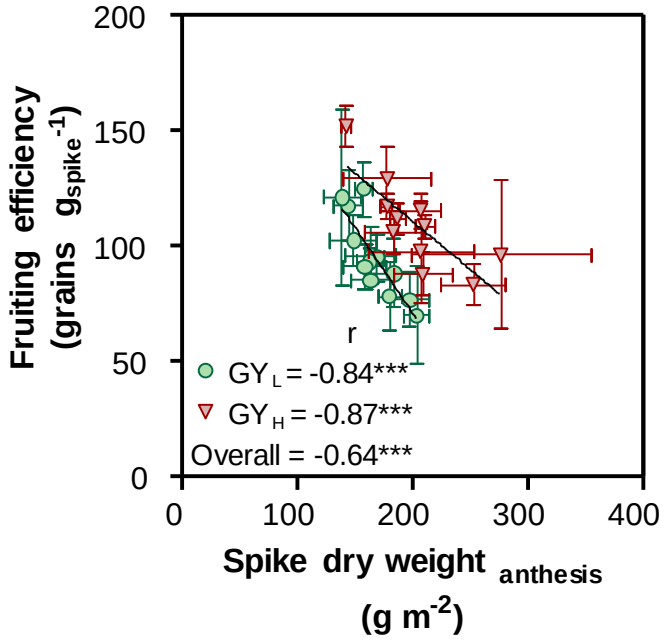


Fig. S3.6. Relationship between spike dry weight at anthesis and fruiting efficiency for the two sub-groups of low and high yielding lines (GY_L and GY_H, respectively). Coefficients of correlations are shown for each sub-group individually and for the overall data across both sub-groups. Significance level: *** $p < 0.001$.

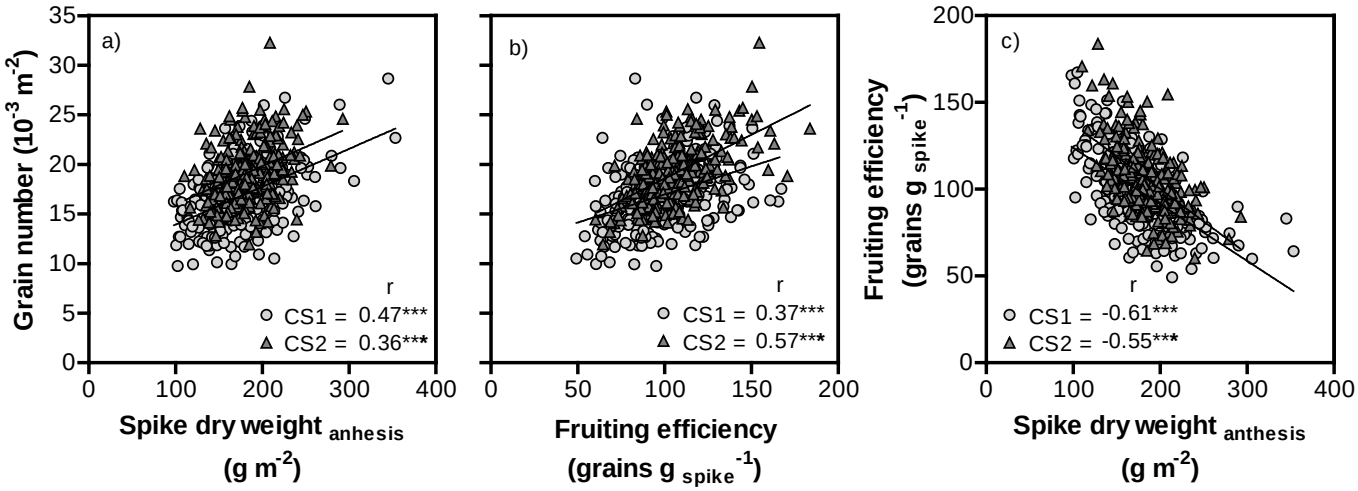


Fig. S3.7. Relations between grain number and two of its physiological determinants: spike dry weight at anthesis (a) and fruiting efficiency (b); relation between spike dry weight and fruiting efficiency (c) in the sub-set of 231 lines. Significance level: *** $p < 0.001$.

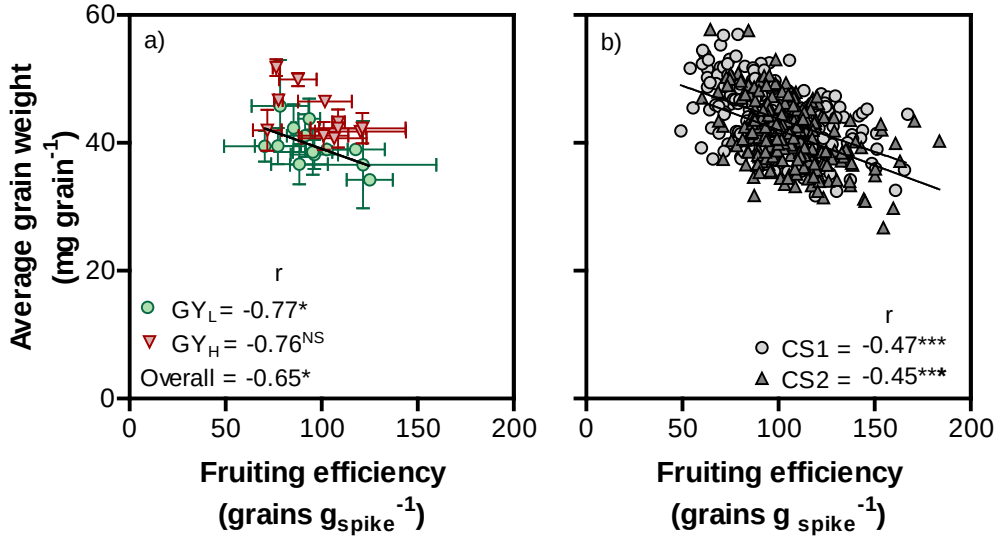


Fig. S3.8. Relation between fruiting efficiency and average grain weight in selected sub-groups of low and high yielding lines (GY_L and GY_H, respectively) with coefficients of correlations are shown for each sub-group individually and for the overall data across both sub-groups (a) and in sub-set of 231 lines (b). Significance level: * $p < 0.05$; *** $p < 0.001$; NS= non-significant.

Chapter IV: Developmental traits, primordia initiation and spike fertility in wheat as affected by two new *Eps* QTLs (*Eps-7D* and *Eps-2B*) under field conditions

4. Chapter IV: Developmental traits, primordia initiation and spike fertility in wheat as affected by two new *Eps* QTLs (*Eps-7D* and *Eps-2B*) under field conditions

4.1. Abstract

Earliness per se (*Eps*) genes are reported to be important in fine-tuning flowering time in wheat independently of photoperiod (*Ppd*) and vernalization (*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 the cross between two vernalisation insensitive lines, Paragon (late flowering) and Baj (early flowering) to study the detailed effects of two newly identified QTLs, *Eps-7D* and *Eps-2B* and interactions between them under field conditions. Although the effect of both QTLs were minor their effect was modulated by the allelic status of the other. Although magnitude of effect on anthesis is similar for these QTL they are associated with very different profiles of pre-anthesis developmental which also depends on their interaction. *Eps-7D* affected both time to TS and late reproductive phase (LRP) while not affecting a change on 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 only altered time TS and slightly altered FLN without affecting the leaf appearance rate. Both *Eps-7D* and *-2B* affected the spike fertility by altering the rate of floret development and floret mortality. The effect of the latter was very small but consistent in that the *-late* allele tended to produced more fertile florets than the *-early* allele.

4.2. Introduction

Wheat adaptation to diverse environments has been possible by coarse tuning time to anthesis (Kamran et al., 2014; Snape et al., 2001). Moreover, fine tuning time to anthesis is relevant to improve crop adaptation and performance within a specific target environment (Araus et al., 2002; Richards, 1991; Zheng et al., 2015). 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 time (Nazim Ud Dowla et al., 2018; Worland, 1996); and will be instrumental in adapting better to climate change (Craufurd and Wheeler, 2009; Semenov et al., 2014).

Wheat development from sowing to anthesis encompasses various stages that make up two major component phases, namely 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 survival (Slafer and Rawson, 1994b). 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 numerical components of grain yield (González et al., 2005a; Whitechurch and Slafer, 2002). It has been hypothesised that developmental phases prior to anthesis can be adjusted optimally with little or no changes in time to anthesis to keep improving adaptation along with stable or improved yield (Miralles et al., 2000; Slafer et al., 2001)

Wheat responds to various environmental stimuli like vernalisation and photoperiod and genetic factors responsible for these sensitivities are mainly *Vrn*-1 and *Ppd*-1 genes, respectively (Snape et al., 2001). Responses to photoperiod and vernalisation sensitivities can be found in all the three (vegetative, early and later reproductive phases) phases of wheat development (Miralles et al., 2000; Rawson et al., 1998; Slafer and Rawson, 1996, 1994b). The direct effect of different *Ppd* and *Vrn* genes on wheat development as well as their interactions have been 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 *Vrn*- Whitechurch and Snape, 2003; Allard *et al.*, 2012; for *Ppd*- González *et al.*, 2005; Shaw *et al.*, 2012; Pérez-Gianmarco *et al.*, 2018; for combination of *Ppd* with *Vrn* or *Eps* or both- (Alvarez et al., 2016; Gomez et al., 2014; Griffiths et al., 2009; Kamran et al., 2013; Steinfort et al., 2017). In addition to differences in photoperiod- and vernalisation-sensitivity, there are residual differences in phenology after the photoperiod and vernalisation requirements have been completely satisfied, and are 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 segregate (Zikhali et al., 2014b). Having much smaller effect in comparison to *Ppd* and *Vrn*, *Eps* genes are ideal for fine-tuning wheat development (Griffiths et al., 2009). The direct effect of *Eps* is known to be on heading or anthesis time, although studies by Lewis et al., (2008) and Alvarez et al., (2016) (*Eps-A^m 1-l* of T. monococcum) have shown effects of *Eps* on development of certain organs like spikelets. Therefore, it can be speculated that the indirect effect of *Eps* on grain yield could be the reason for their indirect selection in the present cultivars (Alvarez et al., 2016). Current cultivars may have a reasonable variation of *Eps* alleles, providing some opportunities to study these alleles in order to improve our understanding on their effects on phenology as well as on yield components, if any.

While much knowledge has been produced on how wheat phenology is affected by *Ppd* and *Vrn* genes, 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; Griffiths et al., 2009; Sukumaran et al., 2016; Zikhali and Griffiths, 2015), and each potentially having a different mode of action (the only thing all have in common is that they produce relatively minor changes in time to anthesis). Dissecting the *Eps* effect into vegetative or/and reproductive phases could be crucial when considering likely effects on yield (Slafer, 2003), not only *Eps* effects on particular component phases but more so on how development of organs are altered. The reports on effects of *Eps* have been inconsistent so far as in some studies have reported that the effects of *Eps* may be limited to earlier phases (Alvarez et al., 2016; Le Gouis et al., 2012; Lewis et al., 2008; Slafer and Rawson, 1995) and others to that of LRP (Ejaz and von Korff, 2017; Ochagavía et al., 2019). The studies mentioned above 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 (Ochagavía et al., 2018a) and epistatic interaction with respect to *Ppd* and *Vrn* genetic factors (Alvarez et al., 2016; Kamran et al., 2013) along with different magnitude of interaction with the growing temperature (Ochagavía et al., 2019). Therefore, it is important to understand the detailed effect on developmental phases, and on the dynamics of organogenesis during these phases, of *Eps* genes to exploit such in wheat breeding. Furthermore, as all 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 (Ochagavía et al., 2018a; Prieto et al., 2018a), but the interaction between different *Eps* genes has never been analysed (i.e. whether the effect of a particular *Eps* gene is altered depending on the particular allelic condition of another *Eps* gene)this is important as many *Eps* genes are acting simultaneously in any genotype.

The present Chapter was aimed to analyse for the first-time effects on different developmental traits of two newly identified *Eps* QTLs on chromosomes 7D and 2B ascertaining not only their direct effects but 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 (i) phenology, not only determining time to anthesis but also quantifying whether they mainly affect development before or after terminal spikelet; (ii) rate of leaf appearance; (iii) dynamics of leaf, spikelet and floret primordia development; and (iv) spike fertility.

4.3. Materials and methods

4.3.1. Plant material, field conditions and management

Two field experiments were carried out 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 m^2. 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 ºC (Fig. 4.1a) and accumulated precipitations for the whole growing period were 263.6 and 286.9 mm for CS1 and CS2, respectively (Fig. 4.1b). 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).

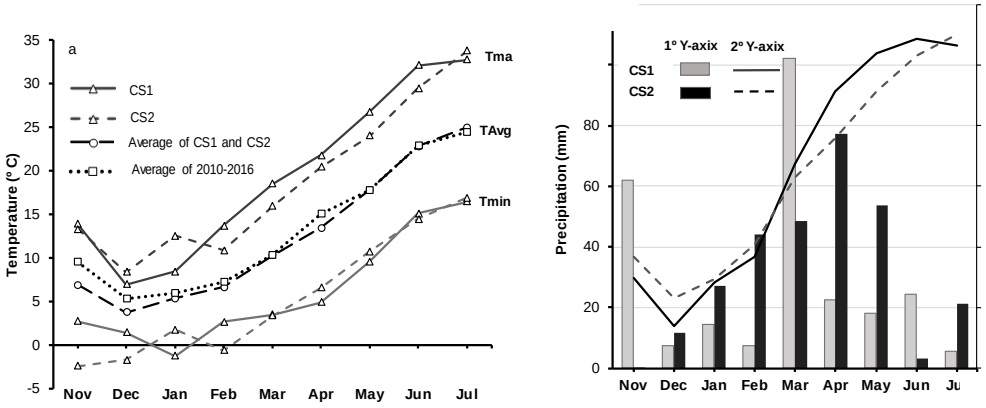


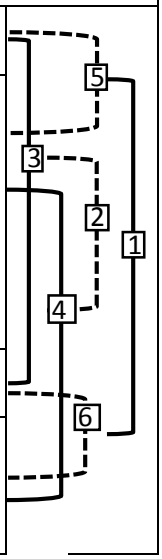
Fig. 4.1. Monthly minimum (Tmin), maximum (Tmax) for first (CS1) and second (CS2) cropping season and mean of the monthly average temperature (TAvg) of CS1 and CS2 as well as monthly TAvg of cropping seasons from the past 5 years (2010-2016, a); monthly accumulated precipitation and global radiation for CS1 and CS2 (b).

4.3.2. Treatments and experimental design

Treatments consisted of eight lines (Table 4.1) 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 for the other major genes influencing anthesis time. The genetic mapping of QTLs and QTL analysis was provided by Dr Griffiths`s Lab (please see details in the supplementary material). The heading date QTL identified in Paragon × Baj are shown in Table S4.1 and QTL plots in Fig. S4.1. 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.

Table 4.1. Lines selected for this study, possessing contrasting alleles of both *Eps* genes (*Eps-7D* and *Eps-2B*). For comparing the effects of these alleles, I 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.

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



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.

4.3.3. Measurements and data analysis

4.3.3.1. 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). 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 ºC) 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 (i) this change in phyllochron coincides with Haun stages between 6 and 8 (Calderini et al., 1996; González et al., 2005a; Jamieson et al., 1995; Slafer and Rawson, 1997) and (ii) using that threshold for the change in slope of the bi-linear regression produces excellent outputs in a wide range of environmental (e.g. Miralles *et al.*, 2001) and genotypic contexts (e.g. Ochagavía et al., 2018b, 2017). 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).

4.3.3.2. 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 Appleyard (1987). 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). 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.

4.3.4. 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 initiated and final number of fertile florets at anthesis. Only 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.

4.3.5. Fertile floret mapping at anthesis

Three plants were randomly selected from the marked 1 m of uniform plants 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.

4.4. Results

4.4.1. Phenology

Despite the fact 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 clearly, with a large direct effect (Table 4.2).

The direct effect of *Eps-2B* was not significant; but there was interaction between the two loci (Table 4.2). This means that the magnitude of the effect on time to anthesis of the *Eps-7D* is dependent upon the allelic status of *Eps-2B*.

Table 4.2. ANOVA for time to anthesis. Factors that significantly affected this trait are in bold.

Source of variation	Degrees of freedom	Mean squares (ºC d)	F-ratio	Significance (P-value)
Season	1	422.73	1.71	0.212
Eps-7D	**1**	**28698.98**	**116.38**	**<0.001**
Eps-2B	1	478.60	1.94	0.185
Eps-7D* x *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		

Thus, although duration from sowing to anthesis was affected by *Eps-7D* always (i.e. in both growing seasons and regardless of the allelic constitution of the *Eps-2B*), the magnitude of effect was different depending on the *Eps-2B* allele in the background (Fig. 4.2 top panels). The presence of the *Eps-7D-early* allele shortened the time to anthesis compared with *Eps-7D-late* by c. 90 ºC d consistently across the two growing seasons when the background had the *late* allele of *Eps-2B* (Fig. 4.2, top left panel). Whereas when the background had the *Eps-2B-early* allele, the presence of the *Eps-7D-early* advanced anthesis only c. 45 ºC d in comparison with the *Eps-7D-late,* the difference was in general smaller yet significant in both seasons (Fig. 4.2, top right panel). Although the *Eps-2B* had been characterised to affect time to anthesis , in this study the difference in time to anthesis between lines having the early and late allele of the *Eps-2B* was only significant in the first season if the *Eps-7D* in the background was the *late* allele (in the second season there was a trend for a later anthesis in the lines with the *Eps-7D-late* allele; Fig. 4.2 bottom left panel), with no difference at all between the lines with contrasting *Eps-2B* alleles when in the background the other gene was the *Eps-7D-early* (Fig. 4.2 bottom right panel).

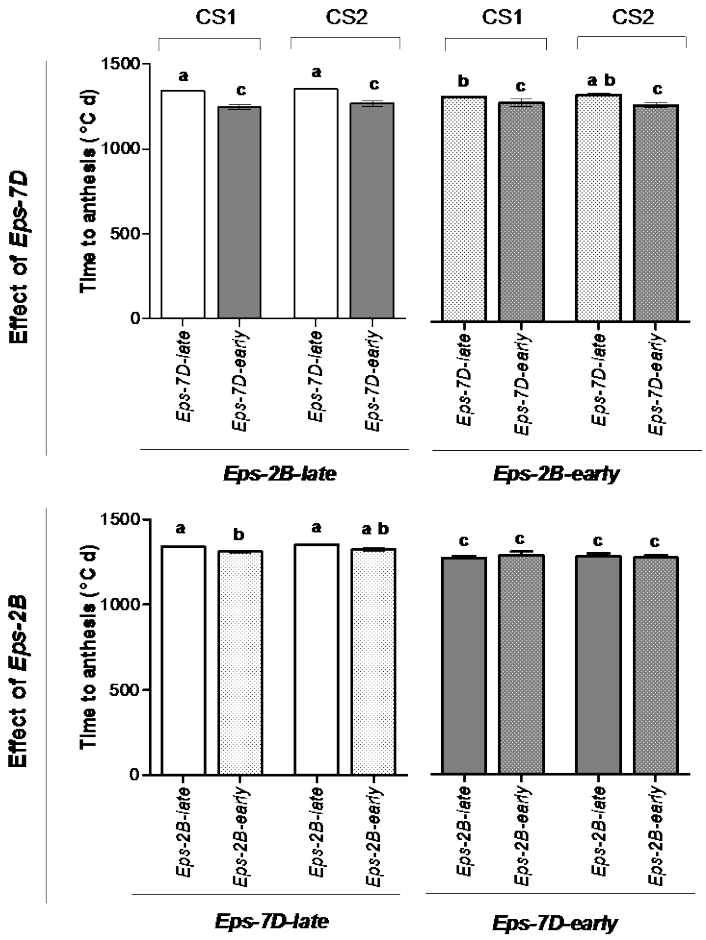


Fig. 4.2. Duration of the crop cycle from sowing to anthesis for the lines carrying (i) the *Eps*-7D-late or early allele (upper panels) on the contrasting background of *Eps*-2B (*Eps*-2B-late and -early, upper panel left and right, respectively); or (ii) the *Eps*-2B-late or early allele (bottom panels) on the contrasting background of *Eps*-7D (*Eps*-7D-late and -early, bottom panel left and right, respectively). Data are shown for each of the two cropping seasons (CS1 and CS2 within each panel). Bars not sharing the letter within the panel are significantly different (α=0.05).

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. 4.3). The effect of *Eps-7D* strongly and consistently affected the duration of the period until TS and only marginally affected the duration of the LRP in the second season when the *Eps-2B* gene in the background was the *late* allele (Fig. 4.3, top left panel). On the other hand, effect of *Eps-7D* when in the background the *Eps-2B* was the early allele, not only the overall effect was smaller (see above) but also it was mainly due to its effect on the LRP with no changes in time to TS at all (Fig. 4.3 bottom

left panel). The *Eps-2B* gene only affected the measured phenology traits when the *Eps-7D* in the background was the *late* allele (Fig. 4.3, right panel). In that case and consistently across both seasons, the effect was seen in the early development to TS with no effects on the duration of the LRP (Fig. 4.3. Top right panel).

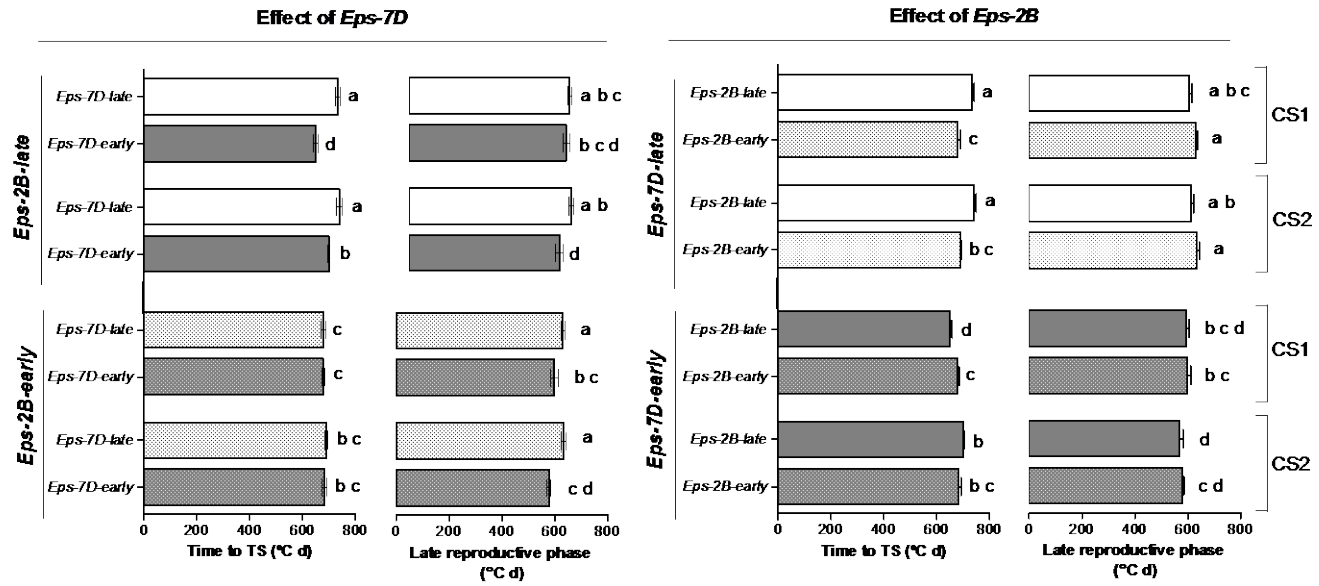


Fig. 4.3. Duration of the two pre-anthesis phases considered: time from sowing to terminal spikelet (TS) and from then to anthesis, the late reproductive phase as affected by *Eps-7D* (left panels) and *Eps-2B* genes (right panels) on backgrounds contrasting in the allelic form of the other *Eps* gene (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. Bars not sharing the letter within a panel are significantly different (α=0.05).

4.4.2. 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. 4.4 left panels). On the contrary presence of the allele *Eps-2B-early* tended to reduce the FLN (Fig. 4.4, right panel). 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*, 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. 4.4, right panel).

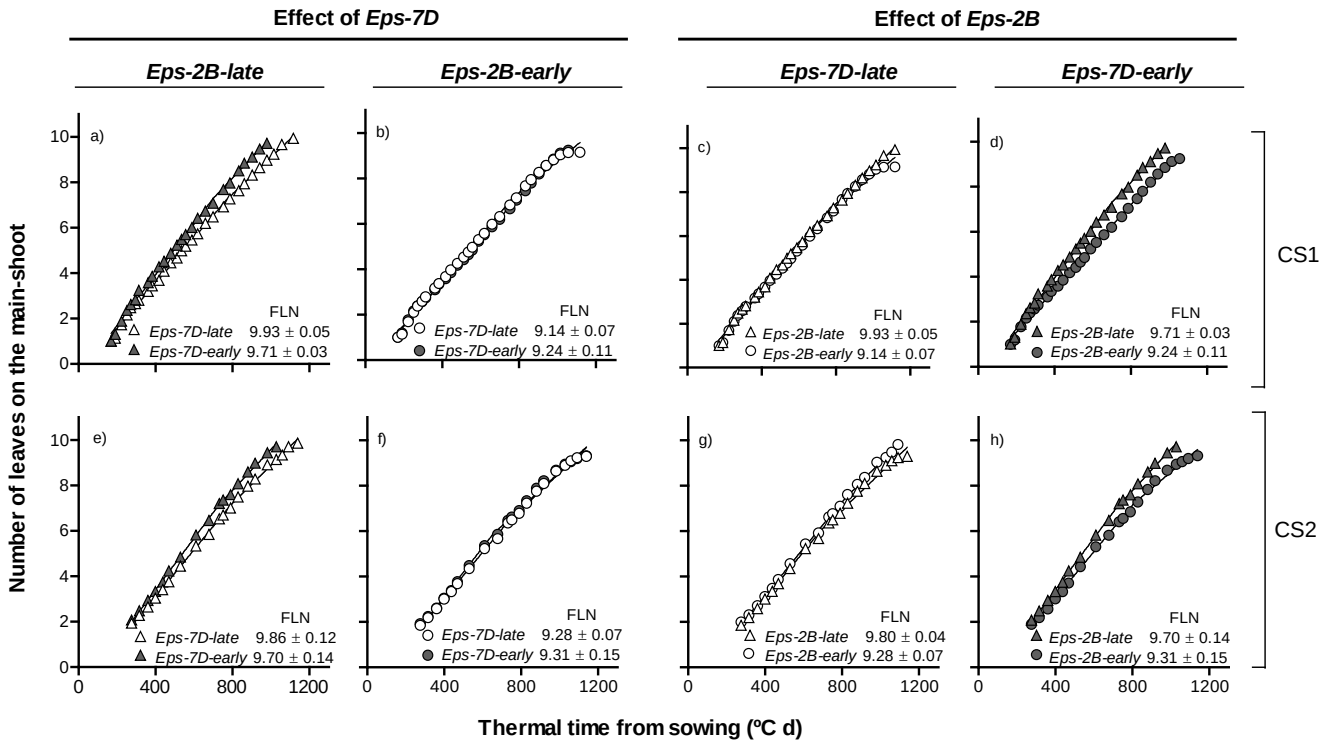


Fig. 4.4. Relationship between number of leaves appeared on the main shoot and thermal time from sowing as affected by *Eps-7D* (left panels, a, b, e and f) and *Eps-2B* genes (right panels, c, d, g and h) on backgrounds contrasting in the allelic form of the other *Eps* gene (left and right panels within 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.

Although in all cases the relationship between the number of appeared leaves and thermal time had a strong linear component (Fig. 4.4), the relationships were actually bi-linear with early leaves appearing significantly faster (and having therefore a shorter phyllochron) than later leaves (Table 4.3). 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 4.3, 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 4.3, bottom half).

Table 4.3. Rates of leaf appearance (leaves [100 ºC d]$^{-1}$; ±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 (ºC d leaf^{-1}) are included between square brackets.

Background	*Eps* allele	CS 1			CS 2		
		RLA-I	RLA-II	R^2	RLA-I	RLA-II	R^2
				***Eps-7D* effect**			
Eps-2B-late	*Eps-7D-late*	0.99±0.01 [100.4]	0.81±0.03 [123.6]	0.998	1.03±0.01 [96.8]	0.84±0.02 [119.4]	0.999
	Eps-7D-early	1.12±0.02 [89.3]	0.89±0.04 [112.4]	0.997	1.13±0.02 [88.2]	0.90±0.03 [110.6]	0.999
Eps-2B-early	*Eps-7D-late*	0.99±0.02 [100.4]	0.69±0.04 [142.9]	0.997	1.02±0.03 [98.0]	0.80±0.03 [124.8]	0.997
	Eps-7D-early	0.95±0.01 [105.6]	0.88±0.03 [114.2]	0.998	1.08±0.04 [91.9]	0.79±0.03 [126.1]	0.996
				***Eps-2B* effect**			
Eps-7D-late	*Eps-2B-late*	0.99±0.01 [100.4]	0.81±0.03 [123.6]	0.998	1.03±0.01 [96.8]	0.84±0.02 [119.4]	0.999
	Eps-2B-early	0.99±0.02 [100.4]	0.69±0.04 [142.9]	0.997	1.02±0.03 [98.0]	0.80±0.03 [124.8]	0.997
Eps-7D-early	*Eps-2B-late*	1.12±0.02 [89.3]	0.89±0.04 [112.4]	0.997	1.13±0.02 [88.2]	0.91±0.03 [110.6]	0.999
	Eps-2B-early	0.95±0.01 [105.6]	0.88±0.04 [114.2]	0.998	1.08±0.04 [91.9]	0.79±0.03 [126.1]	0.996

4.4.3. 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 *Eps-late* allele (Fig. 4.5). As differences in FLN were small or negligible (see above), these differences in number of primordia reflect 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. 4.5). Thus, the differences in the total number of primordia initiated between lines carrying the *late* and *early Eps* alleles was mostly due to the differences in duration of primordia initiation (Fig. 4.5).

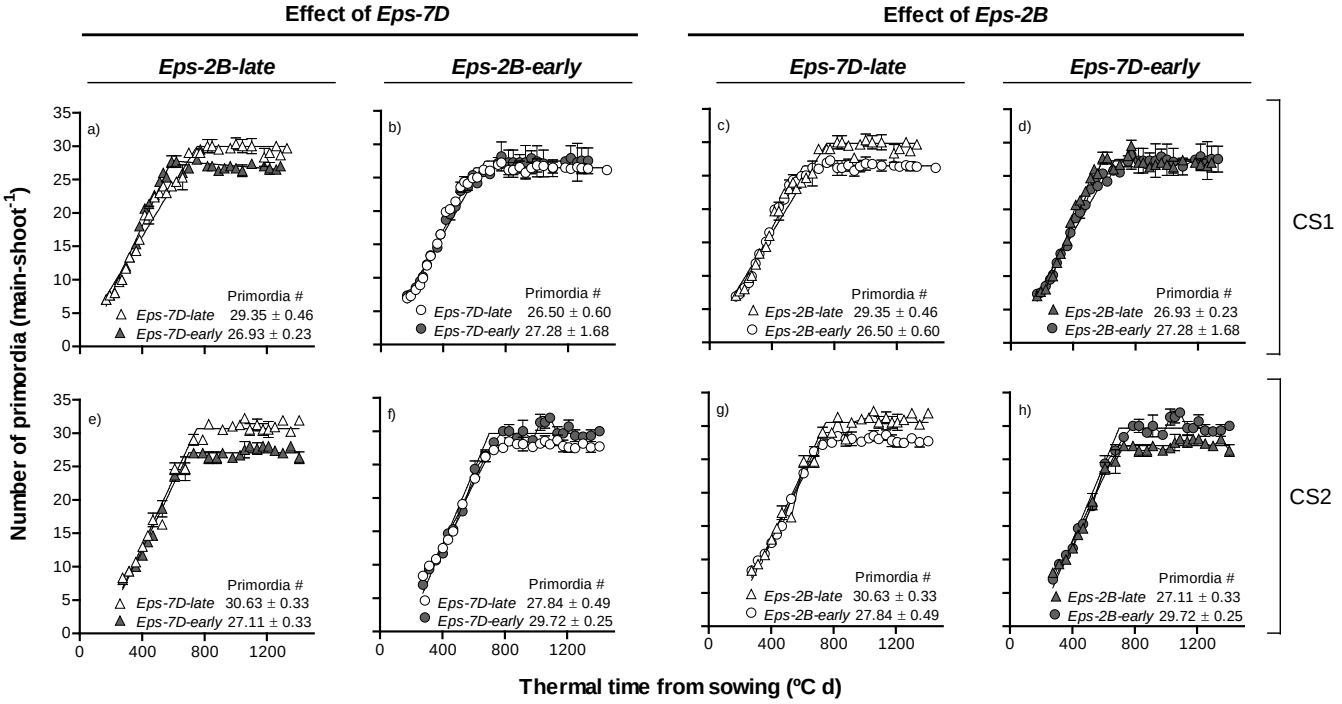


Fig. 4.5. Relationship between number primordia initiated on the main shoot apex and thermal time from sowing as affected by *Eps-7D* (left panels a, b, e and f) and *Eps-2B* genes (right panels e, d, g and h) on backgrounds contrasting in the allelic form of the other *Eps* gene (left and right panels 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 total number of primordia with their SEM.

4.4.4. 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 present the dynamics of florets that reached W4.5 (carpel primordia visible) up to the fifth floret from the rachis. Outside of this range seeds are hardly ever produced. At the other end of the floret survival scale the two florets most proximal to the rachis (F1 and F2) are 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.

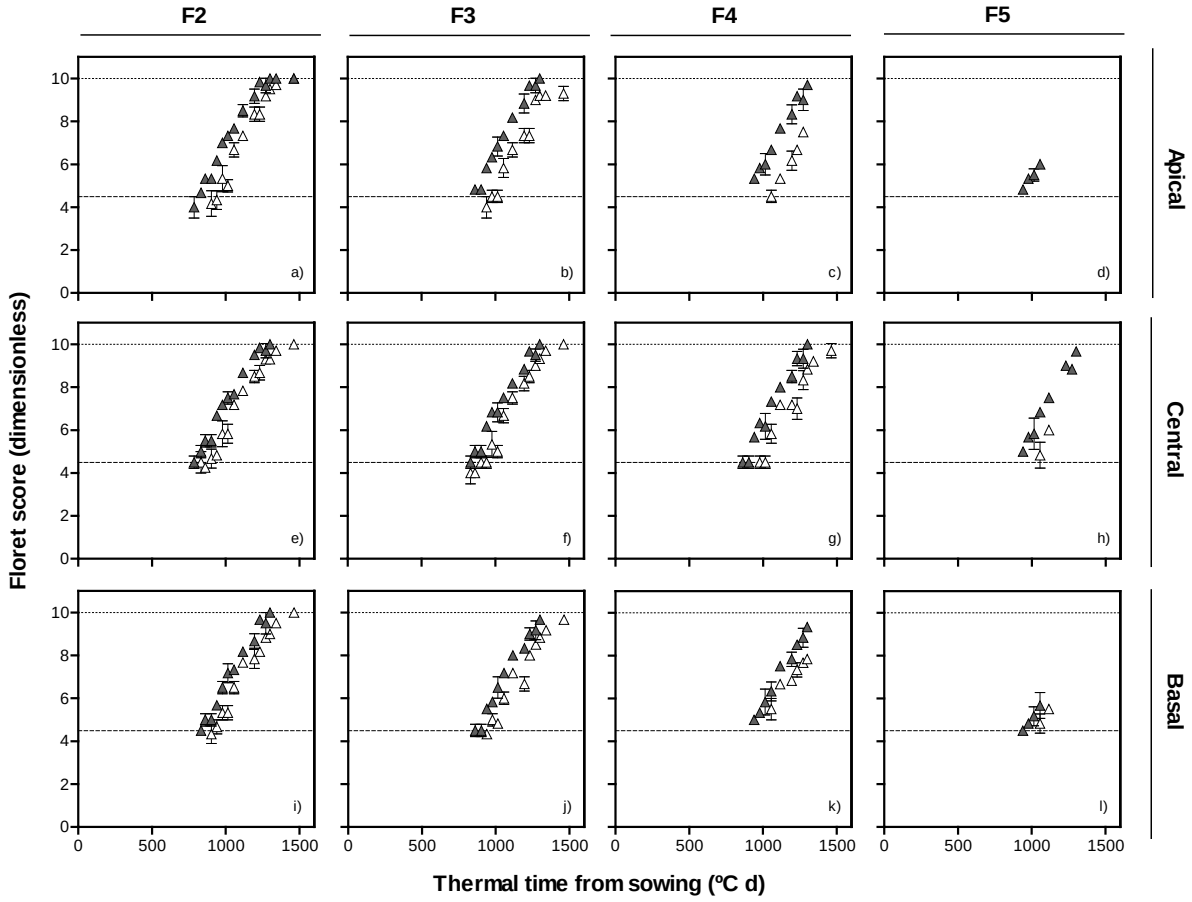


Fig. 4.6. Dynamics of floret development (floret score) in F2, F3, F4 and F5 florets at apical (top panels), central (middle panels) and basal (bottom panels) positions of the spike with thermal time from sowing in lines with *Eps-7D-late* (open symbol) and -*early* (closed symbol) allele with *Eps-2B-late* allele in the background in the first cropping seasons.

For the same reason, as results were consistent across growing seasons, we only included data for the first season in the main text and send the equivalent figures for the second season to supplementary materials.

Regarding the effect of *Eps-7D* on development of florets, the interaction with the allele of *Eps-2B* in the background was evident as well when comparing the differences between lines with *Eps-7D-early* and *Eps-7D–late* between when in the background the *Eps-2B* was the *late* or *early* allele.

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. 4.6 and supplementary Fig. S4.2). 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 (panels a, e and i in both Figs. 4.6 and supplementary Fig. S4.2). However, and 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 (Figs. 4.6a, e and i; supplementary Fig. S4.2a, e and i). The remaining distal florets (F3, F4 and F5) also showed the same trend for a 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 finally fertile in only c. 33% of the plants measured whilst it was fertile in almost all plants of the lines with the *Eps-7D-late* allele (Figs. 4.6b, j; supplementary Fig. S4.2b and 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 (Figs. 4.6c, k and supplementary Fig. S4.3c and k). Floret F5 reached W4.5 in almost all the lines but was fertile (W10) only in the central spikelet of most, but not all, plants with the *Eps-7D-early* (Figs. 4.6h and supplementary Fig. S4.3h).

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* in the background, there were no noticeable differences

in any of the spikelets and floret positions (Figs. 4.7 and supplementary Fig. S4.4). 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.

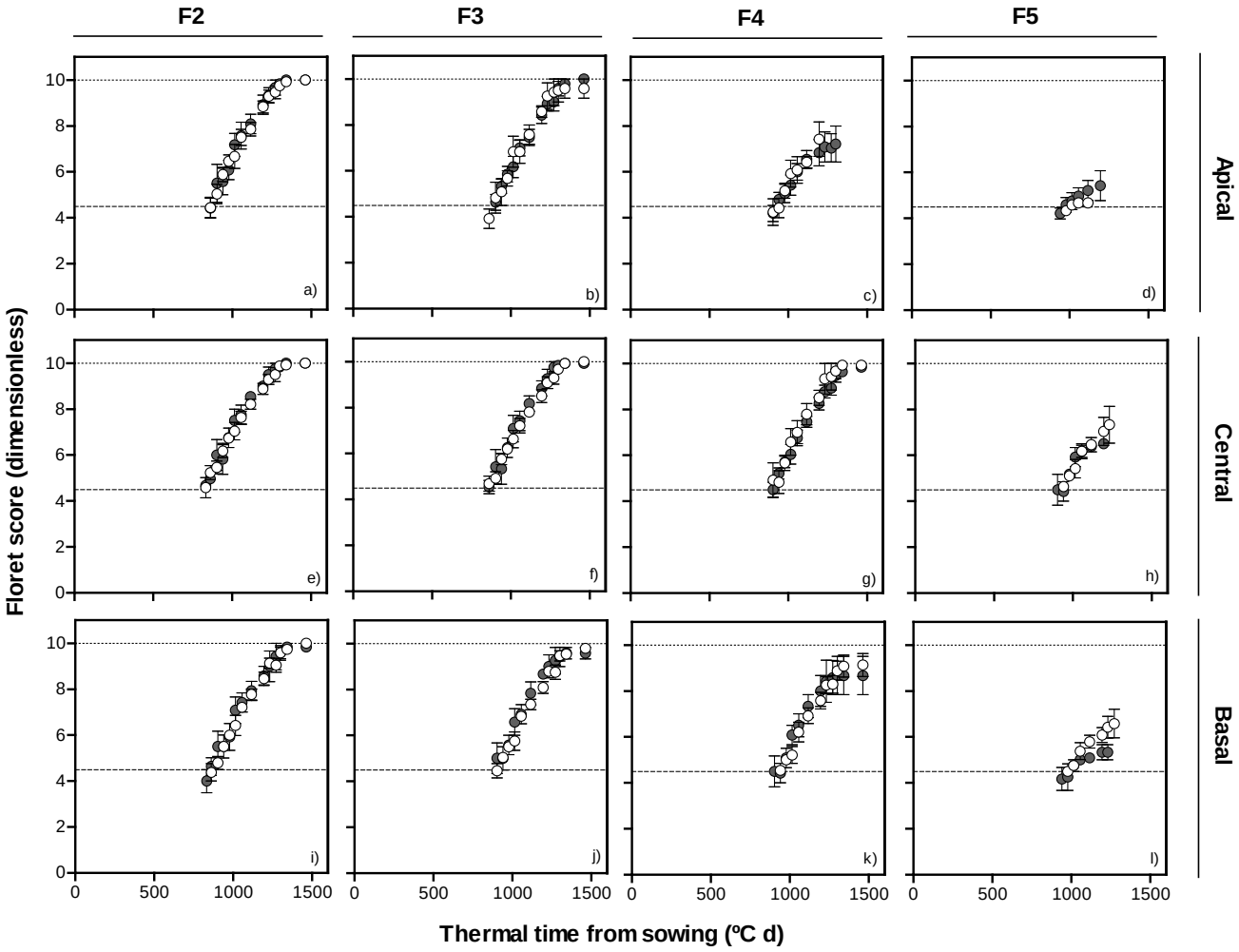


Fig. 4.7. Dynamics of floret development (floret score) in F2, F3, F4 and F5 florets at apical (top panels), central (middle panels) and basal (bottom panels) positions of the spike with thermal time from sowing in lines with *Eps-7D-late* (open symbol) and *early* (closed symbol) allele with *Eps-2B-early* allele in the background in the first cropping season.

Regarding the effect of *Eps-2B* on development of florets, the effects were clearer when in the background the *Eps-7D* was the *early* allele and generally subtler than those of *Eps-7D* (Figs. 8, 9 and supplementary Fig. S4.5 and S4.6). 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 Figs. S4.4).

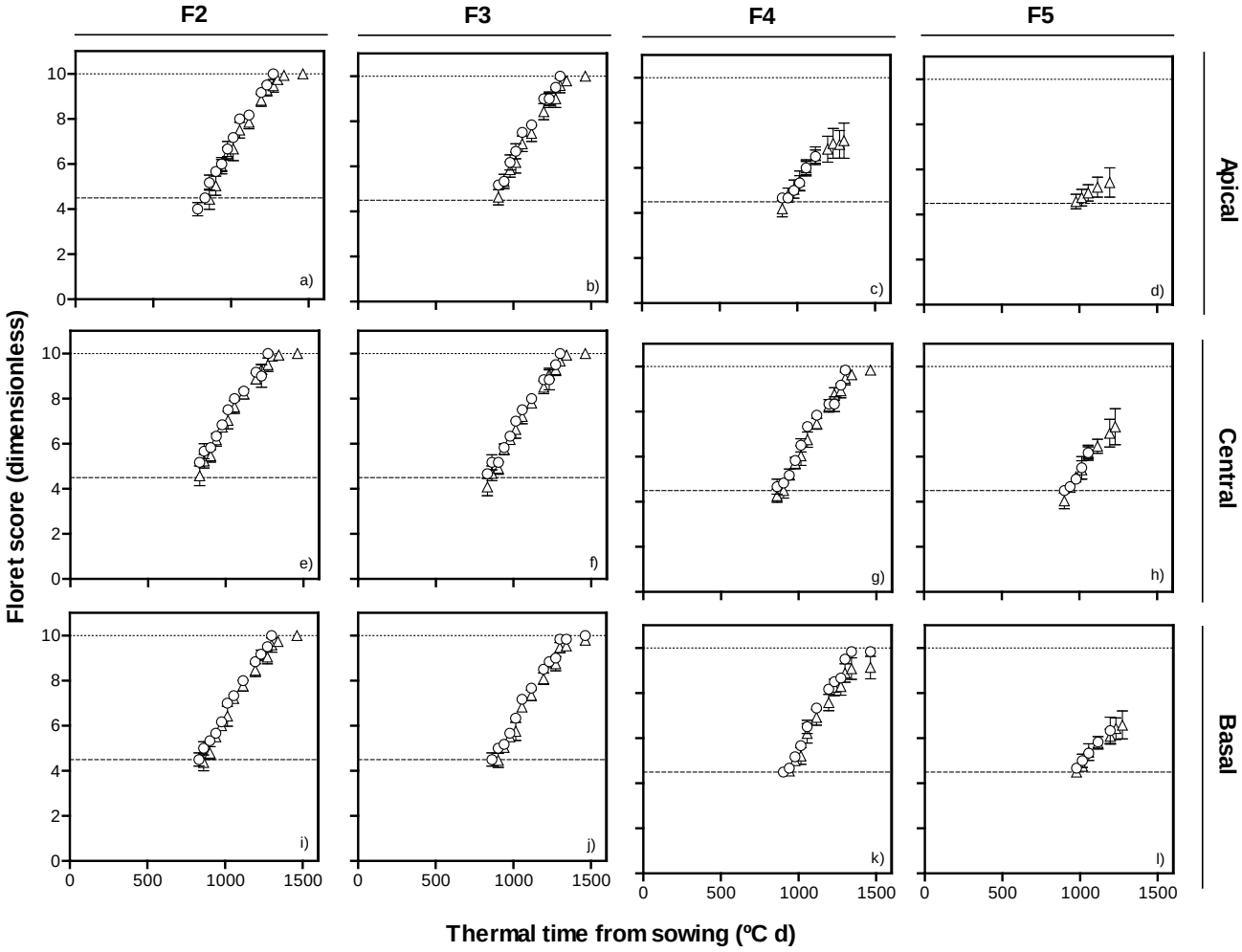


Fig. 4.8. Dynamics of floret development (floret score) in F2, F3, F4 and F5 florets at apical (top panels), central (middle panels) and basal (bottom panels) positions of the spike with thermal time from sowing in lines with *Eps-2B-late* (triangles) and *early* (circles) allele with *Eps-7D-late* allele in the background in first cropping season.

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. 4.9 and supplementary Fig. S4.5).

4.4.5. 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. S4.6 and S4.7 for CS1 and 2 respectively) and therefore we reported here an average of these three spikelets (Fig. 4.10).

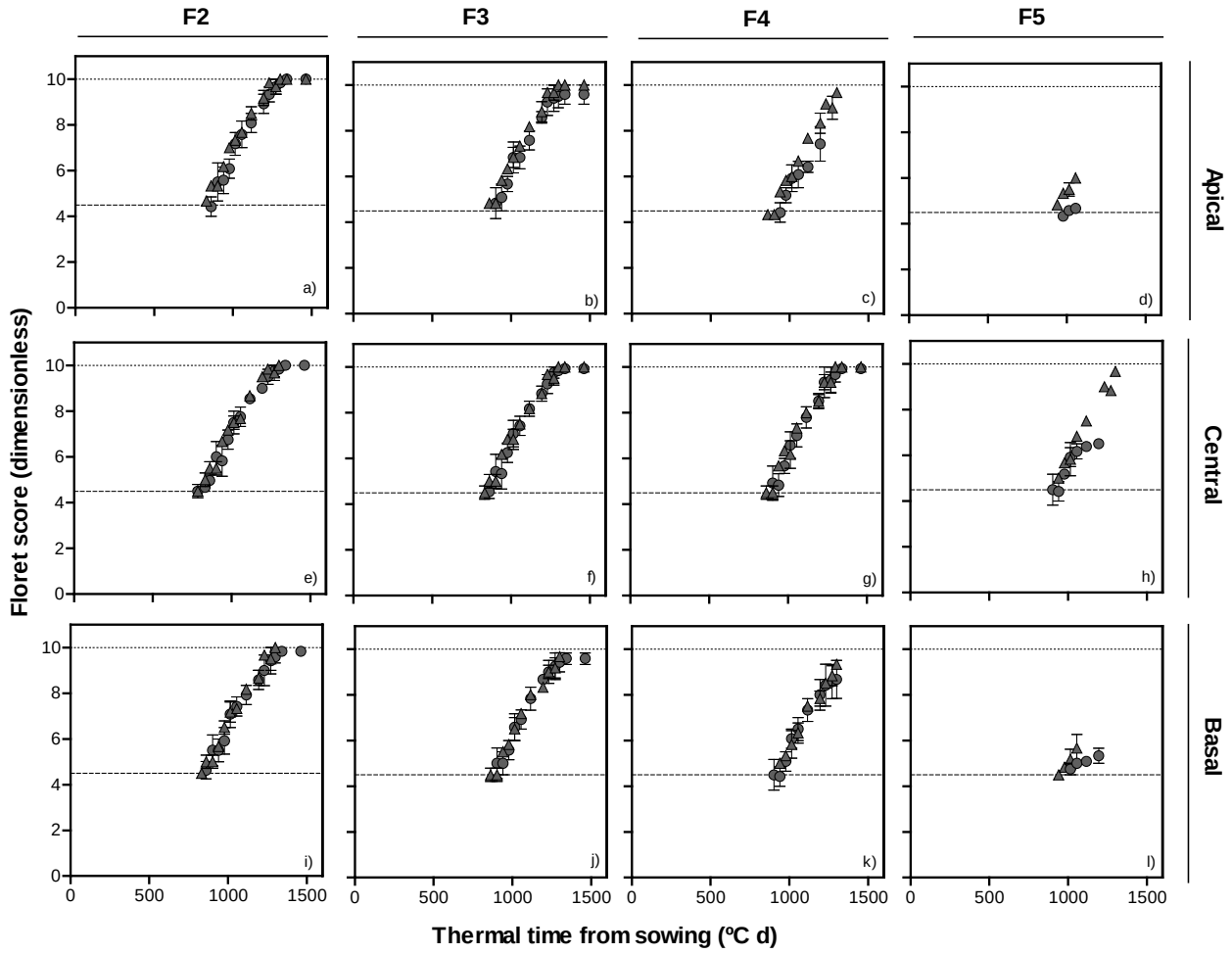


Fig. 4.9. Dynamics of floret development (floret score) in F2, F3, F4 and F5 florets at apical (top panels), central (middle panels) and basal (bottom panels) positions of spike with thermal time from sowing in lines with *Eps-2B-late* (triangles) and *early* (circles) allele with *Eps-7D-late* allele in the background in first cropping season.

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. 4.10a). These *Eps-7D-early* lines had initiated floret development earlier and therefore the duration of the process of floret initiation was not reduced (Fig. 4.10a), although the duration of the whole period from TS to anthesis tended to be shorter (Fig. 4.3, top left panel). When the *Eps-2B* in the background was the *early* allele the *Eps-7D* showed no clear effect one dynamics of floret initiation-mortality (Fig. 4.10b).

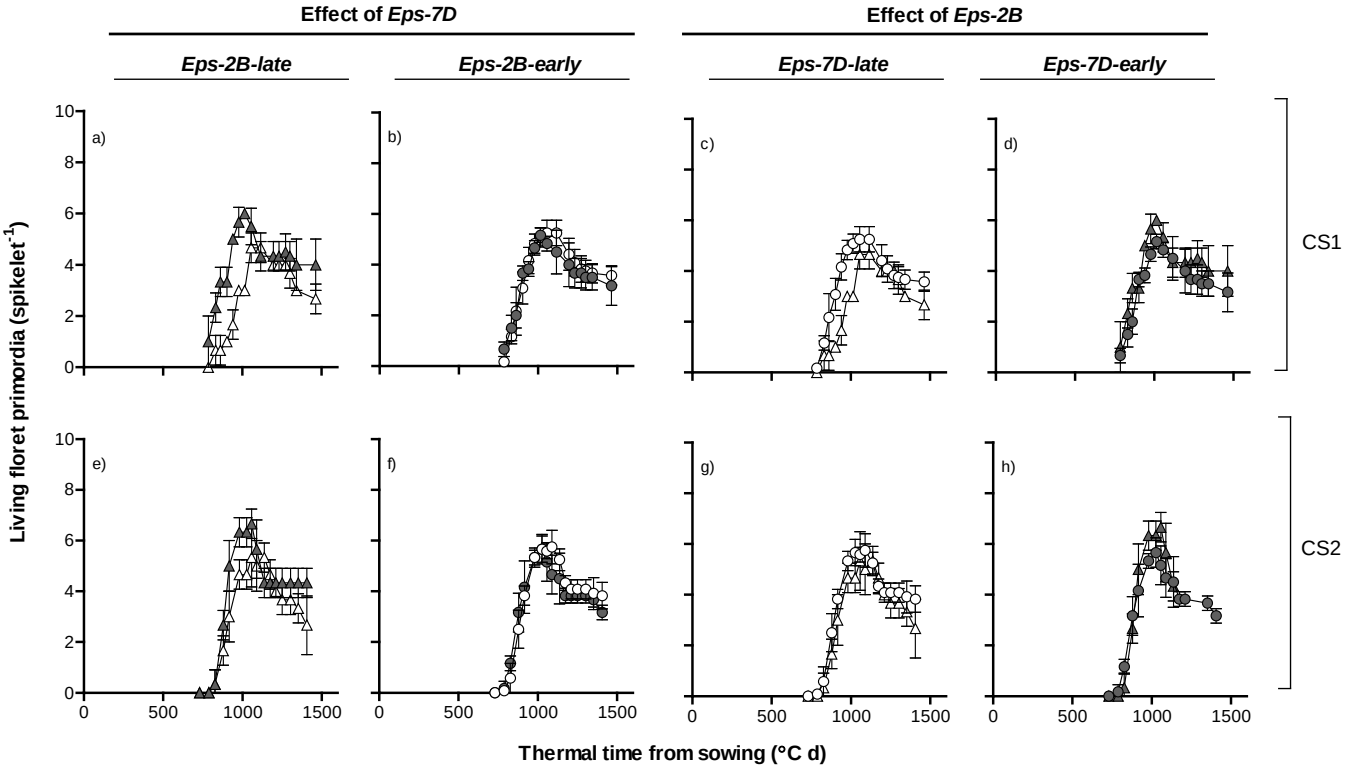


Fig. 4.10. Number living floret primordia per spike and thermal time from sowing as affected by *Eps-7D* (left panels, a, b, e and f) and *Eps-2B* genes (right panels, c, d, g and h) on backgrounds contrasting in the allelic form of the other *Eps* gene (left and right panels within each *Eps* gene) 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).

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. 4.10c). However, when the background had the *Eps-7D-early* the *Eps-2B-late* slightly increased spike fertility with respect to *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. 4.10d).

4.4.6. 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, but not when the *Eps-2B* had the *-early* allele (Fig. 4.11, left panel). Reflecting the interaction between the two *Eps* genes as well, the *Eps-2B* also affected significantly the spike fertility when the *Eps-7D* in the background was the early type, and the magnitude of the difference shrunk when the *Eps7D-late* was in the background (Fig. 4.11, right panels).

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. 4.11, left panels). 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. 4.11, right panels).

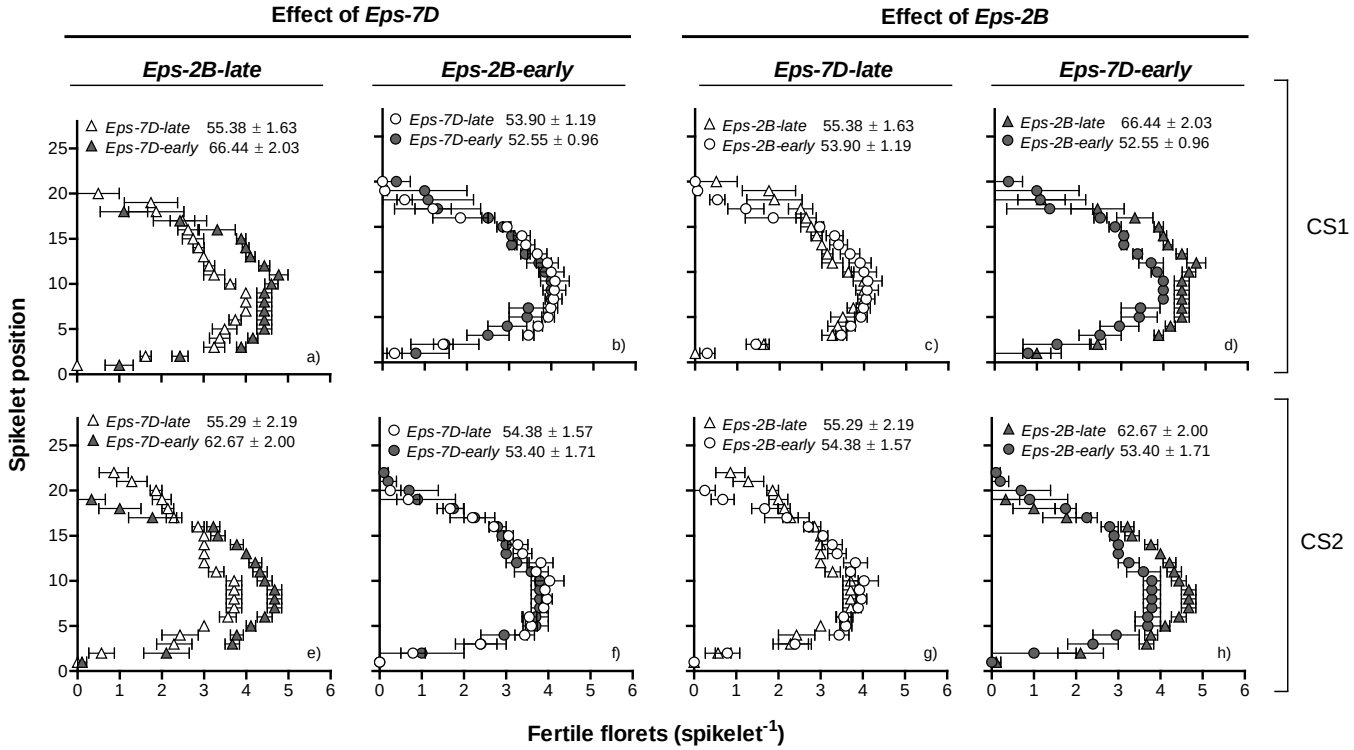


Fig. 4.11. Mapping of fertile florets at anthesis per each spikelet in the spike as affected by *Eps-7D* (left panels, a, b, e and f) and *Eps-2B* genes (right panels, c, d, g and h) on backgrounds contrasting in the allelic form of the other *Eps* gene (left and right panels within each *Eps* gene) 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.

4.5. 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 in general 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. Bullrich *et al.*, 2002; Appendino and Slafer, 2003; Lewis *et al.*, 2008; Gomez *et al.*, 2014; Griffiths et al., 2009; Ochagavía et al., 2018; Zikhali et al., 2014) but effect of *Eps-2B* was relatively very small again such smaller effects were also noted for other *Eps* (Ochagavía et al., 2018a). Therefore, either of them, but particularly the *Eps-7D* due to its more consistent effect, might be exploited to fine-tune time to anthesis of 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 (Miralles and Slafer, 2007). *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 (Lewis et al., 2008). In agreement with Appendino and Slafer (2003) our study provides proof for the possible interaction between different *Eps* in the genome (although in the present study we showed interactions with another *Eps* gene in particular, not only with the genome in general). In addition, in agreement with (Ochagavía et al., 2018a) 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 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 on how important it is to study individual *Eps* from a particular genetic background with 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 abundancy provides an enormous opportunity to work with at the same time most of them being minor genes and 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) 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 the *Eps-7D* affected the rate of leaf initiation in parallel, and consequently the lines with the *Eps-7D-early* allele had the same FLN than lines with the *Eps-7D-late* allele; 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 Ochagavía et al. (2018a) for another *Eps* gene. On the other hand, *Eps-2B* did not affect the phyllochron. This opposite effects on phyllochron, are commensurate with the fact that *Eps-7D* did also affect the duration of the late reproductive phase 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 was rather small. And the differences in the primordia was mainly due to the differences in the duration of primordia initiation as there was no alteration in the rate of primordia initiated (plastochron). Such marginal effect of *Eps* on spikelet 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¨ (Lewis et al., 2008) and ¨minor¨ (Hoogendoorn, 1985; Ochagavía et al., 2018a; Prieto et al., 2018a) *Eps* genes. Among the few studies reporting *Eps* effects beyond time to anthesis the studies by Lewis et al. (2008) in diploid wheat (under field condition) and Alvarez et al. (2016) in tetraploid wheat (under controlled conditions) were exceptions showing effect of *Eps*-Am1 bringing about larger changes in spikelet number per spike but this exception was justified by the unusual larger effect of that *Eps* on time to anthesis/heading. 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 grain yield (spikelet number- Hoogendoorn, 1985; spike fertility- Prieto *et al.*, 2018). 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 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 other *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 had been published for which having a longer stem elongation phase (LRP) would improve spike fertility which has a straight forward explanation that longer duration allows distal florets to become fertile as well as positive effect from improved spike dry weight (Miralles et al., 2000). But this is true only when the genetics involved in

altering the duration does not affect the rate of development and the differences in the duration was big enough to cause variations in spike dry weight. 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 of their differences in the overall 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 for distal florets to improve their rate of survival producing fertile florets. 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 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* genes; (Prieto et al., 2018a); and through extending the LRP manipulating the photoperiod; (Miralles et al., 2000; Prieto et al., 2018b; Serrago et al., 2008). 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 initiated (Miralles et al., 1998; Serrago et al., 2008).

4.6. References

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4.7. Supplementary data

4.7.7. Discovery of QTL on 2B, 2D and 7D and analysis

4.7.7.1. Plant material and field conditions

A segregating recombinant inbred line (RIL) population of 94 individual was developed to generation F4 from a cross between Paragon × Baj in the same way as described in (Wingen et al., 2017). Field experiments were carried at two different sites. The population was grown in 2015-16 at John Innes Centre Field Station (Church Farm, Bawburgh, 52.63' N, 1.17' E) in Norfolk, UK. RILs were grown without replication in 1 m^2 plots. Seed density was 250 seeds per m^2. Plots were sown in late October and harvested in early August. Best local agronomic practices were applied. The heading dates were recorded as the date when 50% of the plants in the plots were flowering.

4.7.7.2. Genetic mapping and QTL analysis

The Paragon x Baj RILs were genotyped using the 35k Axiom Wheat Breeders' array 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. Full genotype files and genetic maps can be found at https://data.cimmyt.org/dataset.xhtml?persistentId=hdl:11529/10996. Employing the genetic map, QTL detection was performed on heading date measurements from the UK trial, using package "qtl" (vs. 1.44–9, Broman et al., 2003) in two st*Eps*, the first scan determining co-factors and the second scan identifying robust QTL, taking the co-factors into account (Wingen et al., 2017).

Table S4.1. Summary of the QTL result for heading date in the UK environment. Abbreviations: chr = chromosome, %var = percentage variance explained by the QTL, start and end marker border the QTL confidence interval.

chr	LOD	%var	add eff	peak marker	start marker	end marker	Increase allele
2B	4.3	6.0	-2.12	AX-94940971	AX-94408000	AX-94940971	Baj
2D	16.4	27.0	-3.87	AX-94603120	AX-95217264	AX-95124335	Baj
7D	10.7	16.2	-3.25	AX-94545759	AX-94935560	AX-94523269	Baj

4.7.7.3. Heading date QTL

Three heading date QTL were identified in the Paragon x Baj RIL population with genomic locations on chromosomes 2B, 2D and 7D (see Table S4.1). 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.

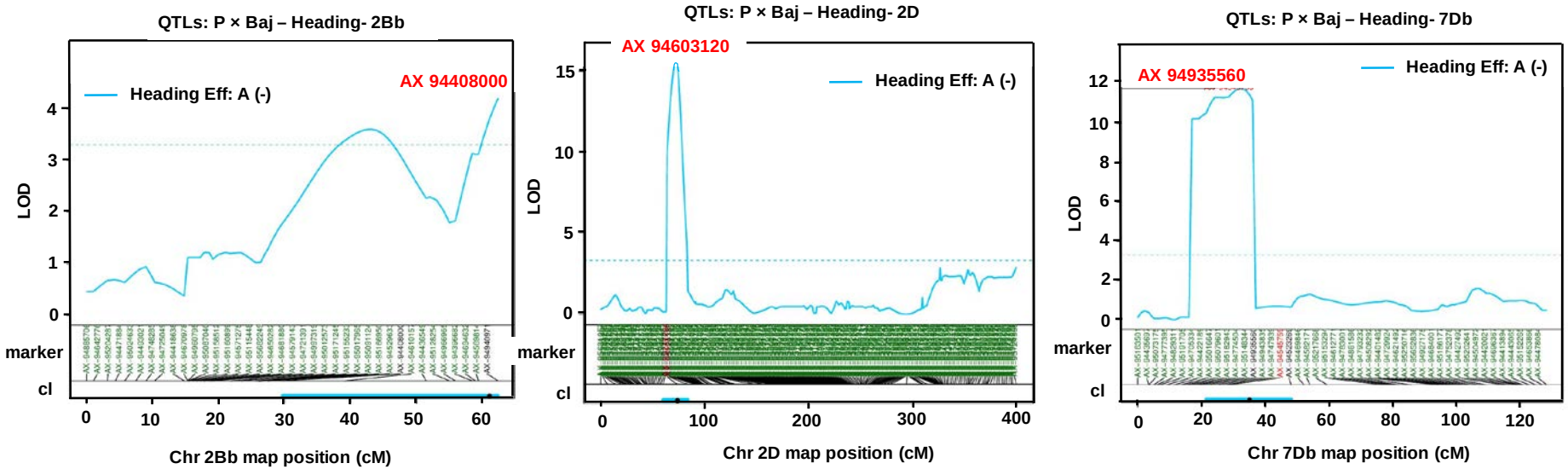


Fig. S4.1. Heading date QTL on chromosome 2B (upper panel), 2D (middle panel) and 7D (bottom panel). LOD scores are plotted along the chromosome axis. Marker names and position are listed underneath, along the chromosome axis. The peak marker is highlighted in red and the markers bordering the confidence interval in black. The extend of the confidence interval is shown as horizontal blue lines underneath the marker names.

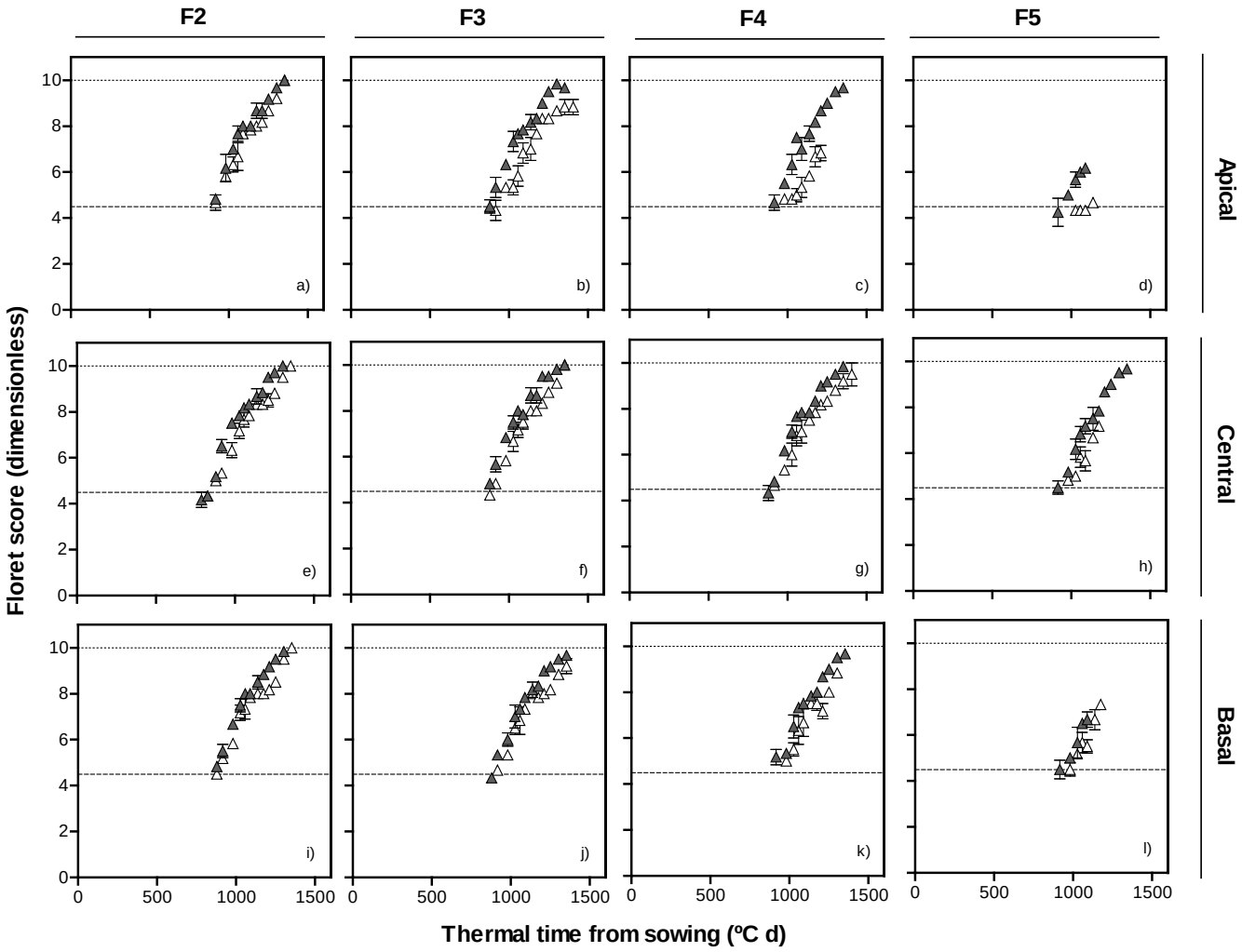


Fig. S4.2. Dynamics of floret development (dimensionless floret score) in F2, F3, F4 and F5 florets at apical (top panel), central (middle panel) and basal (bottom panel) positions of spike with thermal time from sowing in lines with *Eps-7D-late* (open symbol) and -*early* (closed symbol) allele with the -*late* allele of *Eps-2B* in the background in cropping seasons 2.

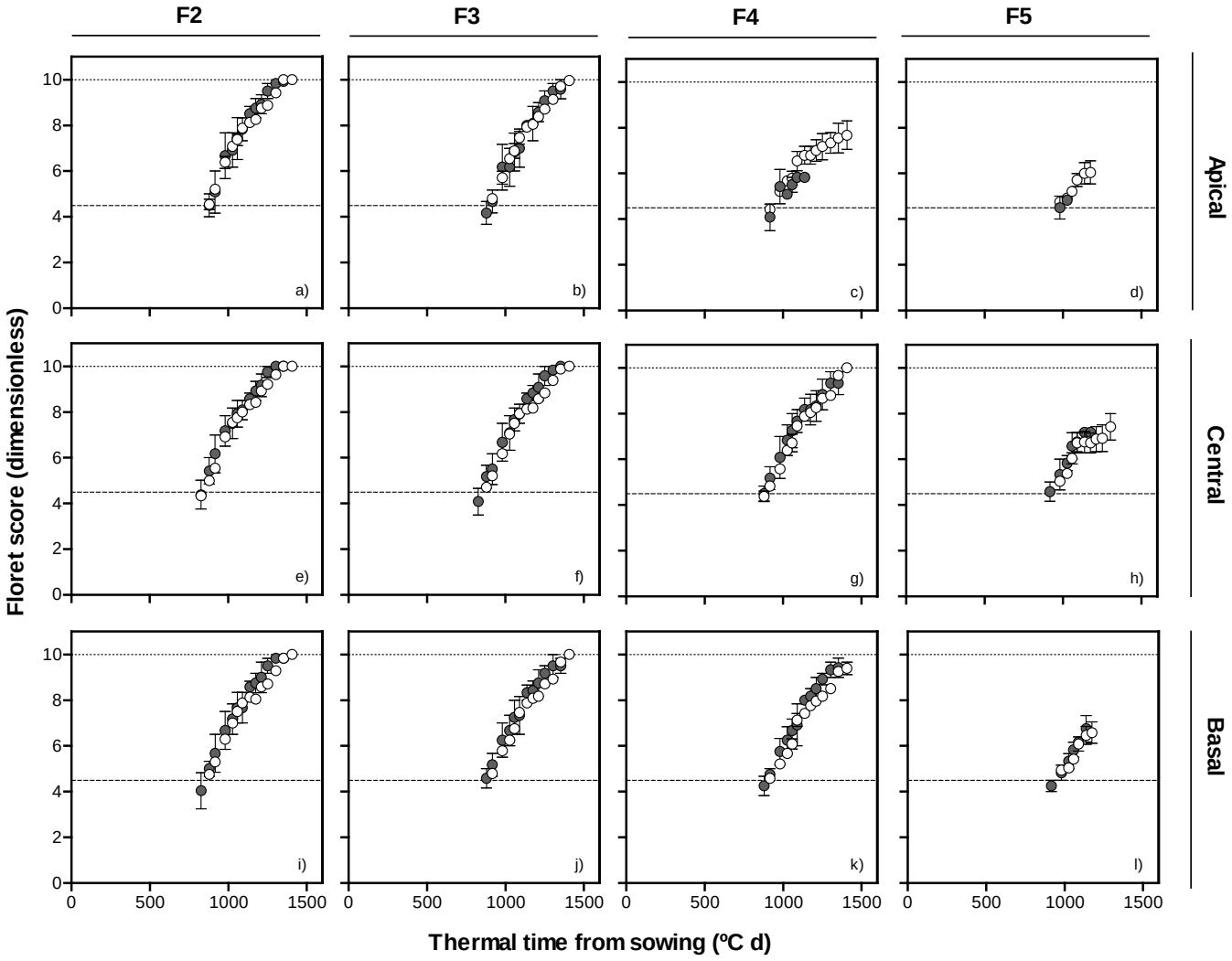


Fig. S4.3. Dynamics of floret development (dimensionless floret score) in F2, F3, F4 and F5 florets at apical (top panel), central (middle panel) and basal (bottom panel) positions of spike with thermal time from sowing in lines with *Eps-7D-late* (open symbol) and -*early* (closed symbol) allele with the -*early* allele of *Eps-2B* in the background in cropping seasons 2.

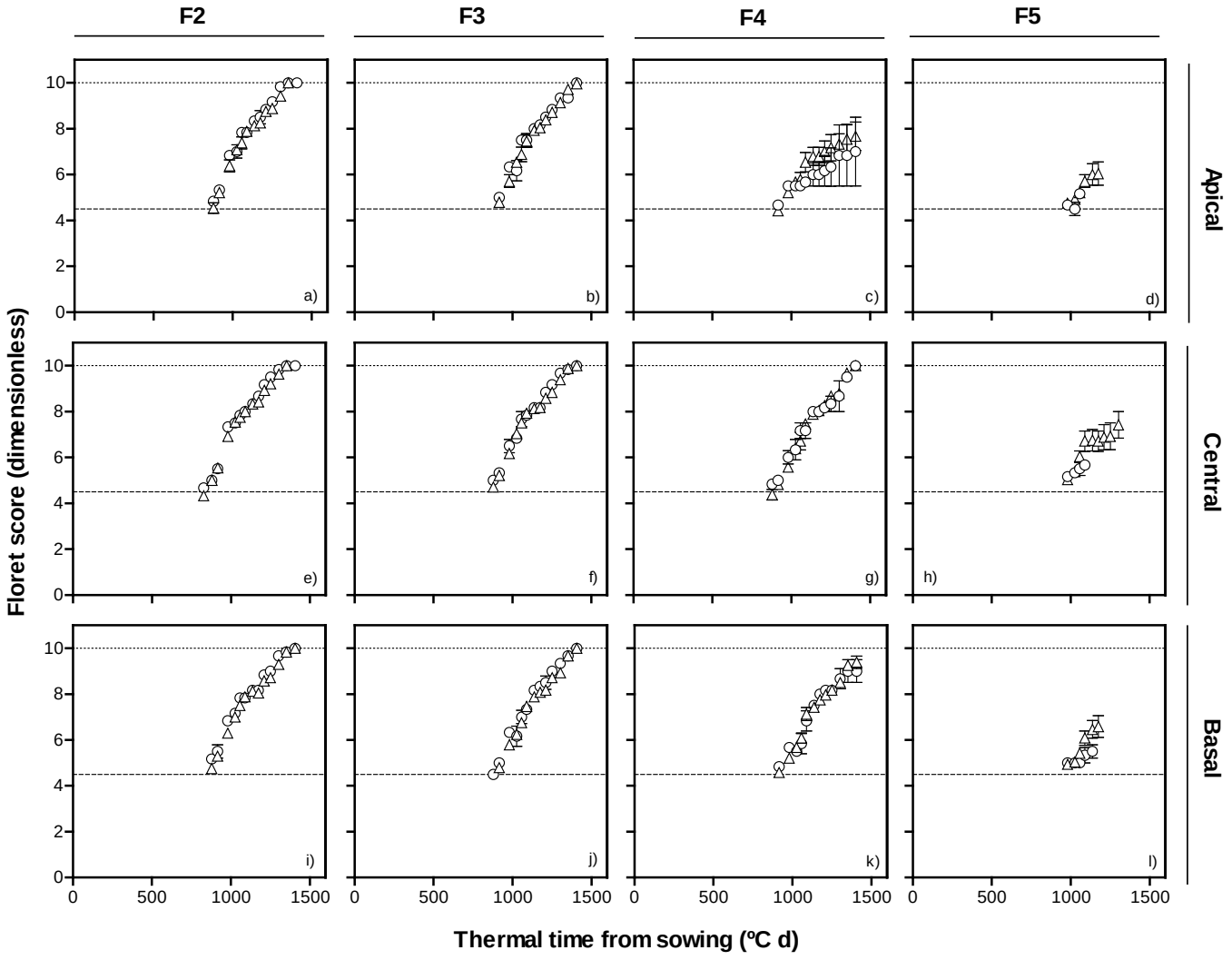


Fig. S4.4. Dynamics of floret development (dimensionless floret score) in F2, F3, F4 and F5 florets at apical (top panel), central (middle panel) and basal (bottom panel) positions of spike with thermal time from sowing in lines with *Eps-2B-late* (triangles) and *-early* (circles) allele with the *-late* allele of *Eps-7D* in the background in cropping seasons 2.

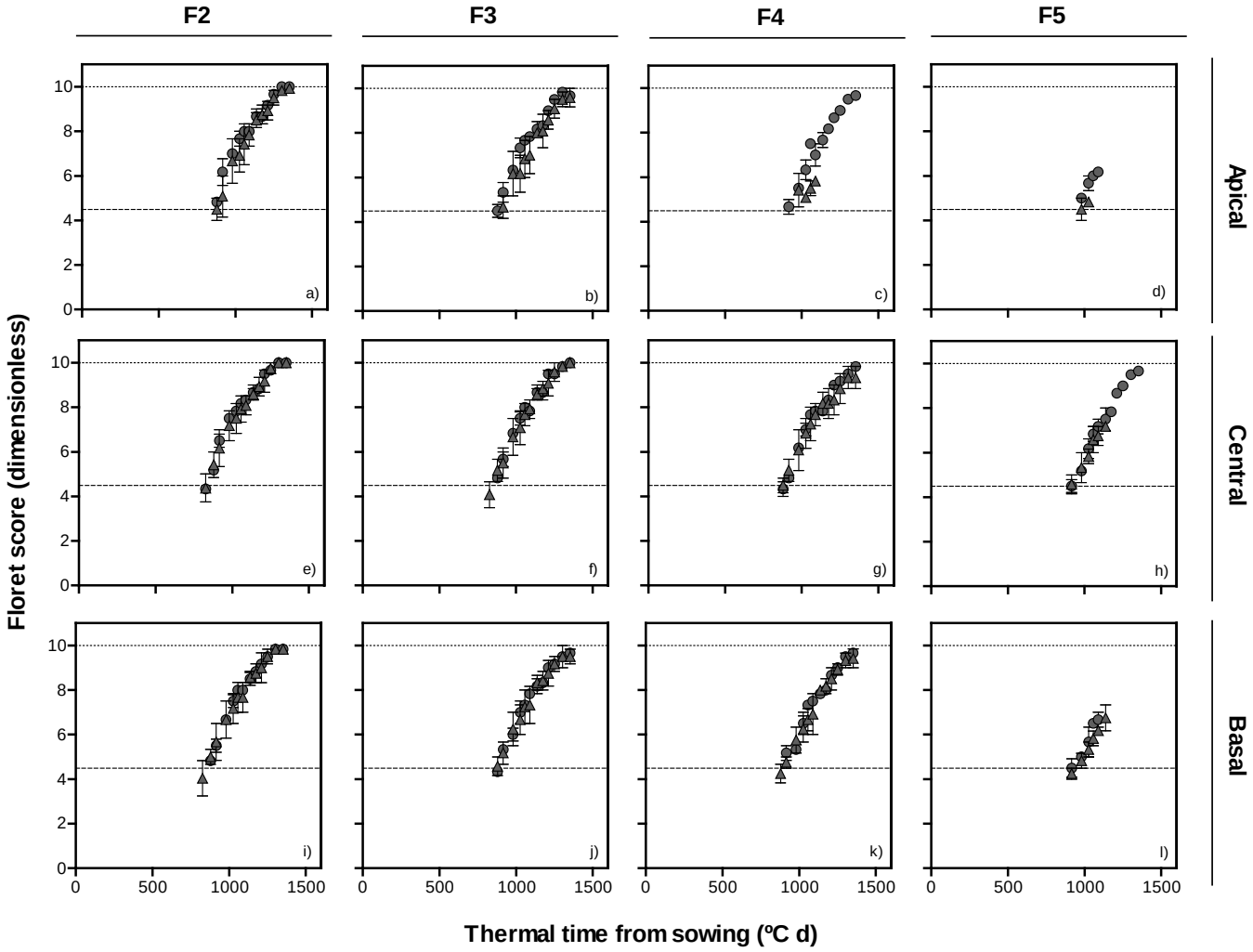


Fig. S4.5. Dynamics of floret development (dimensionless floret score) in F2, F3, F4 and F5 florets at apical (top panel), central (middle panel) and basal (bottom panel) positions of spike with thermal time from sowing in lines with *Eps-2B-late* (triangles) and -*early* (circles) allele with the -*early* allele of *Eps-7D* in the background in cropping season 2.

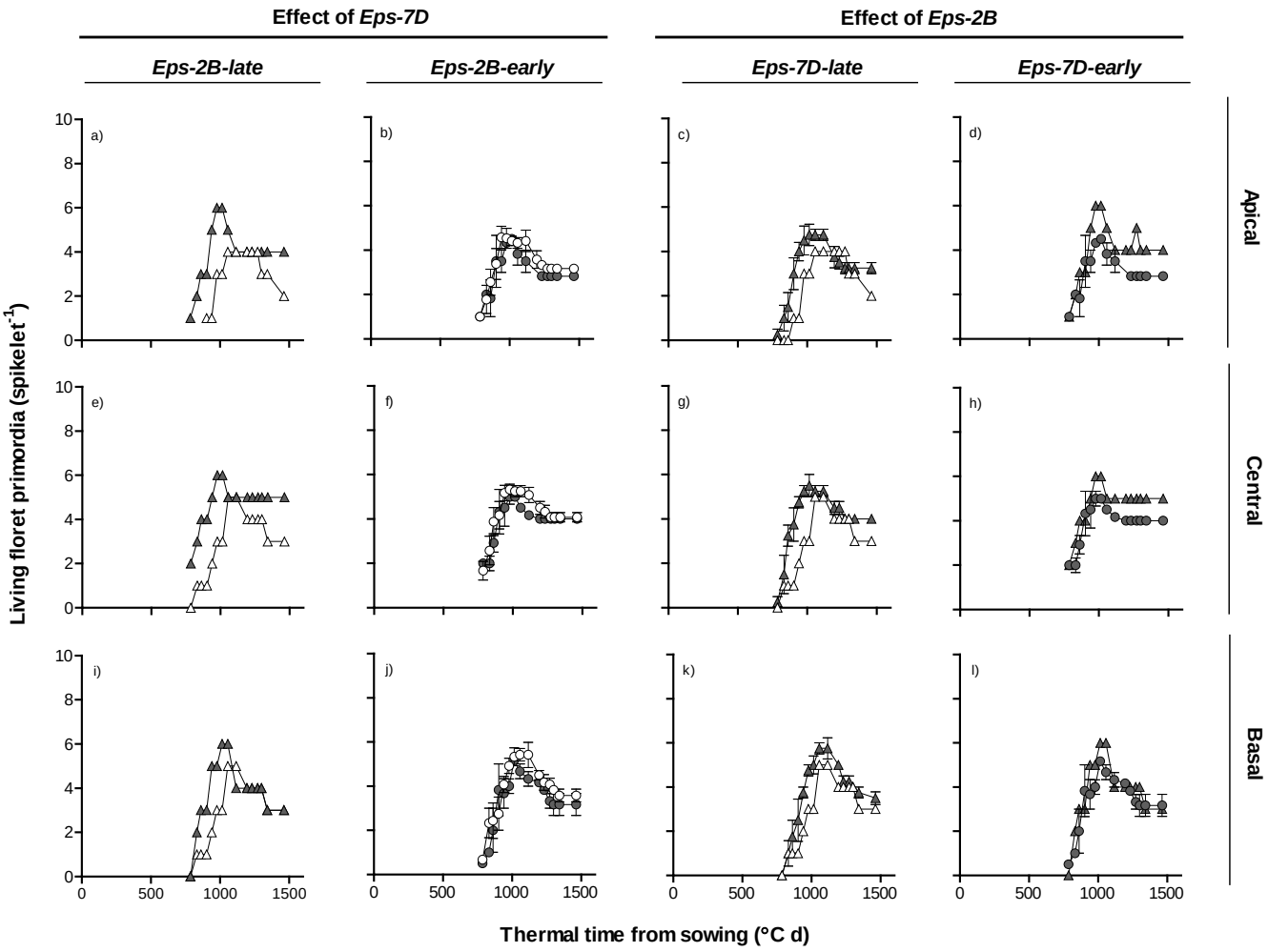


Fig. S4.6. Number living floret primordia at apical (top panels) central (middle panel) and basal spikelet (bottom panel) and thermal time from sowing as affected by *Eps-7D* (left panels) and *Eps-2B* genes (right panels) on backgrounds contrasting in the allelic form of the other *Eps* gene (left and right panels within each *Eps* gene) in cropping seasons 1.

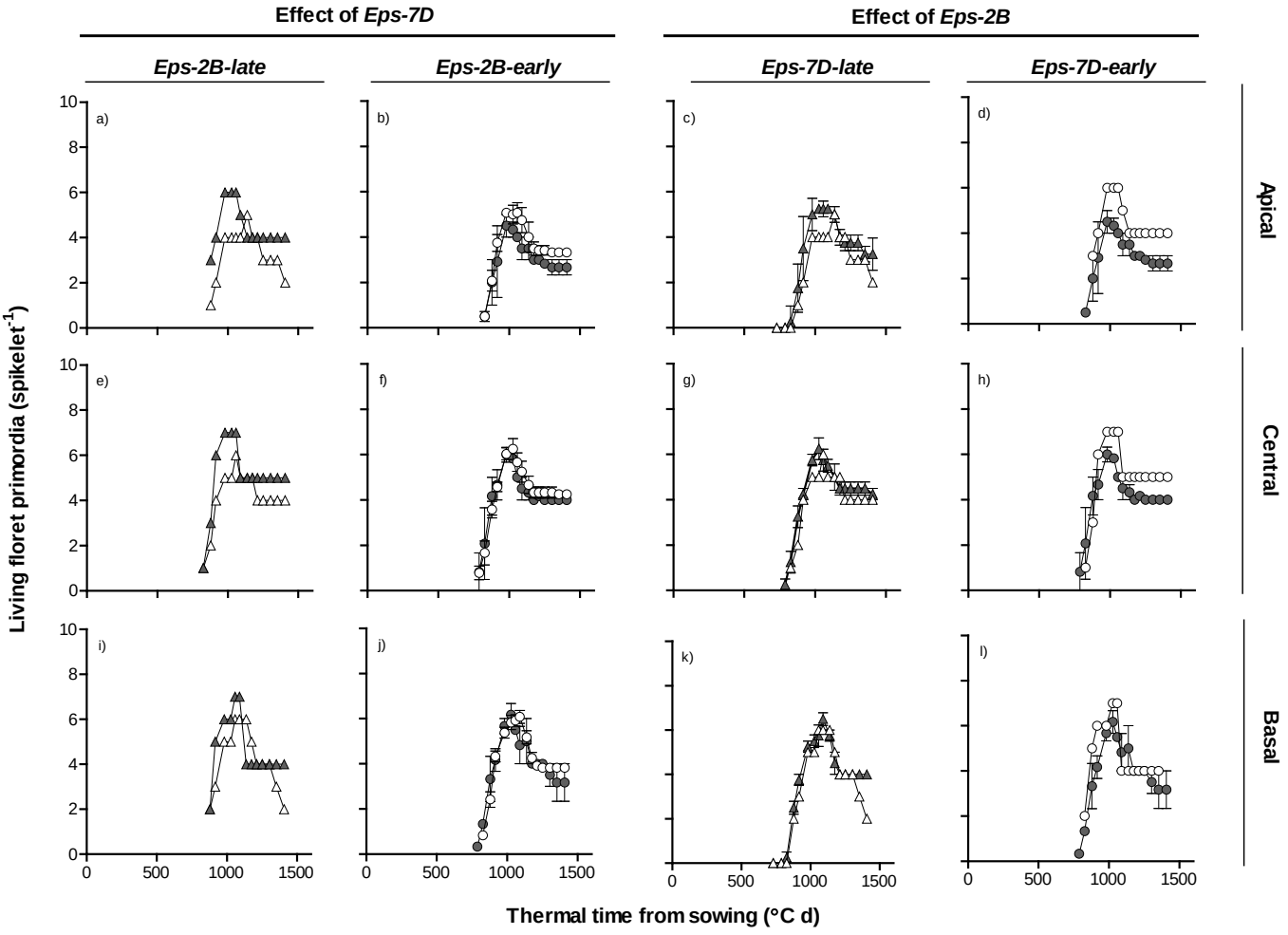


Fig. S4.7. Number living floret primordia at apical (top panels) central (middle panel) and basal spikelet (bottom panel) and thermal time from sowing as affected by *Eps-7D* (left panels) and *Eps-2B* genes (right panels) on backgrounds contrasting in the allelic form of the other *Eps* gene (left and right panels within each *Eps* gene) in cropping seasons 2.

Chapter V: Wheat developmental traits as affected by the interaction between *Eps-7D* and temperature

5. Chapter V: Wheat developmental traits as affected by the interaction between *Eps-7D* and temperature

5.1. Abstract

Earliness *per se* (*Eps*) genes are important to fine tune adaptation, and studying their probable pleiotropic effect on wheat yield traits is worthwhile. In addition, it has been shown that some *Eps* genes interact with temperature. We studied two NILs differing in the newly identified *Eps-7D* but carrying insensitive *Ppd-D1* in the background under three temperature regimes (9, 15 and 18 ºC) and two photoperiods (12 h and 24 h). *Eps-7D* affected time to anthesis as expected and the *Eps-7D-late* allele extended both the period before and after terminal spikelet. The interaction effect of *Eps-7D* × temperature was significant but not cross-over: the magnitude and level of significance of the difference between NILs with the *late* or *early* allele was affected by the growing temperature (i.e. difference was least at 18 ºC and largest at 9 ºC), and differences in temperature sensitivity was influenced by photoperiod. Rate of leaf initiation was faster in NIL with *Eps-7D-early* than with the *late* allele which compensated for the shorter duration of leaf initiation resulting in similar final leaf number between two NILs. *Eps-7D*-late consistently increased spike fertility through improving floret primordia survival as a consequence of extending the late reproductive phase

5.2. Introduction

Wheat development is critical for yield determination as it controls not only adaptation (i.e. the critical stage of anthesis must occur when conditions are best, minimising stresses during grain number determination and grain weight realisation; Fischer, 2011; Reynolds et al., 2012) but also the timing and rate of generation of structures that will become sources and sinks (González et al., 2005b; Whitechurch and Slafer, 2001). Indeed, wheat yield (as well as that of other grain crops) is the consequence of the balance between source- and sink-strength, in turn determined as the result of initiation, degeneration and rate of growth of leaves, tillers, spikelets, florets and grains. Genetic factors controlling the duration of the developmental phases would be expected to have pleiotropic effect on yield traits (Börner et al., 1993; Foulkes et al., 2004). Certainly, a number of studies have shown that modifying the duration of particular developmental phases either through genetic factors (Gawroński et al., 2014; Lewis et al., 2008; Ochagavía et al., 2018b; Pérez-Gianmarco et al., 2018; Prieto et al.,

2018a) or environmental treatments (González et al., 2005b, 2003b, 2003a; Serrago et al., 2008; Steinfort et al., 2017; Wall and Cartwright, 1974) improves spike fertility; which in turn is a major determinant of wheat yield (Slafer et al., 2014; Würschum et al., 2018).

Time to anthesis in wheat encompasses various phases with different degrees of sensitivities towards cold temperature and daylength termed as vernalisation (*Vrn*) and photoperiod (*Ppd*) sensitivities, respectively. And the genetic factors responsible for such sensitivities are referred as *Vrn* and *Ppd* genes. The *Vrn*-sensitivity genes define the growth habit (*Vrn*-sensitive cultivars are winter wheats while *Vrn*-insensitive cultivars are spring wheats), while *Ppd*-sensitivity genes determine whether flowering will be earlier (cultivars with little or no sensitivity) or late (very sensitive cultivars) in spring. However, once the effects of *Vrn* and *Ppd* sensitivity genes are removed (because genotypes have insensitive alleles for all these genes or because plants are gown under long days after having been fully vernalised), genotypes may still exhibit differences in earliness of flowering. These genotypic differences are known as earliness *per se* (*Eps*) or intrinsic earliness (Slafer, 1996). Past wheat breeding has already ventured changing time to anthesis to expand adaptation and to maximise yield by positioning anthesis time to avoid yield penalties due to abiotic stresses (Araus et al., 2002; Richards, 1991). Then, major changes in anthesis time may not be as relevant as fine adjustments. The importance of *Eps* genes may be even higher than that of the major *Vrn* and *Ppd* sensitivity genes when the need is to fine adjust phenology because they normally have a relatively small effect (Bullrich et al., 2002; Griffiths et al., 2009; Lewis et al., 2008; Ochagavía et al., 2018a). Indeed, due to their relatively subtle effect, *Eps* genes may have gone undetected during the course of selection (Zikhali et al., 2014a), and are mostly identified as QTLs (Zikhali et al., 2014a). Although much lesser known, their possible pleotropic effect on yield components might be one of the reasons for their indirect selection (Alvarez et al., 2016).

Most of what is known of the identified *Eps* genes relates to their effects on time to anthesis. The importance of these genetic factors, like any other genes, to be used in breeding programmes is limited by the lack of understanding of their detailed effect on individual phases occurring before anthesis, and their possible influence on different yield attributes along the way. Although yield components are being determined during the whole growing season, some phases are more critical than others (Fischer, 2007; Slafer, 2003). Duration of phase before and after terminal spikelet (TS) may have completely different relevance for yield determination. Indeed, it is during the TS-anthesis phase that

spike development controlling spike dry weight and spike fertility are determined (Abbate et al., 1997; Fischer, 2007; Halloran and Pennell, 1982; Serrago et al., 2008).

Some recent studies have shown the possible relevance of *Eps* genes not only in fine adjusting anthesis time, but also through affecting spikelet number (Alvarez et al., 2016) and grains per spike (Lewis et al., 2008). This is in line with the hypothesis that genes effecting developmental traits might alter the dynamics of organs initiated in response to changes in the duration (Ferrante et al., 2013; González et al., 2005a; Miralles and Richards, 2000; Prieto et al., 2018a, 2018b; Snape et al., 2001). The dynamics of organs such as tillers, spikelets and florets (resulting *a posteriori* in yield components) may well depend, at least in part, upon the time allocated for their development.

Despite *Eps* genes owe their name to the assumption that genotypic differences produced were "intrinsic" (*per se*) and therefore independent of the environment (Slafer, 1996), it was hypothesised to be temperature sensitive genes (Slafer and Rawson, 1995). The speculated *Eps* × temperature interaction (Appendino and Slafer, 2003a; Bullrich et al., 2002; Lewis et al., 2008) was recently proven in few studies (e.g. Ochagavía et al., 2019; Prieto et al., 2020). However, what we collectively call *Eps* genes are consistent in their effect on time to anthesis, but could strongly differ in their effects on other traits. It could be possible that the temperature responses of each *Eps* be different in terms of type and magnitude of the response and this needs to be studied. Understanding whether temperature affects the functionality of each *Eps* is necessary to explore the kind of environment those *Eps* could be effective and beneficial.

Recently an *Eps* QTL on chromosome 7D was identified in wheat which was known to influence time to heading (see Chapter IV). Four NILs were generated from the cross Paragon (a modern UK commercial cultivar; e.g. (Wingen et al., 2017) *Eps-7D* and Baj (a CIMMYT cultivar, used frequently as check; e.g. (Mondal et al., 2016) both of which are spring type with no requirements of vernalisation. Paragon has the *Eps-7D-late* and *Ppd-D1*b alleles while Baj has the *Eps-7D-early* and *Ppd-D1*a alleles. Thus, the four NILs comprised the four combinations of both alleles *Eps-7D* and *Ppd-D1* had identical mixture of Paragon and Baj in the background. The present study was aimed to evaluate the direct effect of the *Eps-7D* alleles (comparing the performance of the NILs having always the *Ppd-D1a* allele) and the interaction with temperature at two contrasting photoperiods to quantify mainly the effect of *Eps-7D* on phenology as well as dynamics of organ development. The NILs were grown under three constant temperatures (9, 15 and 18 ºC) and two very contrasting photoperiods (12 and 24 h).

5.3. Materials and methods

The experiments were conducted under controlled conditions in growth chambers (GER-1400 ESP, Radiber SA, Spain) at the University of Lleida, Spain. The pots (200 cm^3) were filled with approximately 120-125 g of mixture of 70% soil and 30% peat. Two seeds were sown in each pot at uniform depth and were kept under dark at room temperature until seedling emergence. And only one seedling was retained per pot before shifting the pots to the growth chamber. Extra pots were sown to select 54 pots per NIL for each chamber which had uniform seedling emergence to avoid differences in plant development before the start of the experiment. Pots were watered once or twice a week based on the growth stage/water requirements/treatment. Micro and macro nutrients were provided through irrigation at 4-leaf stage in all growing conditions. Pots were rotated once a week within each chamber throughout the experimental period to eliminate any spatial variation causing differences in micro-environment within the chambers.

Treatments consisted of a factorial combination of two near isogenic lines (NILs) differing in the allele of *Eps-7D* (*Eps-7D-early* and *-late*); two photoperiod conditions and three temperatures regimes. The NILs were derived from the cross Paragon and Baj carrying either *Eps-7D-late* from Paragon or *Eps-7D-early* from Baj. The plants were grown under either 12 or 24 h photoperiod (short day, SD and long day, LD, respectively), the treatment of LD having only half of the lights on so that daily radiation was the same for both photoperiod conditions. Three constant temperature regimes (9, 15 and 18 ºC) were imposed under each of the two photoperiods from seedling emergence to anthesis.

Nine randomly chosen plants per NIL in each of the six temperature × photoperiod conditions were marked at one leaf stage to record the dynamics of leaf appearance until the flag leaf was fully emerged. These plants were arranged in a completely randomise design with nine replicates (each replicate being an individual plant). The leaf appearance was recorded three times a week for plants under LD and at least twice a week for plants under SD at all the temperatures following the scale proposed by Haun et al. (1973). The same plants were used to map the fertile florets (number of fertile florets at each spikelet) per spike at anthesis where florets that had either hanging anthers or were at least at the green anther stage were considered to be fertile. On all plants we measured (i) the phenological stages of flag leaf emergence (DC39), heading (DC59) and anthesis (DC65) by visual observation following the scale of Zadoks et al. (1974). The dates for each stage were recorded after observing the stage in number of representative plants in each NIL. The rest of the unmarked plants (45 in each combination of NIL × photoperiod × temperature) were also arranged in a completely randomised design and were

sampled at regular intervals (depending on temperature and photoperiod treatment) to dissect and record the apex stages and number of primordia until the stage of terminal spikelet (TS), and from then to anthesis dissecting particular spikelets to determine the number and stages of each floret primordia. Three plants (replicates) per NIL within each treatment were sampled every time. Number of spikelet primordia was calculated *a posteriori* by subtracting final leaf number from the total number of (leaf and spikelet) primordia recorded until TS. For the determination of stages of development of the spike and florets we used the scale proposed by Waddington et al. (1983).

Nine plants per NIL in every treatment which were marked and maintained for recording the leaf appearance were sampled at anthesis and final number of fertile florets in each spikelet of the main shoot spike was determined. The florets were numbered F1 to Fn based on their position with respect to rachis, F1 being the most proximal to, and Fn the most distal from, the rachis. Wheat displays asynchronous development of florets across different spikelets of the spike, so dissection was carried out in three spikelets positions: apical, central or basal spikelets of the spike. Floret score (dimensionless) was recorded at each sampling for each individual floret developing in each of the three spikelet positions. We only considered for the quantitative analysis of traits determining spike fertility in this paper the floret primordia that reached at least the stage W4.5 (stage when stamen, pistil and carpel primordia are present). For the dynamics of the number of living florets (floret initiation followed by floret death) we only took into account florets that at least reached the stage of W4.5 and a floret was considered dead when it did not show development progress (advancement in the floret score) in the following consecutive dissections.

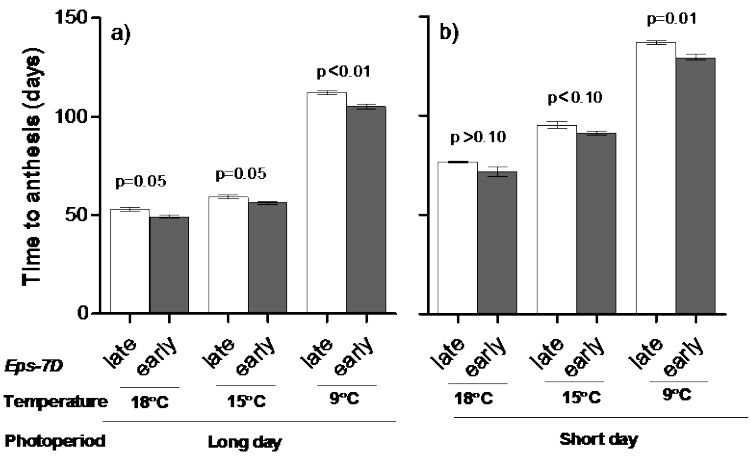
For the purpose of presenting more valuable results we averaged the floret scores of particular floret positions across all the three spikelets (apical, central and basal). While the development F1 in all the three spike positions was very similar (smaller error bars) the distal florets (F2 to Fn) had slower development in apical and basal position compared to that of the central spikelet. So, most of the variation observed due to *Eps-7D* or the temperature and photoperiods were mostly visible in florets F2 and F3.

To determine the overall effects of the *Eps-7D* allele, temperature, photoperiod and their interactions we subjected the data to a full factorial model (a three-way ANOVA) using JMP Pro version 14.0 (SAS Institute Inc., Cary, NC, USA). As the main focus of the paper was to analyse in detail the effect of the *Eps-7D* gene under each of the six growing conditions, we also carried out one-way ANOVA to determine whether the differences between NILs in phenology were significant within each

combination of temperature and photoperiod. As the effects of *Eps* genes are expected to be small, for these analyses we included, in addition to the most conventional levels of probability for significance (i.e. $P<0.05$; $P<0.01$; $P<0.001$) the P-values in each comparison indicating also whenever differences had a $P\leq0.10$ (i.e. significant only at 0.1 probability level) and used $P>0.10$ and $P>>0.1$ whenever $0.1>P<0.2$ and between 0.21-0.99, respectively.

5.4. Results

Time to anthesis was inversely related to both growing temperature (longest at 9 ºC and shortest at 18 ºC) and photoperiod (longest at 12 h and shortest at 24 h) (Fig. 5.1), the latter even though all lines carry the insensitive photoperiod allele in chromosome 1D (*Ppd*-D1a). Although these two direct effects of temperature and photoperiod are expected we also found a significant interaction between them (Fig. 5.1c), that was not simply a reflection of the temperature effect on development as the difference between short and long photoperiod was largest in the intermediate temperature. This interaction reflects the fact that sensitivity to temperature was stronger under long than under short photoperiod (*cf.* Fig. 1a and 1 b). The interaction was significant but not cross-over: the NIL with the *Eps-7D-late* allele was always later to flower than that with the *early* allele (Fig. 5.1), but the magnitude and level of significance of the difference between NILs with the *late* or *early* allele was affected by the growing temperature (i.e. difference was least, and non-significant under SD, at 18 ºC and largest and clearly significant at 9 ºC; Fig. 5.1a, b). The effect of the *Eps-7D* gene did not show any interaction with photoperiod (Fig. 5.1c) and therefore the magnitude of difference between *Eps-7D-late* and *early* NILs were similar at both photoperiods, but when considered within each particular environment, the differences were more significant under long than under short days (Fig. 5.1a and b).



c)

Source of variation	Time to anthesis
Temperature	22412.67***
Photoperiod	6889.00***
Temperature × Photoperiod	268.67***
Eps-7D	235.11***
Temperature × *Eps-7D*	22.89*
Photoperiod × *Eps-7D*	2.78^{NS}
Temperature × Photoperiod × *Eps-7D*	0.22^{NS}

Fig. 5.1. Duration of whole phase from seedling emergence to anthesis for the lines carrying *Eps-7D-late* (open bars) or *-early* (closed bars) under three growing temperatures at long day (a) and short days (b). Error bars indicate the SEMs of the mean and the "P" values stand for the level of significance exclusively due to the action of the *Eps-7D* gene within each temperature and photoperiod condition. The output (mean squares) of the three-way ANOVA for time to anthesis (days) is included on the right (c). Significance level * $p < 0.05$; ***$p < 0.001$; NS= non-significant.

The effects of temperature and photoperiod on time to anthesis were also seen for the two component phases considered here: both time from seedling emergence to TS (when all leaves and spikelets are initiated) and from then to anthesis (i.e. the late reproductive phase of stem elongation, LRP) were longer under low temperatures and short photoperiod than under warm temperatures and long photoperiod (Fig. 5.2). However, (i) even though both phases were clearly sensitive to the growing temperature, their sensitivity was not the same: duration from seedling emergence to TS responded to temperature less markedly than duration of the LRP (cf. differences between Fig. 5.2a and b with Fig. 5.2c and d, taking into account the different scales); and (ii) alike for the whole period to anthesis the sensitivity to temperature was stronger under long than under short days for both phases (Fig. 5.2). Regarding the specific effect of the *Eps-7D* gene, the NIL with the *Eps-7D-late* allele tended to have longer phases both from seedling emergence to TS and from then to anthesis across all growing conditions (Fig. 5.2).

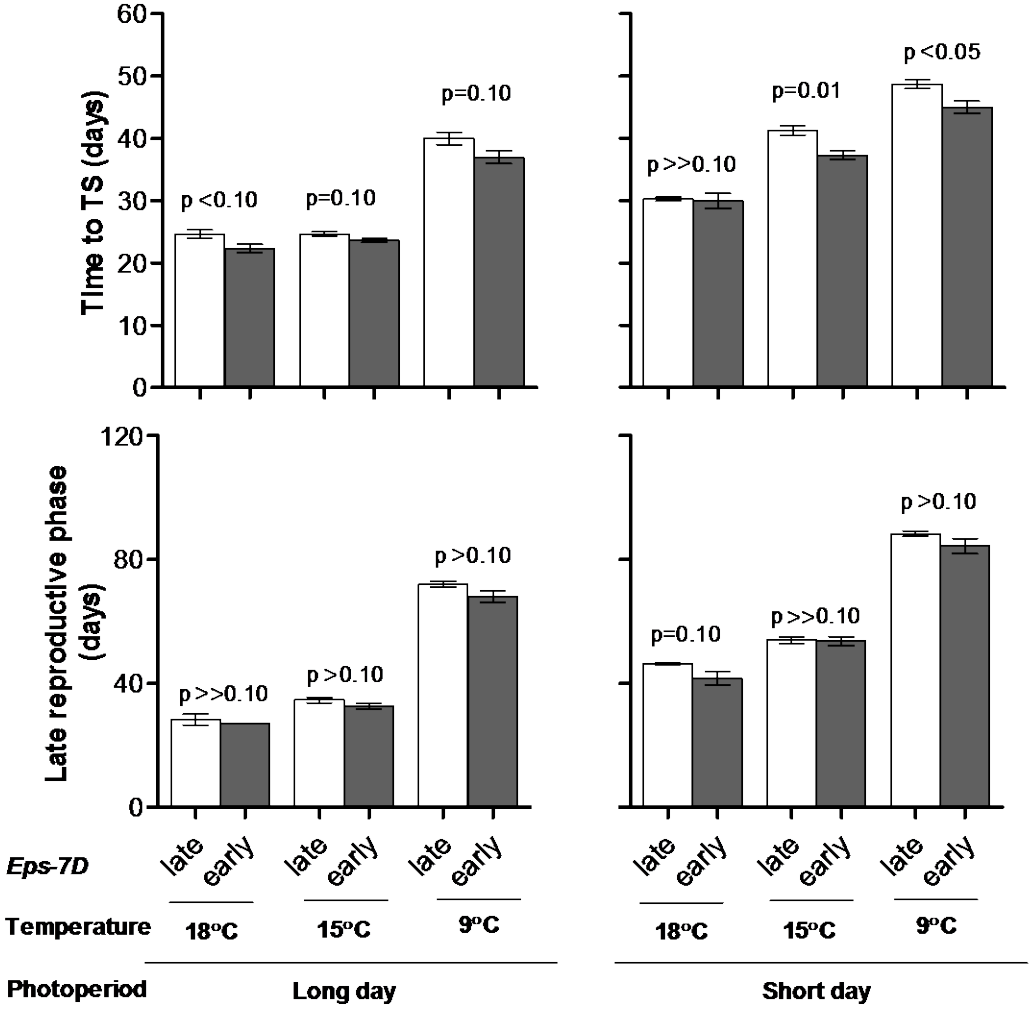


Fig. 5.2. Duration of phase from seedling emergence to TS (upper panels) and time from then to anthesis, late reproductive phase (lower panels) for the lines carrying *Eps-7D-late* (open bars) or -*early* (closed bar) under long (left panels) and short day (right panels) at three temperatures. Error bars indicate the SEs of the mean and the "P" values stand for the level of significance exclusively due to the action of the *Eps-7D* gene within each temperature and photoperiod condition.

However, as the effect on the whole period from seedling emergence to anthesis was subtle, that on the duration of each of its component phases was naturally even smaller and most differences became non-significant with the two-way ANOVA analyses done for each growing condition; particularly for the LRP (Fig. 5.2). But looking at the relationship between the duration of the total time to anthesis and its component phases it seems clear that both were at least equally important, not only reflecting the differences between growing conditions but also the effects of the *Eps-7D* gene (Supplementary

Fig. S5.1). Thus, even though most differences between NILs with *Eps-7D-early* and -*late* alleles were non-significant for the LRP (Fig. 5.2c and d), it can be seen that the magnitude of the shortening of the phases produced by the effect of having the *Eps-7D-early* allele was similar in relative terms for both phases (averaging across the six growing conditions the duration of the phase to TS and that of the LRP was 2.5 and 3 d earlier, respectively in the NIL with the *Eps-7D-early* than with the -*late* allele).

Final leaf number was not significantly affected by temperature or the *Eps-7D* gene (Table 5.1). Thus, any effects of these two factors on the duration of the vegetative phase of leaf initiation (virtually from sowing to seedling emergence or soon after it; see below) would have been compensated by opposite effects on the rate of leaf initiation. Photoperiod effect on FLN was small but clear; averaging across temperatures and *Eps-7D* alleles plants developed slightly less than 1 additional leaf if grown under short photoperiod. This means that when plants were exposed to long days they immediately reached floral initiation at seedling emergence (as there would be 4 leaf primordia in the embryo and a couple would have been initiated between sowing and seedling emergence) whilst at short days it took an additional plastochron to reach floral initiation, a difference that was very slight as expected (all plants were insensitive to photoperiod regarding the major gene *Ppd*-D1).

Table 5.1. Effects of the *Eps-7D* gene on final leaf number (FLN), rate of leaf appearance (RLA; estimated as the slope of the linear regression of leaf number vs thermal time), and the coefficient of determination for that regression (r^2), when grown under two contrasting photoperiods (12 and 24 h) and three temperatures

Growing conditions		Allele at *Eps-7D*	FLN	RLA (leaves d^{-1})	r^2
Long day	18 ºC	*Late*	6.2 ± 0.1	0.142 ± 0.003	0.953***
		Early	6.0 ± 0.0	0.149 ± 0.005	0.923***
	15 ºC	*Late*	6.0 ± 0.0	0.122 ± 0.001	0.986***
		Early	6.0 ± 0.0	0.131 ± 0.001	0.983***
	9 ºC	*Late*	6.0 ± 0.0	0.083 ± 0.001	0.980***
		Early	6.0 ± 0.0	0.083 ± 0.001	0.968***
Short day	18 ºC	*Late*	7.0 ± 0.0	0.126 ± 0.001	0.983***
		Early	6.6 ± 0.1	0.130 ± 0.002	0.975***
	15 ºC	*Late*	7.0 ± 0.0	0.083 ± 0.001	0.985***
		Early	6.9 ± 0.1	0.087 ± 0.001	0.984***
	9 ºC	*Late*	6.7 ± 0.2	0.066 ± 0.001	0.959***
		Early	6.1 ± 0.1	0.072 ± 0.001	0.977***

***All linear regressions of leaf number *vs* time were highly significant ($P<0.001$; n=10-25, depending on the temperatures and photoperiod as leaf number was determined thrice a week)

The initiated leaves always appeared at a reasonably constant pace (as indicated by the very high coefficients of determination of the linear relationship between leaf number and time; $r^2>0.92$, $n\geq10$; Table 5.1). The rate of appearance of these leaves was positively affected by temperature and photoperiod (the higher the temperature or longer the day the faster the rate of leaf appearance; Table 5.1).

As floral initiation occurred at seedling emergence or just 1 plastochron later (see above), we could only collect data revealing the dynamics of spikelet initiation (and estimate from that dynamics the spikelet plastochron). Spikelets were initiated at a more or less constant rate whose actual value was rather similar (and few differences were not consistent) for NILs with the *early* or *late* allele in *Eps-7D*,

and in all cases clearly slower at 9 than at 15 or 18 ºC and slower under short than under long days (Fig. 5.3).

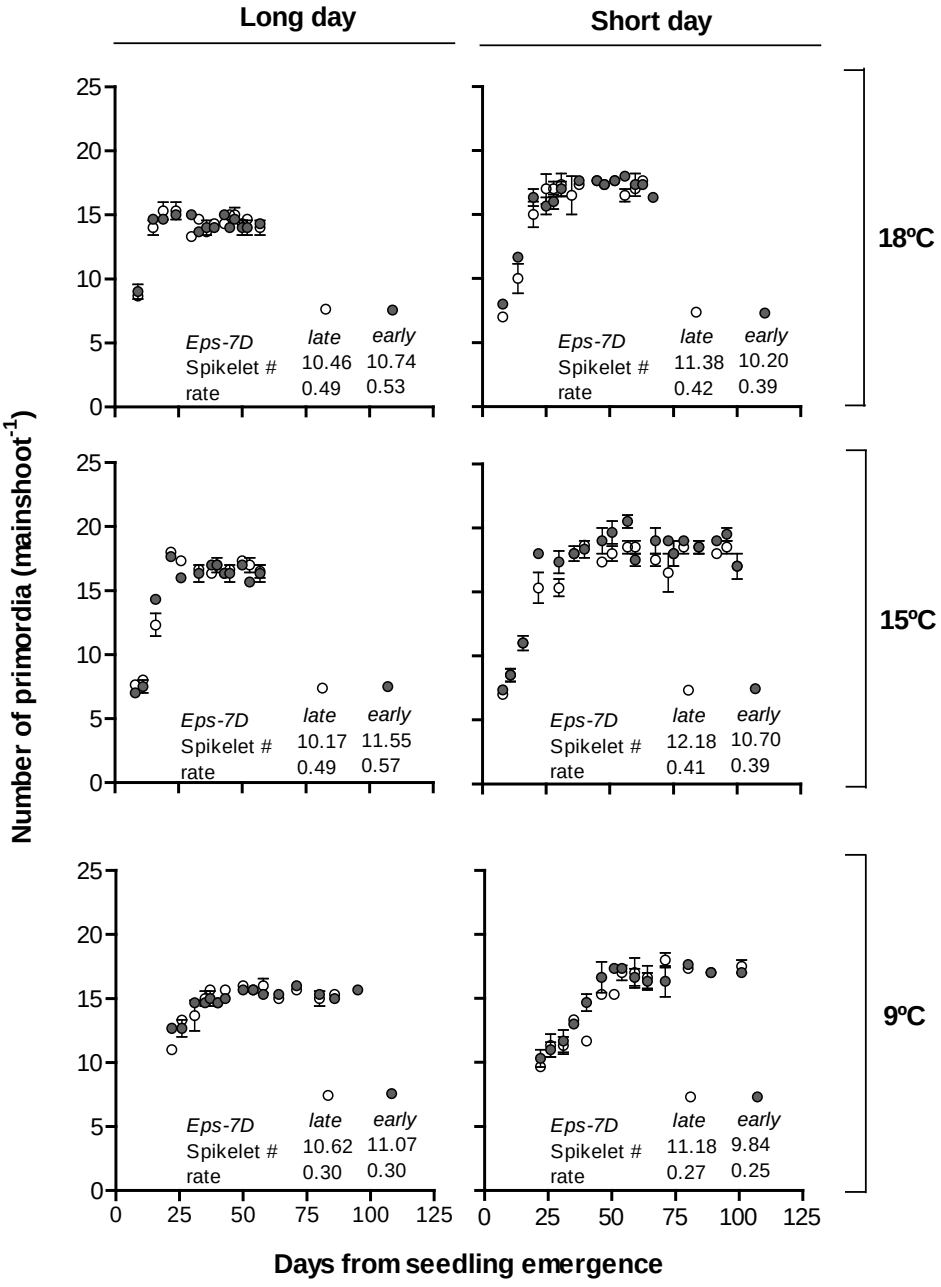


Fig. 5.3. Relationship between number of primordia and days from seedling emergence for *Eps-7D-late* (open circles) and *early* (closed circles) under long (left panels) and short days (right panels) at 18 (upper top panels), 15 (middle panels) and 9 ºC (bottom panels). Inside each panel are the total number of spikelet primordia and rate of spikelet initiation (spikelet primordia per day).

The dynamics of floret development was recorded for all the initiated florets within apical, central and basal spikelets that reached a developmental stage of W4.5 until they either reached W10 (fertile floret) or die. Floret 1 (most proximal floret to rachis) in both *Eps-7D-late* and *early* lines reached the stage of fertile floret (W10) under all three temperatures and two photoperiods, while F4 (the most distal floret consistently reaching at least the stage W4.5) has never reached to a stage close to W10 in any of the growing conditions (Supplementary Fig. S5.2). Then to understand the effects of treatments on spike fertility, we concentrated the results on the fate of the second and third florets from the rachis (F2 and F3 respectively) which were those responsible for the differences in number of fertile florets per spike at anthesis. Alike what was described for the initiation of spikelets, the rates of floret development were affected by the growing conditions. Florets developed much faster at 18 than at 9 ºC but also the opposite was true with the duration of the period of floret development: shortest and longest at 18 and 9 ºC, respectively (Figs. 5.4 and supplementary Fig. S5.2). Photoperiod did not affect noticeably the rate of floret development but did modify the duration of the period of floret development (Figs. 5.4 and supplementary Fig. S5.2).

Regarding the effect of the *Eps-7D* gene, Floret 2 was initiated more or less at the same time for both *Eps-7D-late* and -*early* under long day in all the three temperatures but under short day *Eps-7D-early* tended to initiate the F2 earlier and had faster development compared to *late* allele (Fig. 5.4). Under long day F2 reached W10 at 18 ºC for both *Eps-7D-late* and -*early* alleles, while one third of the florets F2 in *Eps-7D-late* reached W10 under lower temperatures (15 and 9 ºC) and F2 from *Eps-7D-early* aborted when they had reached the W8.5 stage (green anthers). None of the F3 florets reached W10 regardless of whether the lines had the *Eps-7D-late* or -*early* alleles and therefore the effect of the *Eps-7D* gene was inappreciable. Even though the F4 florets did never reach the stage of fertile florets they attained higher floret score when the line had the *Eps-7D-late* allele, especially under short day conditions (Supplementary Fig. S5.2).

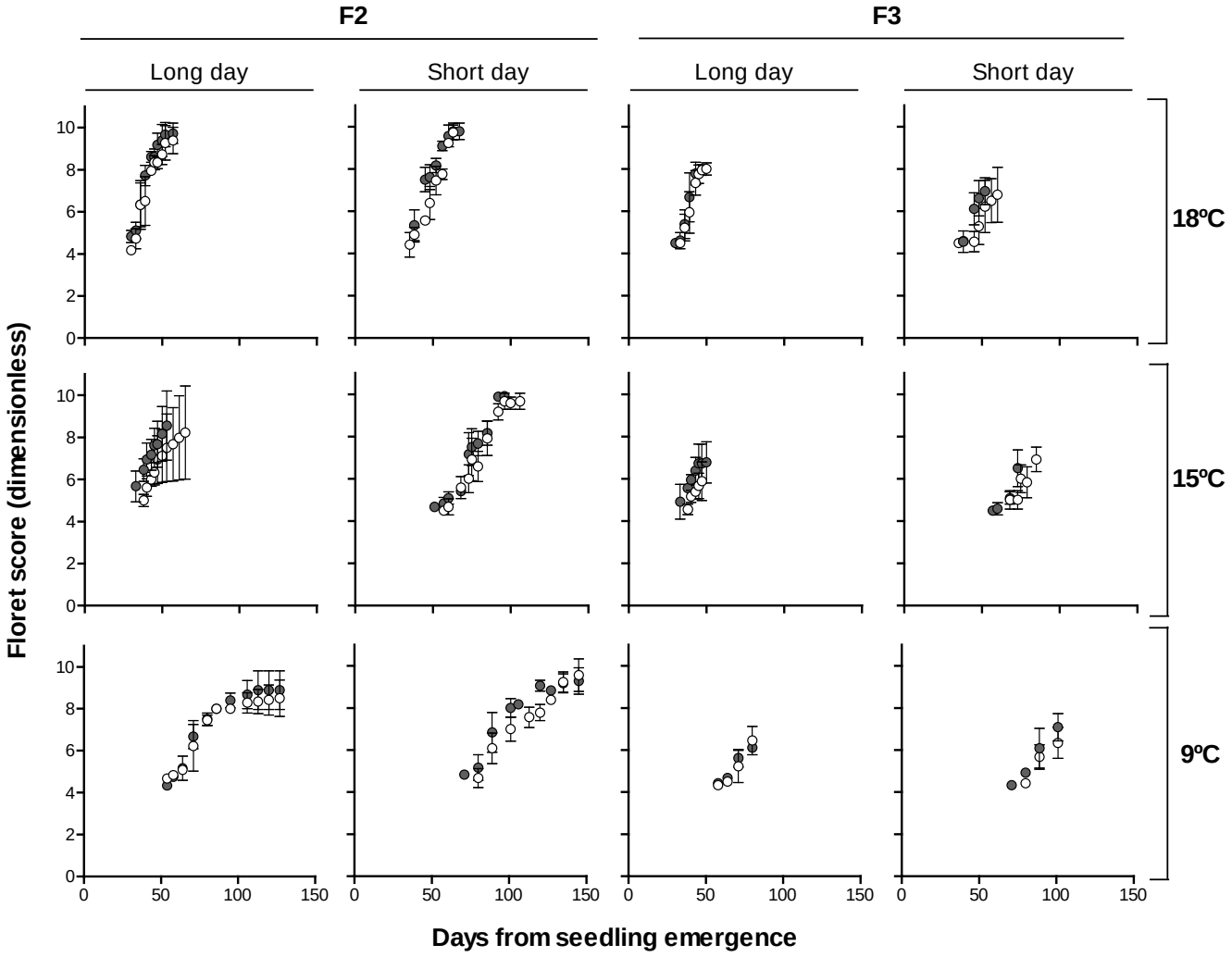


Fig. 5.4. Relationship between floret development (floret score of the Waddington et al., (1983) scale) and days from seedling emergence for *Eps-7D*-late (open circles) and -*early* (closed circles) for floret F2 (left panels) and F3 (right panels) under long and shot day at 18 (upper panels), 15 (middle panels) and 9 ºC (bottom panels). The error bars are SEs of means of floret scores from apical, central and basal spikelets.

Spike fertility was not consistently affected by temperature (because of the opposite effects of this factor in the rate and duration of floret development, see above); and was higher in short than in long days by virtue of the photoperiod effect on duration of floret development (Fig. 5.5). The *Eps-7D* gene had an effect on the number of fertile florets per spike as the NIL with the *late* allele showed a consistent trend (though not always statistically significant) to have more fertile florets than the NIL with the *early* allele (Fig. 5.5).

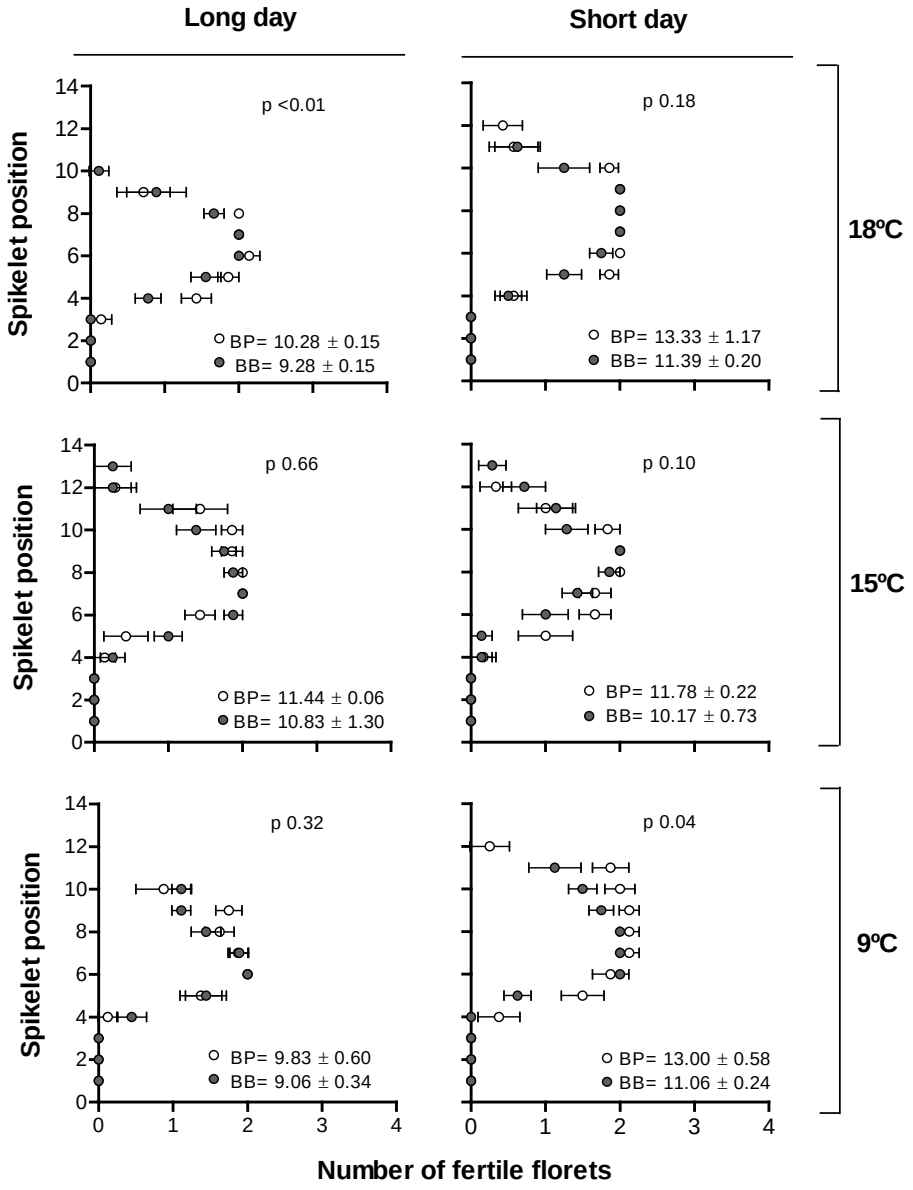


Fig. 5.5. Number of fertile florets at anthesis per spikelet from basal to terminal spikelet for *Eps-7D-late* (open circles) and -*early* (closed circles) NILs under long (left panels) and shot days (right panels) at 18 (upper panels), 15 (middle panels) and 9 ºC (bottom panels). Inside each panel are the fertile florets per spike ± SEs and p value.

The overall direct effect of *Eps-7D* gene on the number of fertile florets was much higher than the direct effect of temperature and *Eps-7D* × temperature interaction effect (F ratio was 8.50, 5.61 and 0.65 for *Eps-7D*, temperature and their interaction respectively). In that the averaging across the temperature the *Eps-7D-late* had almost c. 1 extra fertile floret per spike than that of *early* allele under

LD and the difference doubled under short photoperiod. The huge effect of temperature on the phenology was not reflected in the fertile floret as temperature also affected the rate of floret development (similar to rate of leaf appearance and spikelet primordia initiation explained above) meaning longer duration of floret development due to low temperature did not allow more florets to advance towards fertile stage rather development of each floret was significantly slow (e.g. F1 took 22 d and 74 d at 18 and 9 ºC, respectively under LD to advance from W4.5 to W10 for *Eps-7D-late* allele).

5.5. Discussion

Although the main focus of this study was on the effects of this newly reported *Eps-7D* gene on developmental processes and whether or not those effects were affected by the growing temperature, we also reported the effects of temperature, photoperiod and their interaction on these developmental processes. As the temperature x photoperiod and *Eps-7D* x temperature interactions were significant (but that of *Eps-7D* x photoperiod and the triple interactions were not), we firstly discussed briefly the effects of the environmental factors and then those of the *Eps* and its interaction with temperature.

5.5.1. Temperature, photoperiod and their interaction

In general, developmental rates were faster (reducing the length of both the whole cycle to anthesis and its component phases occurring before and after terminal spikelet) under high than under low temperature conditions. This overall effect is in line with the recognised universal effect of temperature on accelerating developmental processes not only in wheat (Slafer and Rawson, 1994a; John and Megan, 1999); as well as in and other crops (Parent and Tardieu, 2012) and other unrelated organisms (Gillooly et al., 2002). Also the rate of leaf appearance (that was constant for all leaves, as expected when FLN is less than 8; (Ochagavía et al., 2017; Slafer and Rawson, 1997) was positively responsive to temperature; as has been known for a long time (e.g. Miglietta, 1989; Slafer and Rawson, 1997). As temperature accelerated the rate of primordia initiation was compensated with the acceleration of development (i.e. phases are shorter but primordia are initiated faster under higher temperatures). Consequently, not clear effects of temperature were evident for the final leaf number, the number of spikelets per spike or the number of fertile florets per spike, again as expected from this universal effect of temperature on rates of phenological development and of initiation of primordia during the corresponding phenological phases (Slafer and Rawson, 1994a).

There was a direct effect of photoperiod on time to anthesis, that was not restricted to the phase from seedling emergence to TS as the LRP was also affected by the exposure to contrasting day lengths (in line with previous evidences in the literature showing that the LRP can be highly sensitive to photoperiod; González et al., 2005b, 2003; Pérez-Gianmarco et al., 2018). As NILs had the insensitive allele for *Ppd-D1* gene (*Ppd-D1a*), which is the insensitivity gene frequently reported to have the strongest effect (e.g. Langer et al., 2014; Pérez-Gianmarco et al., 2018), we did not expect large differences between growing the plants at short or long photoperiod. However, the NILs might have sensitive alleles in the *Ppd*-1 loci on A and/or B genome. These genes produce responses that are frequently less noticeable than *Ppd-D1*, but still significant (Bentley et al., 2011; Pérez-Gianmarco et al., 2018; Shaw et al., 2013, 2012). Again as expected from the literature, photoperiod effects on the rate of phenological development is not paralleled by concomitant effects on the rate of leaf initiation and therefore the final number of leaves was increased under short days (Slafer and Rawson, 1994b). Long photoperiod not only reduced FLN but also accelerated the rate of leaf appearance (Mosaad et al., 1995; Slafer and Rawson, 1997) both factors contributing to the shortening of the time to anthesis produced by the extended photoperiod.

Beyond the direct effects of temperature and photoperiod discussed above, in the present study there was a clear temperature × photoperiod interaction. For instance, analysing in detail the responses to temperature in the contrasting photoperiods there were particularities that are worth noticing. The length of the phase under long day were similar for 15 and 18 ºC while it differed clearly under short day between these temperatures showing shorter phase at 18 than at 15 ºC indicating that the probable $T_{optimum}$ for development under long days is lower than that under short day. This was all the more so when looking at the time to TS but not so much when LRP was considered, which is in line with the fact that cardinal temperatures would increase with the stage of development (Rahman and Wilson, 1978; Slafer and Savin, 1991; Slafer and Rawson, 1995). The fact that photoperiod affect the temperature response has been described several times not only for wheat (Kiss et al., 2017; Slafer and Rawson, 1996) but also for barley (Hemming et al., 2012; Karsai et al., 2013).

5.5.2. 4.2. Eps-7D and Eps-7D × temperature interaction

In line with the previous knowledge about other known *Eps* genes, the *Eps-7D* studied here also had subtle through consistent and significant effects on time to anthesis (Ochagavía et al., 2019, 2018a;

Zikhali et al., 2014a). This is not surprising as even though each *Eps* gene would have different mechanisms of action, by definition they all result in relatively small differences in time to anthesis or heading (Griffiths et al., 2009; Zikhali et al., 2014a) to the degree that many times may be undetectable if photoperiod and vernalisation requirements are not fully satisfied (Zikhali et al., 2014a). There are very fewer studies on detailed effect of *Eps* genes on pre-anthesis and, unlike with the overall time to anthesis, they vary in their conclusion on whether *Eps* affect early or late stages of development. While the study by Lewis et al. (2008) reported that the effect of *Eps-A^m1* on time to anthesis was mainly due to its effect on the duration of early developmental phases until terminal spikelet, others (Ochagavía et al., 2018) reported varying effect of *Eps-D1* on all the three phases, vegetative, early reproductive and late reproductive, depending on the cross (genetic background). The *Eps-7D* we characterised in the present study (with *Ppd-D1a* in the genetic background) was found to affect the duration of both the early phase from seedling emergence to TS as well as that of the LRP, similarly to what was reported for the *Eps-D1* by Ochagavía et al. (2018).

Considering that the NILs had similar FLN might seem like effect of *Eps-7D* on phenology was realised much later during the development (after flag leaf initiation). Indeed, the dissection of apex stipulated that the *Eps-7D* affected development since early reproductive phase. The rate of leaf appearance was affected by *Eps-7D* allele which resulted in *Eps-7D-early* allele to have similar FLN as that of *late* allele for a shorter duration. This implies a different mechanism regarding leaf development than what was shown for the *Eps-D1*; which affected time to anthesis through mainly affecting time from flag leaf emergence to anthesis (Prieto et al., 2020).

Improvements in spike fertility may be possible with either lengthening the LRP (with no compensation from the change in the rate of development, so that more florets may become fertile) and/or increasing spike dry weight at anthesis (which could be in turn the result of lengthened LRP or increased dry matter partitioning; Slafer et al., 2015). Changes in spike dry weight are uncertain with minor differences in phenology (unless partitioning was altered) and differences in spike fertility would be very subtle which would mainly be the result of the efficiency (Prieto et al., 2020 and references quoted there in). The consistent trend observed in the present study for the *Eps-7D-late* allele to produce more fertile florets per spike than the *early* allele was result of couple of extra florets in the distal position (F2 and F3 in this case) that continued developing for a slightly longer time as a consequence of the slightly lengthened LRP. Effect of *Eps-7D* on the duration of floret development did not alter number of florets primordia produced but altered floret survival which is strongly

supported by other studies where major or minor differences in length of floret development phase resulting in differences in spike fertility was not through number of floret primordia produced (Prieto et al., 2020 and refernecs quoted there in). There was huge difference in duration of floret development between 18 and 9 ºC but this did not generate similar improvement in fertile florets per spike at the low temperature because the driving force for decelerating the rate of development during the LRP was also decelerating the rate of floret development.

Further, in the present study there was clear interaction effect of *Eps-7D* × temperature on the phenology. Although temperature accelerates development of all phases in all crops (see above) that only means that there would be no cases of insensitivity, but genotypic variation in sensitivity has been shown since long time ago (Atkinson and Porter, 1996; Rawson and Richards, 1993; Slafer and Rawson, 1995). At least in part, the genotypic variation in sensitivity to temperature might reflect the interaction of *Eps* genes with temperature (Slafer, 1996). The interaction we found in this study between *Eps-7D* and temperature was not as obvious as to observe the inverse ranking of *Eps-7D-late* and *early* allele at varying temperature, but clear differences in the magnitude of the effect of the *Eps-7D* allele at different temperature. To the best of our knowledge such interaction had been only recently shown in hexaploid wheat for the *Eps-D1* (Ochagavía et al., 2019), although it had been recognised time ago in diploid wheat (Bullrich et al., 2002), and now we expand the concept within commercial wheat germplasm to the new *Eps-7D*. Both the NILs carrying either *Eps-7D-late* and *early* accelerated the rate of development when the temperature was increased but the *Eps-7D-early* had higher sensitivity to temperature than the *late* allele which made *early* allele to have much shorted phenology than the *late* allele. Alleles of *Eps* genes might have different optimum temperatures which shows differences in earliness by *early* or lateness by *late* allele under various temperatures (Appendino and Slafer, 2003a).

5.6. References

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5.7. Supplementary material

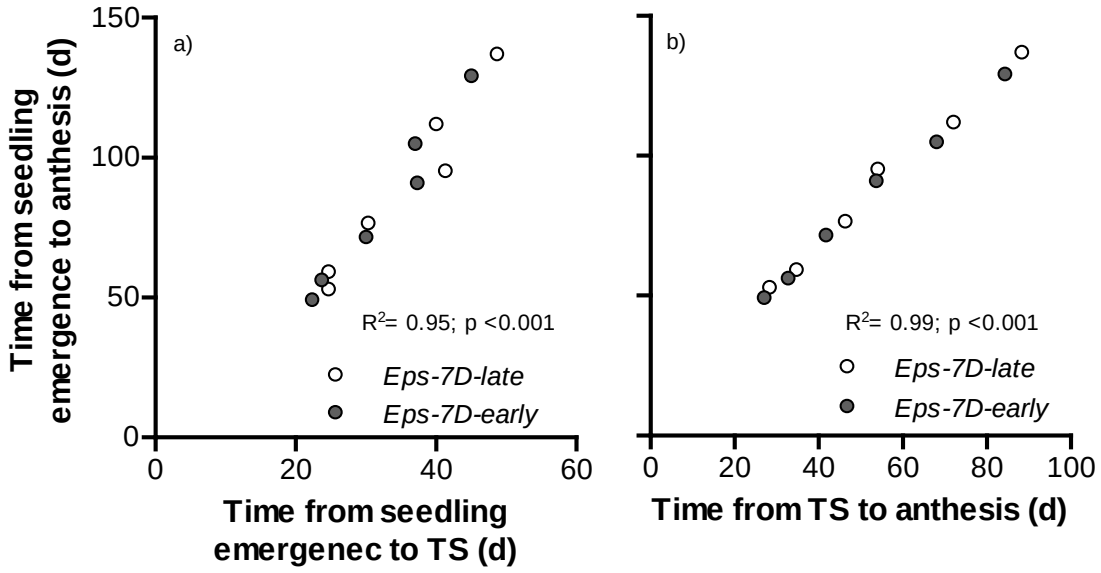

Fig. S5.1. Relationships between time to anthesis and its component phases: time from seedling emergence to terminal spikelet (TS, a) and time from then to anthesis, i.e. the late reproductive phase (b) for the both the NILs carrying either *Eps-7D-late* or *early* allele under three temperature and two photoperiod regimes.

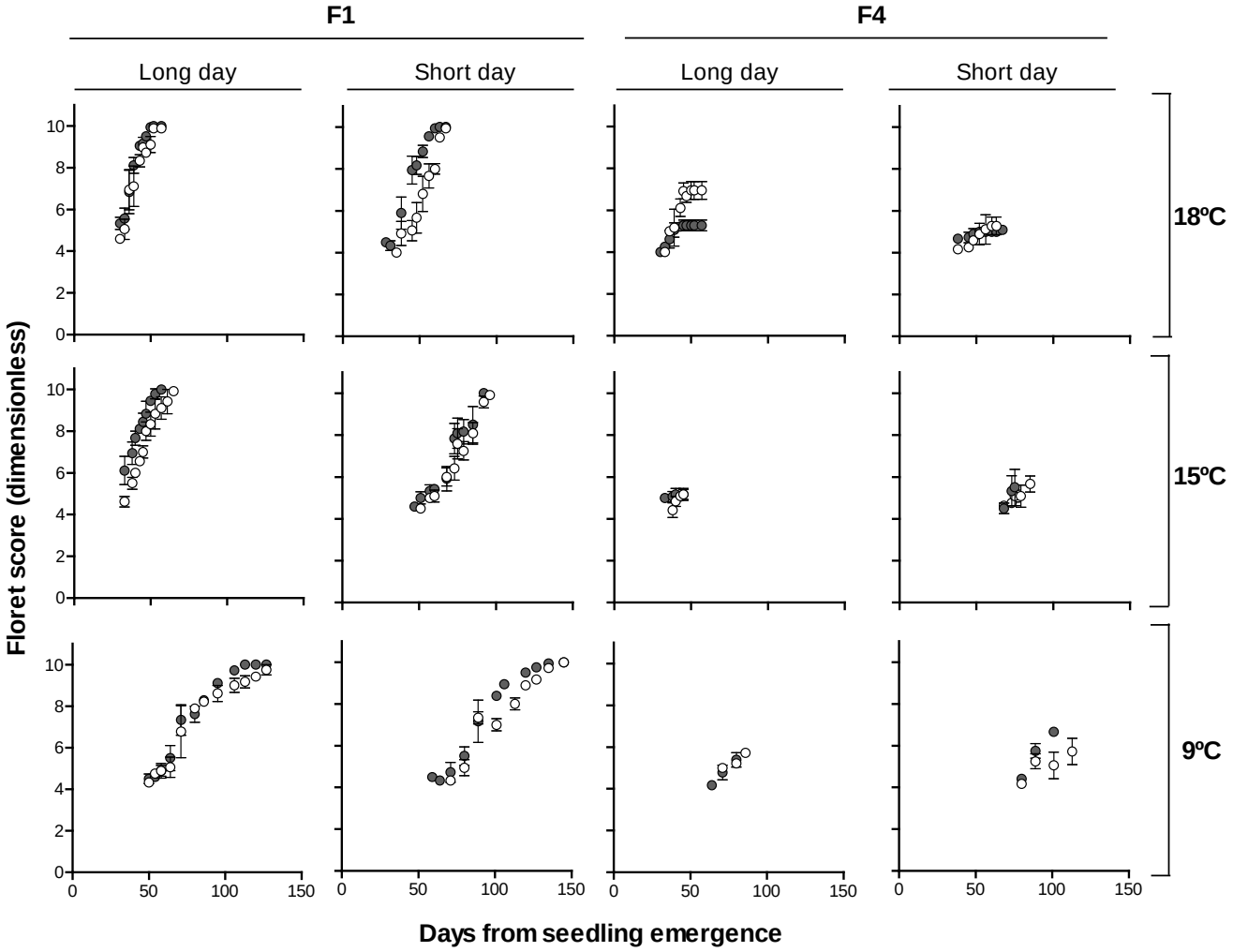

Fig. S5.2. Relationship between floret development (floret score of the Waddington et al. (1983) scale) and days from seedling emergence for *Eps-7D*-late (open circles) and *early* (closed circles) for floret F1 (left panels) and F4 (right panels) under long and shot day at 18 (upper panels), 15 (middle panels) and 9 ºC (bottom panels). The error bars are SEs of means of floret scores from apical, central and basal spikelets.

Chapter VI: General discussion

6. Chapter VI: General discussion

6.1. Background of the study

Reducing plant height and major adjustments in flowering time brought about tremendous improvements in wheat genetic gain in the past (Araus et al., 2002). Further reductions in plant height will hardly bring further grain yield (GY) improvements as the present cultivars have an optimum plant height of 70-100 cm (Richards, 1992); on the contrary further reductions in plant height will likely penalise yield as it would reduce the above ground biomass due to low radiation use efficiency (Miralles and Slafer, 1995b; Richards, 1992). Also, as wheat is well adapted to most diverse environments, so major alterations in phenology are not desirable unless it is to further expand wheat adaptation. Various wheat growing regions have strictly chosen varieties with flowering time to match the optimum environmental conditions (avoiding frost, high temperature and water stress) in the prevailing regions. Genetic factors such as *Ppd* and *Vrn* genes conferring photoperiod and vernalisation sensitivity respectively have been extensively used in breeding to obtain tailored varieties which along with improving adaptation also had pleiotropic effect on GY (Börner et al., 1993; Flood and Halloran, 1986b; Worland, 1996). Number of studies conducted under different photoperiod and vernalisation treatments have pointed out responsiveness of individual phases such as vegetative, early or late reproductive phase to the photoperiod and vernalisation and how changes in the duration of these phases affect developmental rate and number of organs initiated (Flood and Halloran, 1986a; González et al., 2005a, 2002; Miralles and Richards, 2000); some studies have shown that the phenophases can be altered with partial independence from one another (Miralles and Richards, 2000; Slafer and Rawson, 1994b). *Ppd* and *Vrn* genes have also been employed in strategic alteration of various phenophases which has brought about changes in development of organs such as leaves, spikelets and florets and have been known to alter spike development thereby affecting GY (Foulkes et al., 2004; Steinfort et al., 2017; Worland, 1996).

Consequently, to increase wheat genetic gain there is an urgent need of finding critical traits that play important role in GY determination, and those traits should be other than plant height and time to anthesis. A possible way could be that current cultivars with optimum plant height improve the spike fertility (Slafer et al., 2015). While there is very less scope to improve spike fertility through coarse tuning of the time to anthesis, the concept of optimising duration of pre-anthesis phases without

resulting in major changes in time to anthesis should be exploited better with the improving knowledge on genetic factors controlling phenology.

The present thesis was focused on improved understanding of traits and trait combinations beyond plant height and time to anthesis which influence grain yield and effectively doing so in elite material. The study highlights the criticality of identifying or understanding yield attributes in elite lines as such material represents the breeders´ primary choice and along with the improved scientific knowledge the study also provides set of well characterized lines which may be used as parents. I also investigated spike fertility in terms of fertile florets at anthesis and phenology as affected by newly identified *Eps* QTL viz., *Eps-7D* and studied to what degree other *Eps* factor in the background (*Eps-2B*) and temperature modulate its effects.

This last chapter is dedicated in summarising and integrating most important results from the entire Thesis, discussing them with a broader context while highlighting the novelty of the work.

6.2. Novelty of the approach

There are many reports in the literature focusing on the effects of phenology and some of them in yield components involving different genotypes trying to identify and understand traits to increase yield. However, very few concentrated on both duration of particular sub-phases of wheat development or the combination of final leaf number and phyllochron and the effects of floret fertility (a major determinant of the number of grains). Thus, although a large body of work on wheat phenology and yield components is available in the literature, there are still several issues that needed further analysis and were part of the studies carried out in this Thesis:

(i) Identify traits or combination of traits, in lines derived from an elite × elite collection of crosses with similar plant height and time to anthesis. This work involved field evaluation of 1937 lines generated from 13 bi-parental crosses that showed large variation for both plant height and time to anthesis. The stringent selection criteria were applied to reduce the degree of variability in time to anthesis and plant height and out of 1937 lines, 231 lines were selected still maintaining the diversity of crosses they come from. The selected population naturally differed lesser in GY compared to original population after narrowing the range of variability for plant height and time to anthesis but such population portray true nature of the present commercial cultivars. The research is one of a kind in its approach and size of the population

evaluated to identify and understand traits to further improve genetic gains in future (Chapter III).

(ii) Identify the functions of two novel *Eps* QTLs such as *Eps-7D* and *Eps-2B* that are known to affect time to anthesis. The detailed effect of these two *Eps* QTLs on pre-anthesis phases, dynamics of leaf appearance and rate as well as number of primordia (leaf, spikelet and floret) initiated were studied for the first time (Chapter IV and V). The uniqueness of this work is that the interaction between two *Eps* QTLs has been shown for the first time which was only speculated by Appendino and Slafer (2003a). Number of researchers have pointed out probable existence of interaction between *Eps* and temperature (Appendino and Slafer, 2003a; Bullrich et al., 2002; Ochagavía et al., 2018a; Slafer and Rawson, 1995) while there has been only fewer studies in wheat proving this interaction (diploid wheat- Bullrich et al., 2002; hexaploid wheat- Ochagavía et al., 2019; Prieto et al., 2020) and the present Thesis provides proof to such interaction in a commercial hexaploid wheat background.

The work done in this thesis is also quite unique considering the amount of detailed work done in experiments with lines differing in *Eps* gene(s). There have been works analysing the fine development of leaves and/or spikelets as well as others (fewer) determining the rate of development of individual florets across different spikelets. But to the best of my knowledge, this is the first effort done integrating the developmental organogenesis of all primordia.

6.3. Integrating main results from the Thesis

The common objective of the thesis which is discussed across the chapters was to identify variability in pre-anthesis phases among elite lines differing less in time to anthesis and to study spike fertility as affected by changes in the duration of developmental phases and rates of development of particular organs. Selection of lines with similar plant height and time to anthesis allowed identification of physiological traits underlying in the determination of the GY variability in those lines. And lines showed acceptable degree of variation in time from sowing to terminal spikelet (TS) and time from then to anthesis (late reproductive phase, LRP) for similar time to anthesis implying that the further GY improvements might be achieved through optimum allocation of time for individual developmental phases. Lines from the cross Paragon and Baj (late and early flowering type respectively) among 231 lines is shown as an example below depicting the variation in pre-anthesis phases. Two lines, 129 and 138 (marked with red arrow: Fig. 6.1a and b) which had very similar time

to anthesis but differed significantly and consistently in distribution of period from sowing to TS and LRP (Fig. 6.2a and b) showed differences in GN (Fig. 6.2c and d). The line 129 had longer LRP and higher GN than the line 138 supporting the rationale that the longer spike development phase can affect the floret development resulting in higher number of fertile florets at anthesis and thereby determining GN as suggested by Slafer (2003) and Araus et al. (2002). Higher spike fertility in terms of fertile florets or grains per spike as a result of longer spike development duration has been shown in few previous reports (e.g. González et al., 2005b, 2003; Miralles et al., 2000; Serrago et al., 2008); and partial independency of these two pre-anthesis phases (Fig. 6.1d) reinforces the opportunities to alter the distribution of time between leaf + spikelet development phase and floret development phase to further improve the GY without depending on major adjustments in time to anthesis (Guo et al., 2018; Slafer et al., 2001; Whitechurch et al., 2007).

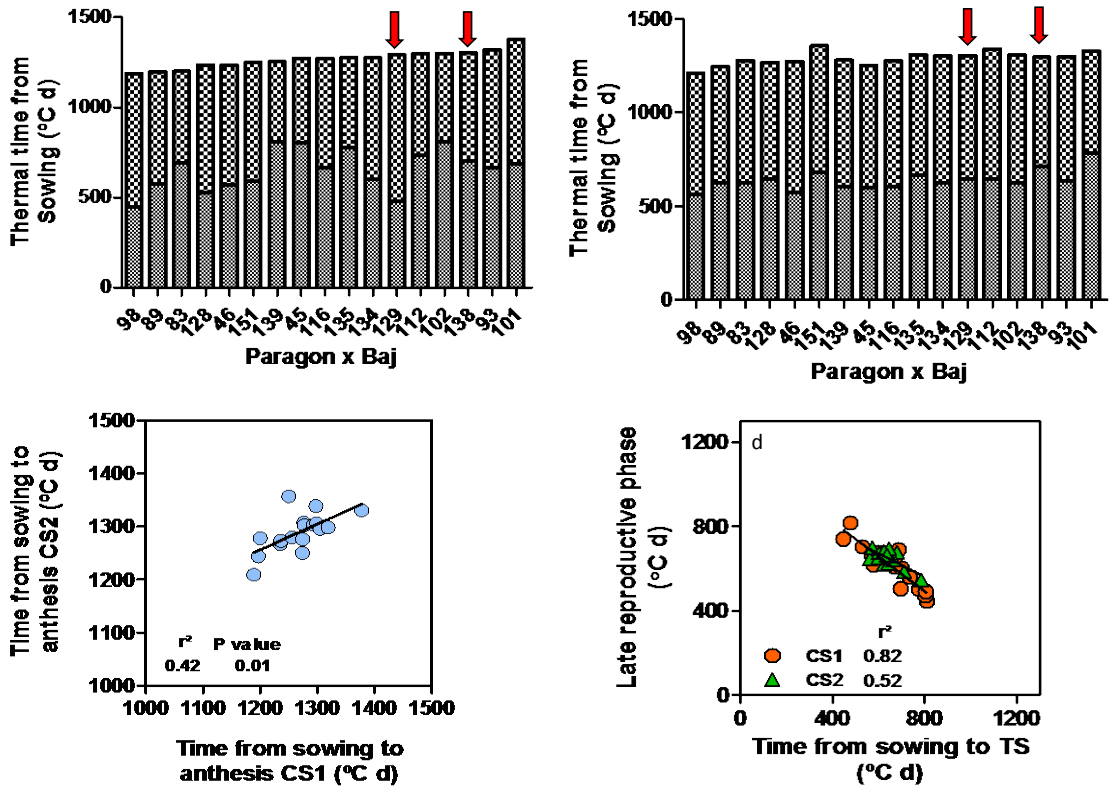

Fig. 6.1. Upper panels-bar graph showing distribution of whole phase from sowing to anthesis in lines with similar time to anthesis in cropping season 1 (a) and 2 (b); lower panels: consistency in time to anthesis of the lines over two cropping seasons, CS1 and CS2, (a) and relation between two pre-anthesis phases with r^2 inside the panel.

The major part of the thesis discusses *Eps* (precisely discovered in the population of Paragon x Baj that was one of the 13 crosses included in the NAM population) which might be critical in fine tuning time to anthesis with subtle influence on GY attributes. The *Eps* QTLs studied here bring about relatively minor though significant and consistent changes in the time to anthesis and they differ in their effect on the pre-anthesis phases. Not only qualitatively but quantitatively speaking the effect of allele of one *Eps* varies in magnitude depending on the allelic status of the other *Eps* (*Eps-2B*) and growing conditions such as temperature and photoperiod. Such differential magnitude of effect was very much evident in phenology but were also extended to floret development. The *late* allele of both the *Eps* QTLs studied here delayed the time to anthesis in comparison with the *early* allele proving the commonality of their function as to alter the phenology but the present study is a proof in itself that each *Eps* needs to be tested under various growing conditions and genetic backgrounds to understand their mechanisms and extrapolate the conclusions to benefit in practical application.

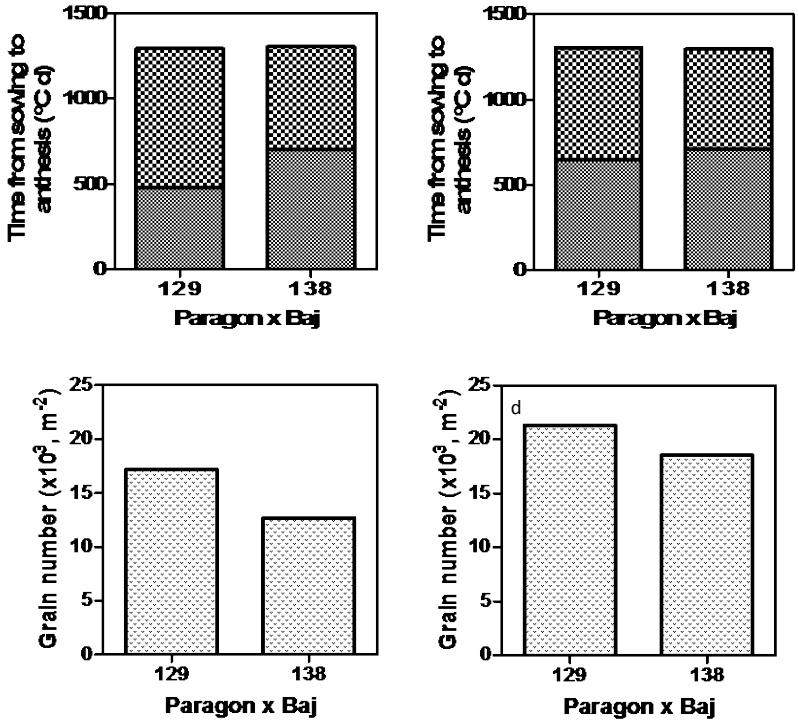

Fig. 6.2. Upper panels-bar graph showing distribution of whole phase from sowing to anthesis in two lines that consistently showed very similar time to anthesis in cropping seasons 1 (a) and 2 (b). in cropping season 1 (a) and 2 (b); lower panels: grain number of the selected lines in cropping season 1 (a) and 2 (b).

6.4. Briefing important contributions

6.4.1. Components of grain yield and their trade-offs

a. Selecting elite wheat lines with similar plant height and time to anthesis allowed us to identify other traits underpinning the grain yield variability- spike dry weight at anthesis and fruiting efficiency defined during the late reproductive phase were both relevant in explaining changes in GN and GY.

b. Improving grain yield through higher grain number did not mandatorily reduce the overall grain size as many lines in high GY sub-group (GY_H) had higher AGW along with higher GN compared to low GY sub-group (GY_L).

6.4.2. Eps-7D and Eps-2B affect the time to anthesis under field condition

a. The *late* allele of both *Eps-7D* and *2B* always delayed time to anthesis with any allele in the background for the other *Eps*, but their effects were different qualitatively and quantitatively speaking; and the magnitude of their actual effects were affected by the allelic status of the other *Eps*; revealing an epistatic interaction between them

b. Part of the epistatic interaction was evident when analysing the effects of these *Eps* genes on the dynamics of organ development, beyond their effect on time to anthesis: *Eps-7D-late* showed noticeable slower rate of leaf, primordia and floret initiation/appearance compared to *Eps-7D-early* allele but was true only when the allele in the background was *Eps-2B-late*.

6.4.3. Interaction between Eps-7D, temperature and photoperiod

a. Differences in developmental traits between *Eps-7D-late* and *early* allele were consistent across the contrasting photoperiods but were clearly affected by temperature

b. Sensitivity of developmental phase was affected by the growing temperature; it was stronger under long than under short day.

Overall, the study reports how variability in individual pre-anthesis phases in a population with similar time to anthesis may be useful in further improving grain yield in well adapted wheats. Evaluation of allelic combinations of two newly identified *Eps* and their epistatic interactions help in tailoring allelic combination to produce a desired phenotype with advantageous distribution of time to important

phenophases. Interaction between two particular *Eps* is reported for the first time here which was only speculated before (Appendino and Slafer, 2003a). The study involved detailed investigation of effect of these *Eps* QTLs on dynamics of development of various organs. While the *Eps-7D* alleles studied here showed noticeable and consistent effect on whole phase, the effects differed on different phenophases, dynamics of leaf and spikelet initiation and fertile florets as result of interaction with growing conditions as well as background allele. The study also provided supporting evidence for some already established understanding on aspects such as trade-off between GN and grain weight that this negative relation does not always mean compensation (Acreche and Slafer, 2006; Miralles and Slafer, 1995a) so the future grain yield improvements might have to depend on further improving GN (Fischer, 2011; Reynolds et al., 2012; Slafer et al., 2014). I found that the lines with higher grain number defining sink size which is set around time to anthesis was driving the post-anthesis growth which is in contrary to usual interpretation of size of the source determining the sink size.

6.5. Major conclusions

- The selected lines had appreciable genetic variability in GY which was not explained by plant height and time to anthesis as expected, and GY was explained by both GN and grain weight while former component explained most of the GY variability. GN in turn was well explained by two of its physiological components spike dry weight at anthesis and fruiting efficiency
- Both *Eps-7D* and *2B* affected time to anthesis but the effect of *Eps-7D* was much stronger.
- The effect of both *Eps-7D* and *2B* were well beyond the phenology, they affected slightly the primordia initiation rate and floret developmental rate. The effect of *Eps-2B* on floret development were consistent in that *Eps-2B-late* allele always had higher fertile florets and slower rate of development.
- Even though *Eps-7D* affected the period of floret development, the total effect failed to reflect in fertile floret at anthesis and differed based on the allele of *Eps-2B* in the background.
- In any of the cases (environmental conditions, genetic background), there was no effect of *Eps-7D* or *Eps-2B* on the maximum number of florets produced.
- Differences between *Eps-7D-late* and *early* allele were very apparent at 9°C under short day.

6.6. References

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