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Effets et types d'action des gènes de vernalisation (Vrn-H1, Vrn-H3) et de photopériode (Ppd-H2) sur la date de floraison chez l'orge (Hordeum vulgaris L.).

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Dédicace

À Mes parents Ma Famille Mes amis Fairouz…

Summary

Heading date is a trait of major importance, as one of the main determining factors of adaptation and yield in cereals. This is especially important in regions with limited water availability. The objectives of this work were to assess the possible effects and type of actions of two vernalization response genes (*Vrn-H1* and *Vrn-H3*) and photoperiod response gene *Ppd-H2*.

Two F_2 populations, Esterel x CN106 and Esterel x CN16, were obtained at the EEAD-CSIC, in the framework of the Spanish national barley breeding programme, which were used for this study. The first population segregates only for *Vrn-H1* and *Ppd-H2*. The second one segregates for *Vrn-H1*, *Vrn-H3* and *Ppd-H2*. Both populations are dominant and monomorphic for *Vrn-H2* and *Ppd-H1*, the remaining major genes controlling vernalization and photoperiod responses in barley. Esterel x CN106 was evaluated under autumn (standard) and winter (late) sowing conditions, whereas Esterel x CN16 was sown only in winter condition

The effects detected were dependent on population and time of sowing. No effect was evident in Esterel x CN106 sown in autumn. Any effects were apparent only under winter sowing conditions. There were significant differences between the two alleles of HvBM5A (*Vrn-H1*)for time to flowering, to stem development, and growth habit. Plants homozygous for CN16 allele were earlier and more erect than the plants homozygous for the Esterel allele.

HvFT1 had large and significant effects on flowering time; plants homozygous for the CN16 allele (AG) were later than those plants carrying the allele from Esterel (TC). This gene showed additive as well as dominance type of gene action, and may play a role for the adaptation of barley to Mediterranean conditions.

In winter sowings, HvFT3 had a significant effect in both F_2 populations on flowering time. The plants null for this gene (recessive *ppd-H2* allele) flowered significantly later than the plants with dominant *Ppd-H2* allele.

Résumé

L´épiaison est un caractère de grande importance, c´est l´un des facteurs déterminant d´adaptation et la production (rendement) chez les céréales, spécialement sous conditions de stress hydrique. L'objectifs de la présente étude consiste à évaluer les effets et types d´actions de deux gène de vernalisation (*Vrn-H1* y *Vrn-H3*) et un gène de photopériode *Ppd-H2*.

Deux populations F2, Esterel x CN106 et Esterel x CN16, obtenues au niveau de l'EEAD-CSIC (Programme National Espagnol de l´amélioration de l´orge) ont été utilisées. La première population possède deux gènes en état de ségrégation (*Vrn-H1* et *Ppd-H2*), alors que la deuxième possède trois gènes en ségrégation ; *Vrn-H1*, *Vrn-H3* et *Ppd-H2*. En plus de ces gènes en ségrégation, les deux populations ont deux autres gènes; dominants et monomorphiques: *Vrn-H2* et *Ppd-H1*.Esterel x CN106 a été évaluée sous deux dates de semis, une précoce normale (automne) et l´autre tardive. Esterel x CN16 a été évaluée seulement en semis tardif (hiver).

Les effets détectés dépendent du type de population et de la date de semis, aucun effet n'a été observé durant le semis d´automne. Les effets ont été observés uniquement en hiver. Une grande différence entre les deux allèles de HvBM5A (*Vrn-H1*) a été révélée pour la date de floraison, la montaison et le port de la plante. Les plantes homozygotes pour l´allèle de la CN16 sont beaucoup plus précoces que celles qui possèdent l´allèle de Esterel.

HvFT1, a révélé une grande signification pour la date de floraison, les plantes homozygotes pour l´allèle de la CN16 (AG) sont beaucoup plus tardives que celles qui possèdent l´allèle de Esterel (TC). Des effets additifs et de dominance ont été détectés pour ce gène qui peut jouer un rôle adaptatif en conditions méditerranéennes.

Pour le semis d´hiver, HvFT3présente un grand effet sur la date de floraison, pour les deux populations. Les plantes possédant l´allèle récessif *ppd-H2* sont tardives par rapport a celles qui possèdent l´allèle dominant *Ppd-H2*.

قص الخ

إن عملية التسنبل هي واحدة من العوامل التي تحدد التكيف والإنتاج عند الحبوب ، وخصوصا في ظل ظروف الإجهاد المائي. تهدف هذه الدراسة إلى تقييم تأثير اثنين من جينات vernalization (*VRN-H1 VRN -H3*) و جين الفترة الضوئية *Ppd-H2*.

تم استخدام اثنين من عشائر الجيل الثاني (F2) Esterel x CN106 et Esterel x CN16 المستحلصتان من طرف CSIC - EEAD (البرنامج الوطني الاسباني لتحسين التمور). إن العشيرة الأولى تمتلك اثنين من الجينات في حالة تمايز وراثي (*Vm-H1* و *Ppd-H2*) , في حين أن الثانية تمتلك ثلاثة جينات في حالة تمايز وراثي (*Vm-H1*، *Vm-H3* ، *Ppd-H2*). إضافة إلى هذه الجينات فإن العشيرتين تمتلكان جينين سائدين و وحيدي الصفة الظاهرة (*Ppd-1H ;Vm-H2*).

CN106 x Estérel تم تقييمها في تاريخين للبذر،الأول طبيعي في وقت مبكر (الخريف) والآخر متأخر (الشتاء). أما CN16 x Estérel لم يتم تقييمها إلا في وقت متأخر من الزرع (في الشتاء).

إن الآثار المكتشفة تتوقف على نوع العشيرة و تاريخ الزراعة، و الدراسة لم تبين أية آثار خلال بذر الخريف، لوحظت الآثار فقط في الزراعة الشتوية، كما يوجد فرق كبير بين صنوي HvBM5A (*VRN - H1*) فيما يخص تاريخ الإزهار،طلوع النبتة و كذلك طول ساقها. أما النباتات المتماثلة اللواقح بالنسبة للصنو CN16 فهي أبكر بكثير من التي تمتلك صنو Estérel.

من جهة أخرى فإن HvFT1 أظهرت أهمية كبيرة بالنسبة لتاريخ الإزهار و النباتات المتماثلة اللواقح للصنو CN16 (AG) فهي أكثر تأخرا من تلك التي تمتلك صنو Estérel (TC). كما أظهرت الدراسة وجود آثار مضافة (additivité) مع سيادة عند هذا الجين الذي يمكن أن يلعب دورا مهما في التكيف في ظروف البحر الأبيض المتوسط.

فيما يخص بذر الشتاء، فإن *HvFT3* يؤثر تأثيرا كبيرا على تاريخ الإزهار للعشيرتين، و النباتات التي تمتلك الصنو المتنحي *ppd-H2* متأخرة الإزهار مقارنة مع تلك التي تمتلك الصنو السائد *Ppd-H2*.

1. Introduction

Barley is the fourth largest cereal crop in the world after wheat, maize and rice, with an overall production of 138.6 million tonnes, over 55.51 million of hectares in 2006. In Spain, barley is cultivated on a total of 3.22 million hectares, giving a production of 8.31 million tonne (FAOStat, 2006). Barley is produced primarily for animal feed. It is the third largest feed grain crop produced, after corn and sorghum. This fact relates to the decreased importance of barley for food production. In addition, increasing levels in the consumption of animal products, together with more intensive methods for raising animals, create the need for high energy feed (Slafer *et al.,* 2002). Following the leading sector of feed, barley, is also used for malt production, whose end use is primarily making beer. Also, in certain regions, mainly in dry land farming systems, barley stands provide possibilities for grazing animals in years when growth in pasture grounds remains insufficient.

Cultivated barley, *Hordeum vulgare* L. ssp. v*ulgare,* belongs to the genus *Hordeum*, tribe Triticeae, family Poaceae. Besides *H. vulgare*,there are another 31 species in this genus (Bothmer *et al*., 1995). The progenitor of cultivated barley is *Hordeum vulgare* L. ssp. *spontaneum*. Both forms are diploid (2n=14) (Molina-Cano *et al.* 2002).

The mode and the location of barley domestication have been the subject of vigorous scientific disputes (Orabi *et al*., 2007). Several hypotheses have been proposed. The evidence of early barley domestication and cultivation, in the area of the Fertile Crescent (which geographically corresponds to a region extending from Israel and Jordan, through Syria, Lebanon and Southern Turkey, into Iraq and Iran) dates back approximately 10 000 years (Badr *et al*., 2000, Bothmer *et al.,* 2003). Alternative hypotheses propose that additional independent domestication events have occurred, in Morocco (reviewed in Molina-Cano et al. 2002), in Tibet or in Ethiopia (Orabi *et al.,* 2007). Genetic evidence for a second domestication of barley in Central and Southern of Asia has been recently proposed by Morrell and Clegg (2007), and Saisho and Purugganan (2007).

Barley can be considered a model genome system for Triticeae species (Francia *et al.,* 2004), as its study presents several advantages. First, it is a self-fertile, diploid species. Also, the barley chromosomes are homeologous to those of cultivated wheat and rye (Hori *et al.,* 2003). The genome of barley is approximately 5000 million base pairs (Mpb) in size, much smaller than that of hexaploid wheat (*Triticum aestivum*) with 16,000 million base pairs (Gupta and Varshney, 2004). It has been estimated that the average distance between genes is 240 kb (based on an estimated genome size of 5000 Mpb and 21 000 genes, Yu *et al.,* 2000). However, the average gene density in some gene-rich regions is approximately one gene every 20 kb (Druka *et al.,* 2002).

A number of morphological, adaptive and agronomic characters are used in the classification of barley genotypes, such as earliness, vernalization requirement, reaction to day length and growth habit (Bothmer *et al.,* 2003). Recent diversity studies using molecular markers led to groupings dominated by the geographical origin of the accessions and that may have happened as a consequence of adaptation to specific environments. These studies also support the agro-ecological classification of cultivated barley. The most comprehensive diversity study so far, by Malysheva-Otto *et al*. (2006), subdivided a large

World collection into three groups: Occidental barley, Oriental barley and naked barleys of Central Asia and the Caucasus.

The inflorescence of barley has either one or three fertile florets per rachis node. The former leads to a “two-row” inflorescence and the latter to a “six-row” inflorescence. These two inflorescence types define the major germplasm groups of barley (Jui *et al.,* 1997). The difference in spike type is controlled primarily by a single gene *Vrs1* with the two row type being dominant (Judith *et al.,* 2001). *Vrs1* encodes a transcription factor and its expression is strictly localized in the lateral-spikelet primordial of immature spikes. Loss of function of *Vrs1* resulted in complete conversion of the two row barley in the six rowed phenotype (Komatsuda *et al.,* 2007).

The growth habit forms of barley are spring, facultative and winter. Agronomically speaking, spring forms are planted in the spring and harvested late summer (Fowler *et al.,* 2001, Yan *et al.,* 2005, Limin *et al.,* 2006). Winter forms are planted in the fall and harvested the following summer, and the facultative forms can be planted in the fall or in spring. Ancestral types of cereals were winter genotypes, and the development of spring growth habit was a prerequisite for expansion of the cultivation area for barley out of its centre of origin (Bothmer *et al.,* 2003). Winter types can withstand low temperatures, and this characteristic is usually associated with a prostrate growth habit. Spring types show an erect growth habit.

Barley is globally grown over a wide range of habitats, including a huge range of altitudes and latitudes (Graner *et al*., 2003). In high latitudes and in mountainous regions, barley is sown in spring to avoid damage by severe cold winters. At low latitudes, on the other hand, the temperatures are too high to induce vernalization in a winter type and therefore spring type cultivars prevail in these regions. In mid-latitudinal regions including North Africa and southern Europe, both spring and winter barleys are sown in autumn. In temperate areas, winter cultivars are preferred because of the longer growth cycle and higher yield potential (Bothmer *et al.,* 2003).

Barley production regions all over the world usually present some kind of environmental stress. Freezing temperatures and drought are the most relevant stress factors. In Spain, barley is often grown under semi-arid conditions. Adjustment of crop phenology to the resource available is the main factor for the determination of grain yield (Cuesta-Marcos et al., 2008). Plant growth habit and heading time can be considered as the basic traits involved in adaptation, since they allow synchronisation of plant cycle to the prevailing environmental conditions.

1.1. Heading date or flowering time

The sessile nature of plants limits their ability to isolate themselves from harsh environmental conditions. These organisms have adjusted their growth cycles to cope with climatic constraints, by evolving the ability to alter their developmental program in response to environmental stimuli. A major switch in the developmental program is the transition from vegetative growth to the reproductive stage, which is a highly regulated process (Schmitz et al., 2007). In many species, the timing of this transition, commonly known as transition to flowering, is determined by seasonal changes that are sensed by the plant (Sung and Amasino, 2004).

Genetic studies of early vs. late heading in barley were first reported in 1907. The first models of genetic control of heading time were simple Mendelian models, involving one, two or three genes that explained the segregation observed in most studies (Salisbury, 1963). Investigations of the role of environmental conditions on the timing of heading were initiated following the discovery by Garner and Allard in 1920 that flowering, in many plants, was influenced by day length. Since the 1830s, it was known that exposure to low temperature promoted flowering in some plants, phenomenon later known as vernalization (Boyd *et al.,* 2003).

Ear emergence (heading date) is an important factor in the adaptation of barley varieties to environmental conditions, and in maximising yield potential (Bezant *et al.,* 2003, Cockram *et al.,* 2007a). A correct flowering date ensures the greatest chance of pollination, seed set, dispersal and, therefore, reproduction of the species (Faure *et al.,* 2007). Flowering is regulated by environment, but also has a strong genetic basis. The variation in flowering time is mainly due to variation in genes regulated by day length (photoperiod) or long period exposures to low temperature (vernalization) (Laurie *et al.,* 1995, Trevaskis *et al.,* 2003, Dubcovsky *et al.,* 2005). In order to expand the potential area of adaptation of fall-sown cereals, winter hardiness has been the subject of intensive research in the Triticeae (Skinner *et al.,* 2005). The three components of winter hardiness are low temperature tolerance, vernalization requirement, and photoperiod sensitivity (Skinner *et al.,* 2005).

1.2. Flowering in barley

In barley, as in other temperate cereals, the date of heading is a highly variable phenotypic trait which bears major implications for adaptation to geographic regions and crop management practices (Boyd *et al.,* 2003). It is not surprising that selection for an appropriate heading time is a primary objective of all breeding programs around the world, and that the genetics and physiology of heading time have been very active research areas over many years.

Pollination usually takes place in barley just before or during head emergence from the boot. Pollination begins in the central portion of the head and proceeds toward the tip and base. Since pollen is sensitive to stress, water deficit and inadequate temperature at this time decrease the number of kernels formed and may reduce yield.

1.2.1. Factors contributing to variation in heading time

Heading time in barley is governed by two major factors: photoperiod sensitivity and temperature. This last factor acts in two different ways. One is the phenomenon of vernalization, a hastening of growth following a period of cold temperatures; and the other is the advancement of growth in response to increasing temperatures, which are active at all developmental stages. Photoperiod sensitivity and vernalization requirement vary among cultivars. Responses go from plain non-responsiveness to very large variations in the duration of the life cycle. Responses to temperature other than vernalization may vary among cultivars, but there is no cultivar totally insensitive to this factor (García del Moral *et al*., 2002).

A winter variety sown in autumn requires vernalization to promote flowering, and commonly displays a strong promotion to flowering in response to growth under long day.

A spring variety lacks a vernalization requirement and can display a weak response to long day.

1.2.1.1. Temperature

The timing of heading in barley is advanced by an increase in ambient temperature. All genotypes respond to increasing temperature, reflecting the general effect of temperature on the rate of biological processes. The rate of advance is approximately linear up to an optimum, after which further increases in temperature delay heading (Roberts *et al.,* 1988). The genotypes differ in their sensitivity to, and interaction with, temperature and these details vary with the phenophase for barley (Slafer *et al.,* 2002, Boyd *et al.,* 2003).

Ellis *et al*. (1989), reported that the rate of progress to flowering is a linear function of mean temperature and photoperiod, according to the following equation:

1/f = a + bT + cP with;

F = is the number of days from sowing to first flowering.

T = is mean temperature.

P = is photoperiod, a; b and c are genotype constants.

Temperature during the growing period of plant strongly affects its time of heading. Response of a barley plant to high temperature varies with its grade of spring habit, and at the same time depends on the day length. When grown under long day conditions, the time of heading of a highly spring variety is generally hastened with a rise of temperature (Takahashi and Yasuda, 1971), whereas a winter type plant may show a retarding effect in the same conditions, because of the interaction between temperature and photoperiod.

1.2.1.2. Photoperiod

Length of day affects apical morphogenesis, leaf production, tillering and other developmental processes in cereals. Long day accelerates floral initiation and heading by reducing the number of leaves in vernalized or spring habit plants (Mahfoozi *et al.,* 2001a). The transition point from the ‘vegetative’ to the ‘floral’ state in the shoot apex of wheat and barley is identified by the formation of ‘double ridge’. Growth of the leaf primordium (ridge) is suppressed and the floral bud primordium (ridge) begins to grow out, giving a ‘double ridge’ appearance to the dissected shoot apex when viewed under the microscope. At this time the plant apex no longer produces leaves but is ‘florally initiated’ and only flower parts will subsequently develop. Under short day regimes, ‘double ridge’ formation is delayed.

Following the discovery of its dependency on photoperiod, barley has been classified as a quantitative long-day species, implying that time of heading is advanced by an increasing photoperiod. Boyd *et al*., (2003), proposed the classification of some genotypes that do not respond to increase in photoperiod, or with a minimal advance, as photoperiod insensitive, whereas other genotypes show a photoperiodic response which varies over the life cycle of the plant. These last genotypes do not respond to the influence of photoperiod immediately after germination (period defined as the pre-inductive period) and a period immediately preceding anthesis (post-inductive period). The interval between these two periods is the inductive phase, or photoperiod sensitive period.

1.2.1.3. Vernalization

A vernalization requirement is an extended period of low temperature required for transition from the vegetative to the reproductive state. Without vernalization, genotypes that have a requirement will eventually flower, but the flowering time will be delayed by months and flowering will be erratic (Karsai *et al.,* 2001). The vernalization requirement ensures plants will be in a vegetative state during the winter. As maximum cold tolerance is achieved when plants are in a vegetative condition (Fowler *et al.,* 2001), this trait also enhances cold tolerance.

The term *vernalization requirement* is misleading, as even winter growth habit barley genotypes do not require vernalization as they will eventually flower under warm temperatures. Szűcs *et al.* (2007) utilise the term *vernalization sensitivity* to indicate the delay in flowering time attributable to a lack of vernalization, and *vernalization response* to indicate the difference in flowering time between unvernalized and vernalized growth conditions.

Most winter barleys must be vernalized by a period of low temperature. The effective temperature for vernalization range from -5 to 16 ºC, with a maximum effect between 0 and 8 ºC, and an optimum around 7 ºC (Boyd*et al.,* 2003). The vernalization mainly affects the length of the vegetative phase (Slafer *et al.,* 2002). Growth habit is usually considered synonymous with vernalization response. Exposure of the germinating seeds of a winter variety to vernalization converts its winter morphology (prostrate habit) into a spring one (erect growth habit). Salisbury (1963) postulated that exposure to low temperature led to the synthesis of a substance called *Vernalin.* As of today, this substance has not been yet identified, though very important advances on the genetic control of vernalization requirement and on the metabolic pathways involved have been achieved, as will be shown in the next section.

The vernalization does not affect the rate of leaf initiation, thus, the effect of this factor on the length of the initiation phase brings about conditions that change the final number of leaves. In fact, the number of leaves is generally used as a surrogate measure of vernalization response (Slafer *et al.,* 2002).

1.2.1.4. Interaction between vernalization/photoperiod

Many studies have found empirical evidence that response to vernalization and photoperiod sensitivity do not act independently in most barley plants (Evans, 1987). The interaction between photoperiod and vernalization response determines the final number of leaves (NLF) on the main stem (Mahfoozi *et al.,* 2001b). The plant development rate can be influenced by photoperiod during vernalization process. Takahashi and Yasuda (1971) suggested that photoperiod response is concealed by winter growth habit in cereals and determination of a photoperiod response of winter type must be preceded by vernalization saturation. Photoperiod and vernalization interact with temperature from early stages of plant growth to influence the rate of phenological development.

Among winter varieties, a comparable acceleration of anthesis in long days is evident with fully vernalized plants in most cases, but with unvernalized plants, the response to long days is less. Also, Evans (1987) noticed that, in the absence of vernalization, ear emergence was fastest when long days were preceded by short days

1.2.1.5. Intrinsic earliness

It is clearly recognized that, when spring or vernalized winter barley plants are grown under long day photoperiod, some varieties head earlier that others. This internal factor is usually

called intrinsic earliness or earliness *per se* "*eps* **"** (Takahashi and Yasuda, 1971). Kato *et al.* (1997) reported that earliness factor seems to be of polygenic nature and exhibits a continuous variation.

Hay and Ellis (1998) concluded that the number of leaves initiated on the main stem, combined with differences in development rate, were the major factors contributing to variation of flowering in this trait. Other authors have found evidences that the regulation of the genes implied in the intrinsic earliness can differ according to photoperiod conditions (Kato *et al*., 1997), with which the genetic control of the environmental factors that influence the date of flowering would appear interrelated.

1.3. Genetic control of heading time

The control of flowering time is central to reproductive success in plants, and has a major impact on grain yield (Francia *et al.,* 2004). Flowering time is a complex trait that shows almost continuous variation in cereals. Unraveling its molecular intricacies in species such as barley, with large and complex genome, has benefited from the comparative use of floral pathways from the model plant *Arabidopsis thaliana* (Cockram *et al.,* 2007a). Likewise, research in rice (*Oryza sativa* L.) has extended our knowledge of flowering processes in a model grass species. The identification in barley of orthologous genes with analogous function to genes found in rice and Arabidopsis illustrates the usefulness of the candidate gene approach (Trevaskis *et al.,* 2007).

Variation in flowering time in barley is mainly due to variation in genes regulated by day length (photoperiod) or long period exposure to low temperature (vernalization) (Laurie *et al.,* 1995, Dubcovsky *et al.,* 2005).

1.3.1. Genetic control of vernalization

Takahashi and Yasuda (1971), proposed a three loci epistatic model for barley vernalization requirement, in which winter growth habit genotypes have the allelic architecture *Sh- sh2sh2 sh3sh3.* All other allelic configurations lack vernalization requirement and yield the spring and facultative growth habit. Based on wheat and barley orthology, we can use the standard Triticeae nomenclature, with an *H* to indicate the *Hordeum* genome: *Sh2* = *Vrn-H1* (isolated by map-based cloning in diploid wheat (Yan *et al*., 2003); *Sh* = *Vrn-H2* (identified by positional cloning, Yan *et al*., 2004), and *Sh3* = *Vrn-H3* (identified by homology to a known gene of *Arabidopsis thaliana*, Yan *et al*., 2006).

1.3.1.1. *Vrn-H1*

Vrn-H1 is induced by vernalization and promotes the transition to reproductive development. This gene regulates the identification of the shoot apical meristem to determine which organs are produced by the shoot apex (Trevaskis *et al.,* 2007). The *Vrn-H1* gene encodes an APETALA1 like MADS box transcription factor (Trevaskis *et al.,* 2003; Yan *et al.,* 2003; Fu *et al.,* 2005; Cockram *et al.,* 2007b). HvBM5A has been identified as a candidate gene for *Vrn-H1* (Yan *et al.,* 2005).

The basis of allelic variation within the orthologous genes at the collinear *VRN1* loci of *Triticum aestivum* and barley has been studied; spring growth habit conferred by *VRN-*

A1, *VRN-B1* and *VRN-D1* genes is associated with changes in the promoter or a deletion in the first intron (Yan *et al*., 2003; Cockram *et al.,* 2007b). Comparison of wheat and barley sequence and deletion breakpoints has identified a 436 bp region of *Vrn-H1* intron 1 as representing a putative vernalization critical region (Fu *et al.,* 2005, von Zitzewitz *et al.,* 2005). Mutations in the first intron of *VRN1* are not the only ones associated with the dominant spring allele. Different deletions in the promoter of the *VRN-A1* in *T. monococcum* gene have been correlated with the spring growth habit (Yan et al., 2003; Fu *et al.,* 2005).

High basal levels of *Vrn-H1* expression can substitute for the vernalization requirement. This expression without vernalization is associated with mutations in both the first intron or in the promoter (Trevaskis *et al.,* 2007).

1.3.1.2. *Vrn-H2*

Vrn-H2 is a floral repressor that delays flowering until plants are vernalized. It was first mapped to 4HL by its linkage with morphological and biochemical markers (Takahashi and Yasuda, 1971). The candidate genes for *VRN-A2* in wheat are ZCCT1 and ZCCT2 (Zinc-Finger motif; Yan *et al.,* 2004). In barley; there are three ZCCT genes, designated Ha*,* Hband Hc(Dubcovsky *et al.,* 2005, Karsai *et al.,* 2005). In wheat, natural mutations, expression studies and transgenic experiments were used to demonstrate that the *VRN-2* gene was ZCCT1and not ZCCT2(Yan *et al.,* 2004). Regarding barley, ZCCT-Ha and ZCCT-Hb were more similar to thewheat gene that ZCCT-Hc*,* and might be better candidates to *Vrn-H2*. Dubcovsky *et al.* (2005) proposed that ZCCT-Ha was the most likely candidate for *Vrn-H2*, although Szűcs *et al.* (2007) showed that deletion of this gene retained winter habit, and presented evidence that ZCCT-Hb could be the candidate gene. The deletion of the three loci results in the creation of recessive spring allele (von Zitzewitz *et al.,* 2005).

The varieties that lack a functional copy of *VRN2* do not require vernalization to flower. In barley, there are naturally occurring deletions of *Vrn-H2* and cause early flowering in long day, but not in short day (Trevaskis *et al.,* 2007). These facts suggest that *VRN2* acts to block the promotion of flowering in long day.

1.3.1.3. *Vrn-H3*

Two additional vernalization genes have been reported in barley (*Vrn-H3*) and wheat(*VRN-B4*). *Vrn-H3* was initially assigned to chromosome 1H (Takahashi and Yasuda, 1971), whereas *VRN-B4* was mapped on the short arm of wheat chromosome 7B. Actually*, Vrn-H3* is located on barley chromosome 7HS (Yan *et al.,* 2006, Faure *et al*., 2007).

Vrn-H3, encodes an orthologue of the *Arabidopsis thaliana* floral pathway integrator FT (*FLOWERING LOCUS T*) gene (Yan *et al.,* 2006; Cockram *et al.,* 2007a; Faure *et al*., 2007). In Arabidopsis, FT expression increases in leaves when plants are exposed to inductive day length. In barley, expression of FT is induced by long day and promotes flowering (Hemming *et al.,* 2008). Long day induction of FT requires the photoperiod gene (*Ppd-H1*), and varieties with an inactive version of *Ppd-H1* show reduced sensitivity to long day (Faure *et al.,* 2007).

In barley, FT alleles have polymorphisms in the first intron. Yan *et al.* (2006) suggested that these regions may play an important role in the regulation of the gene. These regions are important for repression before vernalization, and might contain binding sites for repressor proteins (Trevaskis *et al.,* 2007).

1.3.1.4. Epistatic interaction between vernalization genes

Vrn-H2 is a repressor of flowering time down-regulated by vernalization (Yan *et al.,* 2004, Trevaskis *et al.,* 2006) and short day, which negatively regulates *Vrn-H1* and *Vrn-H3* before winter (Trevaskis *et al*., 2007). *Vrn-H3* is a promoter offlowering time up-regulated by long day, which positively regulates *Vrn-H1*, and the increase of the *Vrn-H1* expression down-regulates the expression of *Vrn-H2* (Yan *et al.,* 2004; Dubcovsky *et al.,* 2006). Under short day conditions, all three genes show low expression levels. In the absence of vernalization, when plants are transferred from short to long day, rapid up regulation of *Vrn-H3* and *Vrn-H1* takes place (Yan *et al*., 2006). This model explains the strong epistatic interaction between these three genes. Takahashi and Yasuda (1971) reported that *SH2* and *SH3* are epistatic to the dominant winter gene *SH*, and the recessive spring gene *sh* is epistatic to both *sh2* and *sh3* for winter habit.

The growth habit of plants homozygous for the *Vrn-H2* allele for spring growth habit, is independent of variation of the *Vrn-H1* and *Vrn-H3* loci (Yan *et al.,* 2003, 2006). When the *VRN2* repressor is not produced, it cannot interact with the other genes, independently of the variations present in the other *VRN* genes, having no effects on flowering time (Fu *et al.,* 2005). Conversely, plants homozygous for *Vrn-H1* and *Vrn-H3* dominant alleles had spring growth habit and showed no effects of the *Vrn-H2* gene on flowering time (Szűcs *et al.,* 2007). *Vrn-H2* has no effect since there is no recognition site within the first intron in *Vrn-H1* (Yan *et al.,* 2003: von Zitzewitz *et al.,* 2005).

1.3.2. Genetic control of photoperiod response

Plants commonly use photoperiod (day length) to control the timing of flowering during the year, and variation in photoperiod response has been selected in many crops to provide adaptation to different environments and farming practices.

The control of flowering by photoperiod is understood best in the long day dicot Arabidopsis and the short day monocot cereal rice (Turner *et al.,* 2005; Cockram *et al.,* 2007a). Two photoperiod response genes were found in barley; *Ppd-H1* on chromosome 2HS, regulated flowering time under long day, while *Ppd-H2* on chromosome 1HL was detected only under short day (Laurie *et al.,* 1995, Faure *et al*., 2007).

Ppd–H1, a pseudo- response regulator (PPR) similar to PPR7 in Arabidopsis, was mapped to chromosome 2HS in a population of double haploid lines from Igri x Triumph (Laurie *et al*., 1994; Turner *et al.,* 2005). Comparative markers show the *Ppd-H1* region is syntenous to a region of rice chromosome 7 that contains the heading date QTL, Hd2 (Dunford *et al.,* 2002).

A second major photoperiod response locus, *Ppd-H2,* has been mapped in the same winter x spring barley cross (Laurie *et al.,* 1995). No equivalent loci have been identified in wheat (Cockram *et al.,* 2007a). HvFT3, another member of the family of Flowering Locus T-like genes in barley, was mapped to the long arm of chromosome 1H, and it has been postulated as a candidate gene for *Ppd-H2* (Faure *et al.,* 2007).

1.3.3. Genetic control of intrinsic earliness

Several studies have investigated the reasons for or causes of variation in the time of heading in the absence of photoperiod and vernalization influences. Numerous "*eps* **"** have been mapped in both barley and wheat by Kato *et al,* (1997). Laurie *et al.* (1995) identified 8 "*eps* **"** loci: *eps2S, eps3L, eps4L, eps5L, eps6L, eps7S and eps7L.* The relatively large

number of *eps* loci, suggest that variation in genes controlling flowering time is common compared with major vernalization (*VRN*) and photoperiod (*Ppd*) genes (Cockram *et al.,* 2007a).

2. Objectives

It is well established that allelic diversity in the genes responsible for vernalization, and epistatic interactions among them, are involved in the occurrence of a continuous variation in barley heading time, under field conditions.

The availability of perfect markers for the vernalization and photoperiod candidate genes (*Vrn-H1*, *Vrn-H2, Vrn-H3, Ppd-H1* and *Ppd-H2*) facilitates the possibility of studying their effect on the variations in flowering time in barley. In this study we use two populations derived from crosses between a typical “winter type” variety with two different Spanish landraces, harbouring different alleles at either *Vrn-H1* or *Vrn-H3.*

The objectives of this study are:

1. To assess the possible differential effect of two different alleles of HvBM5, candidate gene for vernalization response gene Vrn-H1, and two different alleles of HvFT3, candidate gene for Ppd-H2.
2. To assess the possible differential effect of the two alleles described in the literature for HvFT1, candidate gene for vernalization response gene Vrn-H3.
3. To study the type of gene actions of the alleles of the two vernalization genes Vrn-H1 and Vrn-H3.

3. Material and Methods

3.1. Plant material

Two F_2 populations were obtained at the EEAD-CSIC, in the framework of the Spanish National barley breeding programme, and will be used for this study.

There are just three six-rowed parents involved: Esterel (7761 x Plaisant), a French cultivar with a winter growth habit and strong vernalization requirement, which is a common parent for both populations; CN106 (Villarrubia de los Ojos, Ciudad Real) and CN16 (Luna, Zaragoza), two inbred lines from the Spanish Barley Core Collection (SBCC, http://www.eead.csic.es/barley), derived from Spanish landraces, with winter growth habit and an apparently weaker vernalization requirement than typical winter cultivars, such as Esterel. A diversity analysis of the SBCC and a set of reference European barley cultivars using microsatellites clustered these landraces into two different subpopulations. CN106 was included in group I, together with Plaisant, one of the parent lines of Esterel, and many other European winter cultivars, whereas CN16 was assigned to group IV (Yahiaoui *et al.,* 2008), consisting only of Spanish landrace materials. This means that the two Spanish parents may come from different germplasm pools.

Two F2 populations were used: Esterel x CN106 and Esterel x CN16 (Table 1). Out of the five major flowering time genes related to vernalization and photoperiod responses, the first population segregates only for *Vrn-H1* and *Ppd-H2*. The second population segregates for *Vrn-H1, Vrn-H3* and *Ppd-H2.* Both populations are dominant and monomorphic for *Vrn-H2* and *Ppd-H1*.

Population	*Vrn-H1* Intron I size (bp)	*Vrn-H3* haplotype	*Ppd-H2*
Esterel / CN106	5150 / 4850	TC / TC	null / *Ppd-H2*
Esterel / CN16	5150 / 4850	TC / AG	Null / *Ppd-H2*

Table 1. *Polymorphism between the parents of the two F2 populations*

3.2. Experimental setup and data analysis

Esterel x CN106 was evaluated under autumn (standard) and winter (late) sowing conditions, whereas Esterel x CN16 was sown only in winter. Sowing dates were 22/11/2007 and 04/02/2008, respectively. Winter sowings were made with the purpose of revealing possible differences in development due to the segregation of vernalization genes, as the late sowing date provides less cold days for vernalization than standard autumn sowings.

Two hundred seeds were used in each sowing of Esterel x CN106 and, 400 seeds for Esterel x CN16. The F2 populations (plus parents) were germinated and grown for one week under greenhouse conditions (21ºC and 16 h light/8h dark photoperiod), and transferred at the same growing stage (one leaf) to a microplot. This consisted of three rows, 12 m long and 1m wide (Fig. 1). The experiments were carried out at Aula Dei Experimental Station in Zaragoza, in long seed beds commonly used for crossing blocks. Each population was watered every week. Weeds were removed by hand, and general purpose insecticide was sprayed as needed to prevent insect damages.

In an F2 population, it is impossible to have a repetition of the same genotype, since each plant is a distinct and a unique individual. In order to control each individual for genotypic and phenotypic analysis, we identified the primary tiller of each plant with a permanent dye (Fig. 2). Flowering time was recorded when 2 cm of the awns protruded from the flag leaf on the primary tiller, i.e., when it reached stage 49 in the Zadoks scale (Zadoks *et al . ,* 1974). Three others agronomical traits were recorded: initiation of stem elongation (jointing stage), number of ears per plant and growth habit. The numbers of ears per plant and growth habit were scored once, when the expression of the trait was optimum. The initiation of stem elongation and heading of the first tiller were recorded daily for a period of two months, since the end of March until the end of May. Initiation of stem elongation, or joint stage, was checked at each plant by grasping and pressing the base of the primary tiller with two fingers, to feel the bulge caused by the first node (stage 31 Zadoks). This node is sometimes difficult to find without killing the tiller. It is possible that in some cases the second node was found (stage 32 Zadoks) and therefore, this variable may bear a larger error than other.

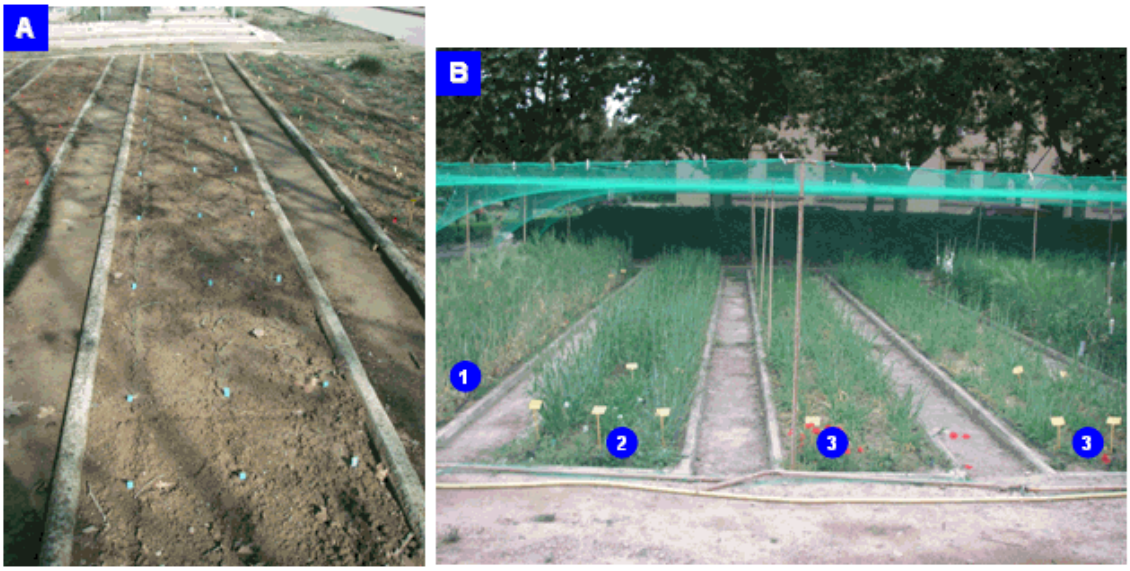

Figure 1*. Planting design in seed beds. A) populations at an early stage, showing the single plant setup; B) populations at an advanced stage; from left to right, autumn sowing of Esterel x CN106 (1), winter sowing of Esterel x CN106, (2) Esterel x CN16 (3).*

Figure 2. *Labelling of primary tillers of each plant, to allow identification of primary tiller over the development period.*

Genotyping was carried out with two markers for the Esterel x CN106 F2 population and three for the Esterel x CN16 F2 population. One or two leaves were harvested from each plant in a 1.5 ml Eppendorf tube and frozen in liquid N2. Tissue was homogenized using a Mixer Mill model MM301 (Retsch) and stored at -20ºC prior to extraction.

DNA was extracted from the ground tissue, according to the protocol described in the NucleoSpin^{R} Plant II Kit (Macherery-Nagel). DNA was eluted in 100 µl PE Buffer and used without further quantification.

The DNA was extracted according to the following protocol:

1. Cell lysis: add to the resulting powder 400uL of buffer PL1 and 10uL of RNAse. Incubate at 65ºC for 30min. Spin at 10,500 g for 5 min.
2. Filter the supernatant through a filter column (violet ring) and centrifuge for 2 min at 10,500 g.
3. Transfer the filtrate to a new tube and add 450 µL buffer PC to adjust DNA binding conditions. Mix by vortexing.
4. Load the DNA binding column with 600 µl and spin for 1 min.
5. To improve the purity of DNA by removing protein and other contaminations, wash the column with 400 µl buffer PW1 and centrifuge for 1min.
6. Wash the silica membrane twice, first with 700 µl buffer PW2, and then dry it with 200 µl buffer PW2, and spin for 2 min.
7. Place the column into a new 1.5 ml tube and elute the DNA with 100 µl buffer PE warm at 70ºC. Incubate at room temperature for 5 min and then spin for 1 min at 10,500 g.

After extraction, DNA amplifications were carried out as follows, for each marker representing a candidate gene:

Candidate genes are HvBM5A for *Vrn-H1* (Yan *et al.,* 2005), *HvFT1* for *Vrn-H3* (Yan *et al.,* 2006; Faure *et al.,* 2007; Hemming *et al.,* 2008) and HvFT3 for *Ppd-H2* (Faure *et al.,* 2007). The markers employed in this study are described in Table 2.

Genes	Diagnostic polymorphism	Candidate gene	Reference
Vrn-H1 (5H)	Size of the first intron	HvBM5A	Szűcs *et al.,* 2007
Vrn-H2 (4H)	Presence/Absence	ZCCT-Ha/-Hb	Karsai *et al.,* 2005
Vrn-H3 (7H)	Two SNPs in the first intron (A/T, G/C)	HvFT1	AM Casas, pers. comm.
Ppd-H1 (2H)	SNP22 (G/T)	HvPPR7	Turner *et al.,* 2005
Ppd-H2 (1H)	Presence/Absence	HvFT3	AM Casas, pers. comm.

Table 2. *Gene specific markers for vernalization and photoperiod genes in barley.*

Differences in the size of the intron I of *Vrn-H1* were detected with primers HvBM5A.88F (5'-gaatggccgctactgcttag-3') and HvBM5A.85R (5'-tctcataggttctagacaaagcatag-3'). *Ppd-H2* was tested using HvFT3 specific primers, HvFT3.1F (5'-atccattggttgtgtggctca-3') and HvFT3.2R (5'-atctgtcaccaacctgcaca-3'). Primers HvFT1.1F (5'-acgtacgtccctttcgatg-3') and HvFT1.2R (5'-atctgtcaccaacctgcaca-3') amplified a 506 bp fragment of the *Vrn-H3* gene. To differentiate the two polymorphic sites in the first intron of the gene, digestion of the amplified DNA was carried out with *Tsp509* I (A/T) and/or *Bcl* I (G/C).

The molecular markers used in the present study were codominant, except HvFT3, allowing to differentiate heterozygous plants, from both homozygous.

Gene	Marker	Esterel	CN16	CN106
Vrn-H1	HvBM5A	520 bp	650 bp	650 bp
Vrn-H3	HvFT1	TC	AG	TC
Ppd-H2	HvFT3	0	431 bp	431 bp

Table 3. *Expected size product (bp) or diagnostic SNPs in vernalization and photoperiod genes of barley.*

PCR reactions were assembled in 15 µl containing 2 µl of genomic DNA, 1x Biotools Standard buffer, 2 mM $MgCl_2$, 0.15 µM of each primer, 0.3 mM of each dNTP and 1 U Biotools DNA Polymerase.

For HvBM5A and HvFT3, amplifications were run using a thermal cycler (Palm Cycler, Corbett or GeneAmp 2700, Applied Biosystems), programmed for 3 min at 95ºC, and then 35 cycles of 45 sec at 95ºC, 45 sec at 56ºC and 1 min at 72ºC, with a final extension of 7 min at 72ºC.

For HvFT1, amplifications were done for 40 cycles, with annealing at 58ºC. After amplification, PCR products were digested with *Tsp509* I at 65ºC or *Bcl* I at 50ºC, respectively. The restriction enzymes (NEB) were diluted at a concentration of 2 U/µl in 1x

Buffer. One µl of enzyme was added to each PCR tube and then incubated at the specified temperature for 2 hours, using a thermal block.

Amplified products were run in 2% agarose gels (MS-12, Pronadisa) and visualized by ethidium bromide staining.

3.3. Statistical analysis

Goodness of fit test X^2 was used to compare the observed and predicted segregation at different vernalization genes. Analysis of variance was performed using the general linear model (GLM) procedure of the SAS System for Windows (SAS Institute Inc., Cary, NC, USA), including all segregating markers and their interactions as fixed variables. Position of the plants in the plot and the degree of damage caused by Barley yellow dwarf virus (BYDV) were included in the analysis of variance as covariates. Contrasts between the homozygotic classes, and between the heterozygotes and the midparent value were used to estimate the additive and dominance effects for HvBM5A and HvFT1. This was not possible for HvFT3, as this marker does not differentiate between one of the homozygotes (product of 431 bp) and the heterozygote.

4. Results

During the experimentation, some differences of growth in individual barley plants were observed. These differences were partially attributed to the effect of plant position in the seed beds, and to reaction to an unexpected virus attack. Parent CN106 was not present in the autumn sowing, as the seed source used was wrongly labeled. This mistake was amended for the winter sowing.

The seed beds, where the plants were grown, were approximately 15 m long by 1 m wide. Only 12 m were used for this experiment. In one end, these seed beds were protected by a perpendicular row of trees, whose shade partially affected the trial. The plants closer to the trees, i.e., most affected by the shade, grew taller and headed later, in general. To account for this effect, each plant was scored for a variable named “position”. This variable was created dividing the seed beds in three areas, each one 4 meters long. The values were assigned using a three points scale (0, 1, 2), each level of the scale representing approximately four meters. This variable represents quite precisely the actual results observed (Fig. 3). The plants localized in the last four meters (closer to the trees) flowered later than the plants in the first four meters (Fig. 3a), especially in the first sowing of population Esterel x CN106, and less clearly in the two late sowings. The number of ears per plant (Fig. 3b) was lower in the last four meters (closer to the trees) than in the first four meters for the two sowings of population Esterel x CN106 but not for Esterel x CN16. There were no differences, however, in time to reach the joint stage. Any differences in heading date of the plants due to differential exposition to shade occurred after the initiation of stem elongation.

Some plants showed a stunted development and some yellowing of the leaves (Fig. 4). It is suspected that there was an attack of barley yellow dwarf virus, which affected some plants. The presence of the virus was confirmed by ELISA in three plants with symptoms sampled for this purpose[1]. A few plants showing severe symptoms were almost killed and did not reached heading. Some plants seemed affected to some extent, but reached heading and produced spikes. A majority of plants showed normal, apparently undisturbed growth. We decided to score the plants for disease severity with a subjective scale from 0 to 2 (Table 5), 2 being assigned to severely stunted plants with heavily altered growth; 1 was assigned to plants with some yellowing and reduced growth compared with neighbours; 0 was assigned to apparently normal plants. Plants with a score of 2 were actually excluded from further analyses.

[1] Kind collaboration of Dr. Miguel Cambra, from the *Centro de Protección Vegetal* of the Government of Aragón, is acknowledged

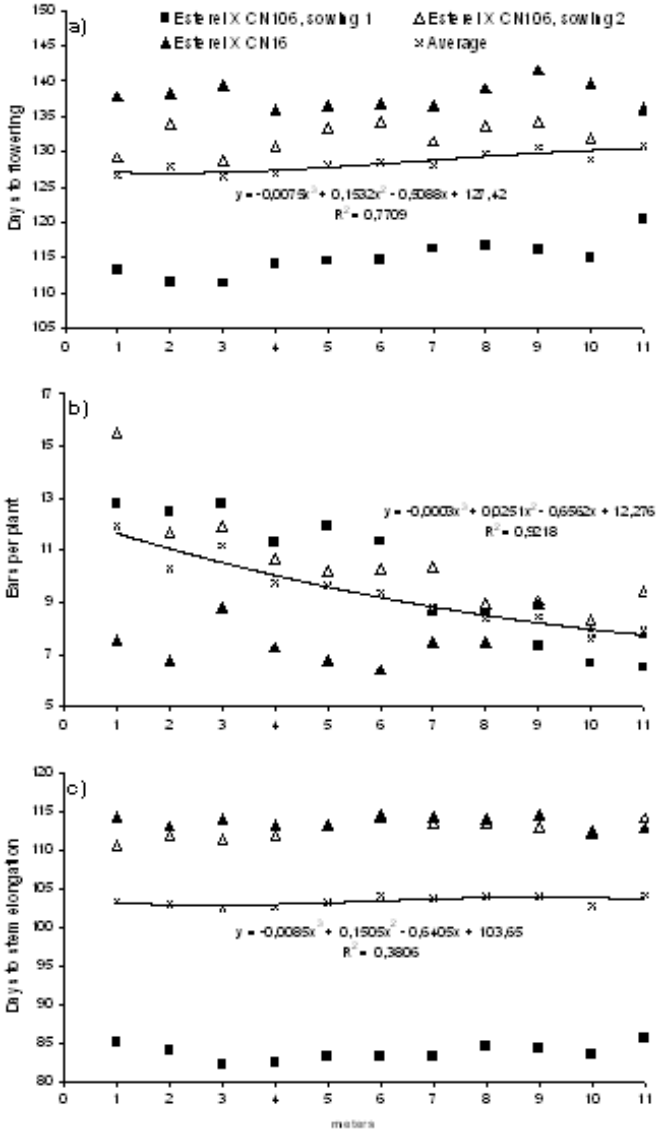

Figure 3. *Evolution of time to flowering (a), N° of ears/plant (b) and initiation of stem elongation (c), which are proportional to position effect.*

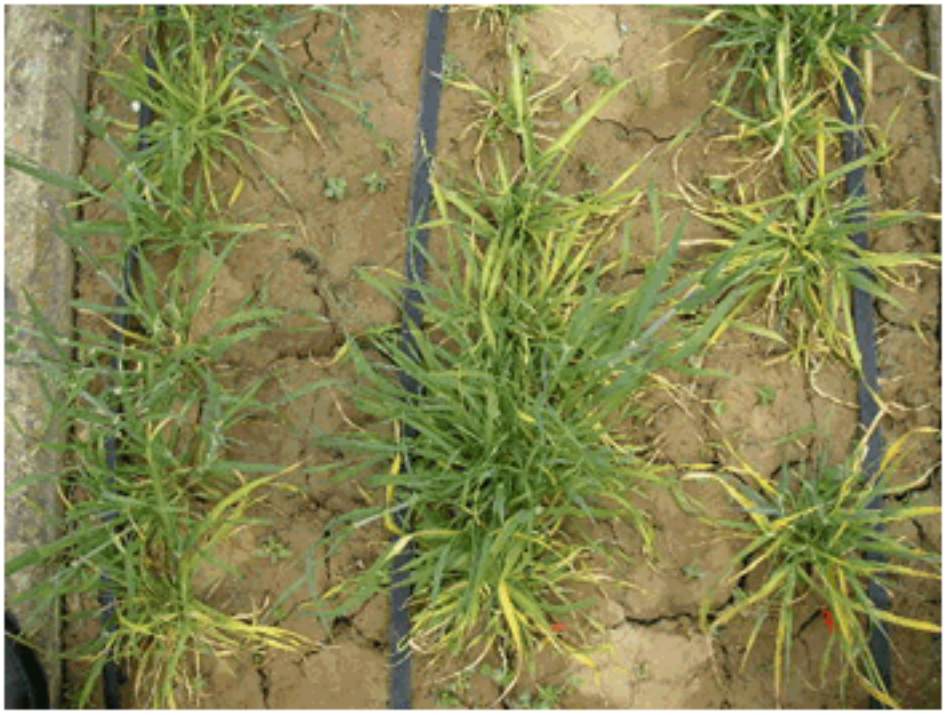

Figure 4. *Yellowing and stunting of some plants due to virus attack.*

Population and sowing	Time to heading	Time to stem elongation	Growth habit	Spikes per plant
	----------Position in seed bed----------			
Esterel x CN106, autumn sowing	28.4 **	1.5	-	17.2 **
Esterel x CN106, winter sowing	8.81 **	6.0	28.2 **	6.6 **
Esterel x CN16	2.1 **	0.1	33.2 **	0.1
	----------Virus damage----------			
Esterel x CN106, autumn sowing	6.5 **	8.1 **	-	2.2
Esterel x CN106, winter sowing	21.8 **	2.7	16.6 **	5.8 **
Esterel x CN16	14.7 **	0.2	4.9 **	11.1 **

*,**,*** significant effect at P=0.05, P=0.01 and P=0.001.

Table 4. *R^2 of each phenotypic variable explained by the covariates "position in the seed bed", and "virus damage".*

When introduced as covariates in the analyses of variance for heading time, days to stem elongation, and number of ears per plant, both position and degree of virus attack

explained sizable amounts in some cases, especially in time to heading and growth habit (Table 4). Plants more affected by shade (position=2) headed later, developed less spikes. Plants affected by virus yellowing were clearly shorter, but also later, had a more prostrate growth habit, and less spikes per plant (Table 5).

Degree of stunting*	n	Days to heading	Days to stem elongation	Growth habit	Spikes per plant
			Esterel x CN106, autumn sowing		
0	96	114.0	83.3	-	10.2
1	59	114.2	83.9	-	10.4
2	10	116.6	87.0	-	8.6
			Esterel x CN106, winter sowing		
0	152	131.6	112.9	2.0	11.0
1	20	139.7	114.6	1.5	7.3
2	0	-	-	-	-
			Esterel x CN16, winter sowing		
0	218	137.1	113.7	1.9	8.0
1	21	143.0	114.5	1.3	4.3
2	3	147.3	-	1.0	2.0

* 0, no stunting; 1, moderate stunting; 2, severe stunting

Table 5. *The effect of virus damage in time to flowering and other agronomic traits.*

The number of plants finally scored for phenotypic traits and molecular markers were 160 and 157 for the standard and late sowings of Esterel x SBCC106 respectively, and 242 plants for Esterel x SBCC016. Only healthy plants which reached flowering were finally kept for further analysis. Also, parent SBCC106 could not be scored at the autumn sowing plot, due to mislabelling of the seed used (which could be corrected for the winter sowing).

4.1. Phenotypic analysis

4.1.1. Heading time

There was also variation for flowering time among the parents and the three F_2 populations. On average, Esterel flowered 6.8 days later than CN106, and 3.9 days later than CN16 when sown in winter (Table 6, Fig. 5). The average days to flowering of the populations was between those of the respective parents for Esterel x CN16, whereas at Esterel x CN106 sown in winter, the F_2 average flowered and reached jointing slightly earlier than either parent (Figure 6, Table 6).

***Figure 5**. Parents of the populations sown in winter. A, CN106 (left) and Esterel (center). B, Esterel (left) and CN16 (right).*

The distributions of days to heading showed a quantitative response and transgressive segregation (Figure 6 a-c). The population Esterel x CN106 sown in autumn flowered over a range of 12 (108-120) days. The range of variation was wider for the winter sown populations, 25 and 28 days for Esterel x CN106 and Esterel x CN16, respectively.

	Days to flowering	Days to stem elongation	Growth habit	No. of ears/plant
		Esterel x CN106, autumn sowing		
Esterel	118.4	85.2	-	7.2
CN106	-	-	-	-
F2 population	114.1	83.5	-	10.5
Range (min, max)	18 (108, 126)	14 (78, 92)	-	21 (1, 22)
		Esterel x CN106, winter sowing		
Esterel	139.3 a	115.4	1.42	11.3
CN106	132.4 b	113.6	1.33	12.3
F2 population	132.0	112.8	2.04	10.5
Range (min, max)	24 (123, 147)	11 (108, 119)	2 (1, 3)	14 (4, 18)
		Esterel x CN16, winter sowing		
Esterel	139.1 a	116.1 a	1.83 a	7.4
CN16	135.1 b	112.4 b	2.25 b	7.4
F2 population	137.7	113.6	1.81	7.6
Range (min, max)	28 (124, 152)	14 (105, 119)	2 (1, 3)	20 (1, 21)

Means of parents followed by a different letter are significantly different for P<0.05

Table 6. *Means and ranges of distribution of agronomic traits in the two populations and the parents.*

The representation of the distribution of flowering time showed that Esterel x CN106 followed a bell shape when sown in autumn (Fig. 6a). But, when sown in winter, this same population presented a bimodal distribution (Fig. 6a). The first group (123-131 days) contained 82 F_2 plants, whereas the second (days 132-147) had 75 F_2 plants, as well as the two parents. Esterel x CN16 (Fig. 6b) also presented a bell shape, apparently following a normal distribution.

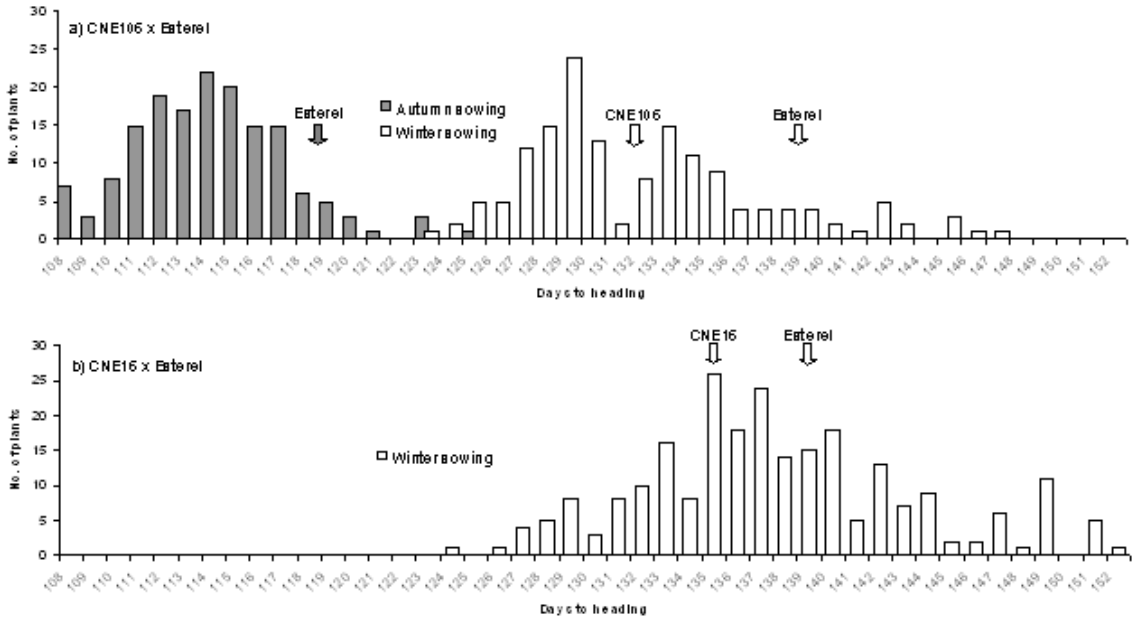

Figure 6. *Distribution of days to flowering in F_2 populations: a) Esterel x CN106 autumn and winter sowings, and b) Esterel X CN16, winter sowing.*

4.1.2. Initiation of stem elongation

Compared to ranges of time to flowering, ranges of variation in time to stem elongation were much narrower, and similar between populations and sowings, varying between 12 days for Esterel x CN106, winter-sown, and 14 days at Esterel x CN106, autumn-sown and Esterel x CN16, winter-sown (Figure 7, Table 6). Overall, there were just slight differences between the two populations sown in winter (differed by less than one day). Esterel was always later than CN106 and CN16 in all winter sowings (around 2 days with CN106 and 4 days with CN16).

The distributions found for this trait tended to be bimodal. In the case of Esterel x CN106 sown in autumn (Fig. 7a), two groups were apparent. The first one ranged from 78 to 81 days, whereas the second covered a wider range. The distribution for this population sown in winter was normal (Fig. 7a). The other population, Esterel x CN16 also showed a hint of bimodal distribution (Fig. 7b), with peaks at 112 and 115 days. But, given the large error associated to this trait, it is not possible to reach any conclusion on the shape of these distributions.

Time from joint stage until flowering was similar for parents Esterel and CN16 as well as for their F_2 (around 23 days, Table 6). This time period was shorter for parent CN106 (19 days in the winter sowing), and the average for its F_2 sown in winter was much closer to CN106 than to Esterel.

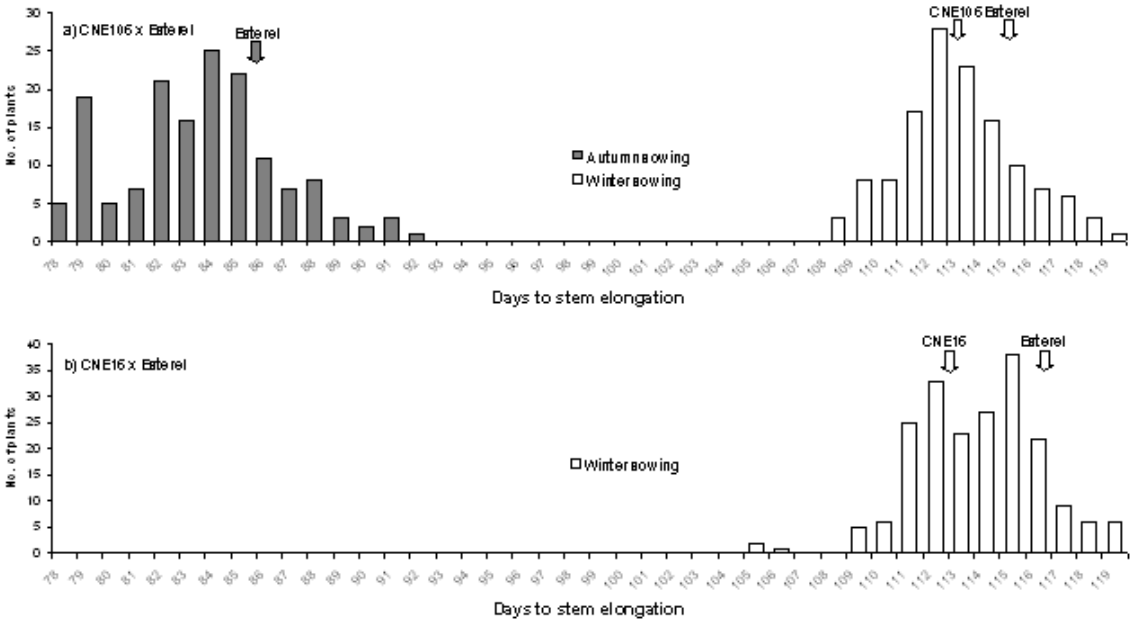

Figure 7. *Distribution of the time to stem elongation in two F2 populations: a) Esterel x CN106, autumn and winter sowings and b) Esterel X CN16, winter sowing.*

4.1.3. Number of ears per plant

There were small differences between the parents of both populations, for each sowing time (Table 6). In the population Esterel x CN106, the number of ears per plant was lower when the population was sown in autumn than in winter (Figure 8a).

For this agronomic trait, the frequency distributions of the populations revealed an apparent transgressive segregation (Fig. 8).

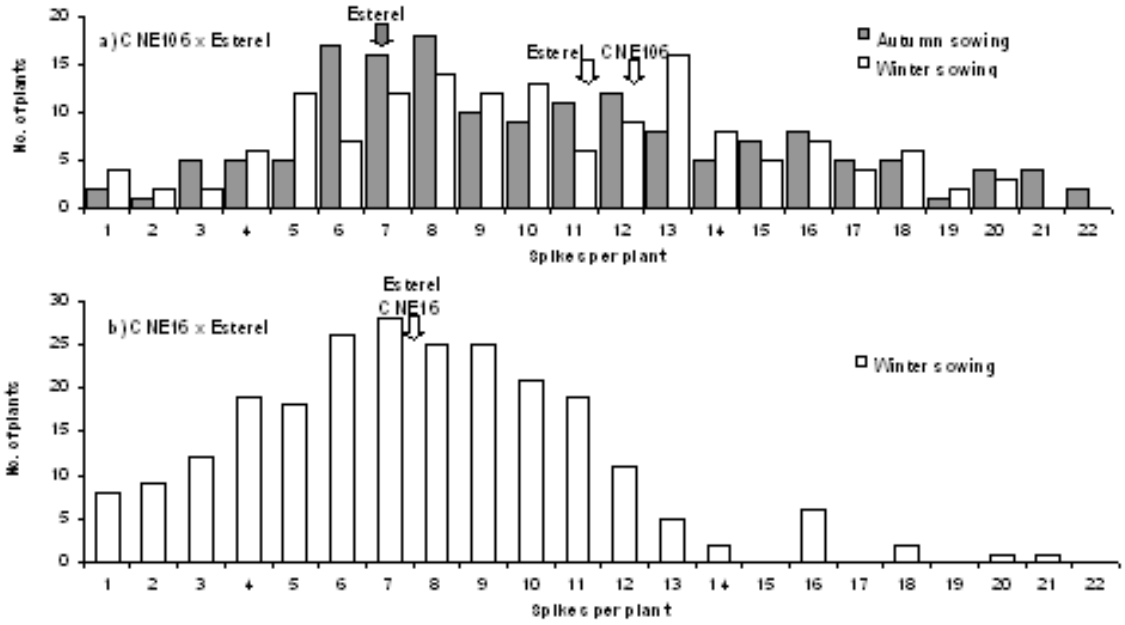

Figure 8. *Distribution of the number of ears per plant in F2 populations: a) Esterel x CN106 autumn and winter sowings, and b) Esterel x CN16, winter sowing.*

4.1.4. Growth habit

To estimate this agronomic trait, in the winter-sown trials, we visually scored growth habit according to three categories: 1) prostrate, 2) intermediate and 3) erect, though intermediate categories (1.5 and 2.5) were also scored, as differences of this degree among plants were visible. This trait was only scored at the winter sown experiment. Prostrate growth habit is typical of winter cultivars, such as the parents of these populations (Table 6). There were slight differences between overall means for populations Esterel x CN106 (2.04) and Esterel x CN16 (1.81). This was probably due to the fact that the Spanish parents of the two populations differed in growth habit, more prostrate for CN106 and more erect for CN16 (Fig. 9).

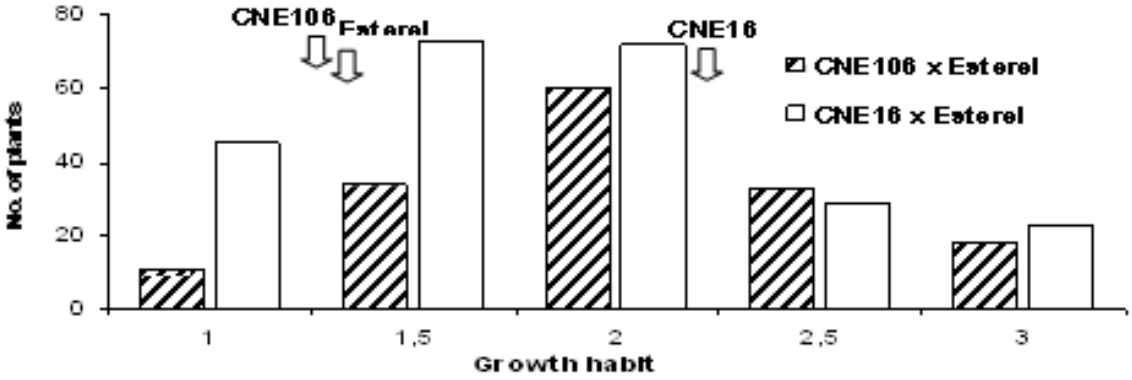

Figure 9. *Phenotypic distributions of the trait growth habit in F2 populations sown in winter: Esterel x CN106, and Esterel x CN16.*

4.2. Segregation

HvBM5A and HvFT3 genotypes were scored for each F2 plant of both populations and sowings. In the case of Esterel x CN16, we also evaluated HvFT1, the candidate gene for *Vrn-H3*, as the parents showed polymorphism for this gene. Two alleles were found for each of the three analyzed genes. HvBM5A segregation in both Esterel x CN106 sown in autumn, or Esterel x CN16, fitted the expected 1:2:1 ratio for a codominant marker (Table 7). However, segregation distortion was found when Esterel x CN106 was sown in winter. 157 plants were genotyped for HvBM5A and the segregation of alleles at this locus showed a deviation from the expected ratio 1:2.1 (P= 0.01), as there was an excess of homozygotes of the CN106 allele (650 bp) and a deficit of homozygotes of the Esterel allele (520 bp) (Table 7). The excess of homozygotes of the CN106 allele was confirmed in the joint segregation analysis for the two sowings of this population.

The second gene analyzed was *Ppd-H2* (HvFT3). The segregation patterns in all crosses agreed with the expected ratio 3:1 (as one of the alleles is null, and thus one of the homozygotes is undistinguishable from the heterozygote) with P=0.58 (Esterel x CN106, autumn-sown), P=0.12 (Esterel x CN106, winter-sown) and P=0.26 for Esterel x CN16 (Table 7).

	HvBM5A					HvFT3				HvFT1				
Sowing	650	650/ 520	520	X^2	P	0	431	X^2	P	TC	TC/ AG	AG	X^2	P
						Esterel x CN106								
Autumn	49	68	43	4.05	0.13	37	123	0.30	0.58	-	-	-	-	-
Winter	54	75	28	8.92	0.01	31	126	2.31	0.12	-	-	-	-	-
Total	103	143	71	9.49	0.01	68	249	2.12	0.14	-	-	-	-	-
						Esterel x CN16								
Winter	59	124	59	0.14	0.92	68	174	1.23	0.26	59	141	42	9.0	0.01

Table 7. *Segregation for HvBM5A, HvFT3 and HvFT1 alleles in two F_2 populations.*

The third major gene, segregating only in the Esterel x CN16 F_2 population, is *Vrn-H3* (HvFT1). The expected segregation of this gene was 1:2:1. However, in the total of 242 plants analyzed, we observed a higher proportion of plants carrying the heterozygote genotype than expected (Table 7). These results deviate significantly from the expected ratio with a $P<0.01$.

4.3. Effect of the vernalization and photoperiod genes on agronomic traits

Data from each population were analyzed separately. A combined analysis of both populations sown in winter was also carried out.

4.3.1. HvFT3

No significant effect or interactions were found, for any of the studied genes, in the Esterel x CN106 F_2 population sown in autumn (Table 8). In this population, the variation was associated with the position of the plants and the degree of BYDV virus infection.

In winter sowings, HvFT3 had a significant effect in both F_2 populations. It had a highly significant effect on flowering time in Esterel x CN106 and Esterel x CN16, which was confirmed by a large effect in the joint analysis for the two populations (Table 8). The plants null for this gene (recessive *Ppd-H2* allele) flowered significantly later than the plants with *Ppd-H2* allele.

Traits	HvBM5A df	HvBM5A MS	HvFT3 df	HvFT3 MS	HvFT1 df	HvFT1 MS	HvBM5A *HvFT3 *HvFT1 df	HvBM5A *HvFT3 *HvFT1 MS	Population *HvFT3 df	Population *HvFT3 MS	Population df	Population MS	Error df	Error MS
Autumn Sown														
Esterel x CN106														
Flowering time	2	2.8	1	16.2	-	-	-	-	-	-	-	-	154	6.8
Stem elongation	2	6.4	1	2.8	-	-	-	-	-	-	-	-	149	8.9
Growth habit	-	-	-	-	-	-	-	-	-	-	-	-	-	
Spikes/plant	2	12.0	1	17.7	-	-	-	-	-	-	-	-	154	19.2
Winter Sown														
Esterel x CN106														
Flowering time	2	39.1	1	596.1***	-	-	-	-	-	-	-	-	147	14.5
Stem elongation	2	15.7*	1	80.4***	-	-	-	-	-	-	-	-	124	4.3
Growth habit	2	1.0**	1	0.9*	-	-	-	-	-	-	-	-	150	0.2
Spikes/plant	2	21.0	1	0.5	-	-	-	-	-	-	-	-	147	22.0
Esterel x CN16														
Flowering time	2	44.6	1	317.2***	2	671.6***	-	-	-	-	-	-	233	20.1
Stem elongation	2	8.4	1	1.3	2	25.4**	-	-	-	-	-	-	193	5.7
Growth habit	2	0.1	1	0.7	2	0.2	-	-	-	-	-	-	232	0.2
Spikes/plant	2	9.5	1	0.1	2	13.9	4	38.2*	-	-	-	-	220	11.7
Populations combined														
Flowering time	2	58.2	1	965.5***	-	-	-	-	1	91.8**	1	1149.8***	384	21.3
Stem elongation	2	22.4**	1	67.8***	-	-	-	-	1	47.1***	1	1.2	321	5.3
Growth habit	2	1.0***	1	1.8***	-	-	-	-	1	0.1	1	0.9**	386	0.2
Spikes/plant	2	1.3	1	0.0	-	-	-	-	1	0.8	1	470.1***	383	16.2

*,**,*** significant effect at P=0.05, P=0.01 and P=0.001.

Table 8. *Significant main effects and interactions of all loci affecting the agronomic traits in the F2 populations (df: degrees of freedom; MS: mean squares).*

	HvBM5A (*Vrn-H1*)			HvFT3 (*Ppd-H2*)		HvFT1 (*Vrn-H3*)		
	520/520	520/650	650/650	0	431/-	TC/TC	TC/AG	AG/AG
Autumn Sown								
Esterel x CN106								
Flowering time	113.6	114.1	113.9	113.5	114.3	-	-	-
Stem elongation	83.2	83.7	83.9	84.1	83.4	-	-	-
Growth habit	-	-	-	-	-	-	-	-
Spikes/plant	10.3	10.1	10.4	10.6	10.2	-	-	-
Winter Sown								
Esterel x CN106								
Flowering time[1]	134.8 a	133.9 ab	132.7 b	136.5 a	131.3 b	-	-	-
Stem elongation	114.4 a	113.4 ab	113.0 b	114.7 a	112.5 b	-	-	-
Growth habit	1.7 b	2.0 a	2.0 a	1.8 b	2.0 a	-	-	-
Spikes/plant	9.6	10.4	11.2	10.5	10.3	-	-	-
Esterel x CN16								
Flowering time	137.8 ab	137.9 a	136.5 b	138.7 a	136.1 b	133.2 c	138.5 b	140.5 a
Stem elongation	114.0	113.4	113.2	113.6	113.4	112.6 b	114.0 a	114.0 a
Growth habit	1.7	1.8	1.8	1.7	1.8	1.8	1.7	1.7
Spikes/plant	8.2	7.4	8.1	7.9	7.9	8.2	7.3	8.2
Populations combined								
Flowering time	136.5 a	136.2 ab	135.1 b	138.0 a	134.0 b			
Stem elongation	114.3 a	113.5 b	113.2 b	114.3 a	113.1 b			
Growth habit	1.7 b	1.9 a	1.9 a	1.7 b	1.9 a			
Spikes/plant	8.9	9.0	9.1	9.0	9.0			

[1] values followed by different letters within traits and genes are significantly different for P<0.05

Table 9. *Means of allelic classes of markers studied.*

In general, those plants headed around four days later in both F_2 Populations (Table 9).

Ppd-H2 also influenced the time to stem elongation, but only in the population Esterel x CN106 sown in winter. Though the effect of HvFT3 was still significant in the joint analysis, this was due solely to the effect in Esterel x CN106, as revealed also by the significant Population x HvFT3 term. In this last population, plants null for *Ppd-H2* reached jointing 2 days later (P<0.01). The effect on growth habit was also significant (P<0.01) in the population Esterel x CN106. The trend was the same in Esterel x CN16, but the difference was not significant. The joint analysis, however, revealed that, though the difference between allele classes was small, it was consistent and significant (P=0.002) across populations.

4.3.2. HvBM5A

The effects of this marker on all traits recorded in population Esterel x CN106 sown in autumn were negligible.

No significant effect of HvBM5A (*Vrn-H1)* on flowering time was observed in the analyses within populations (probabilities were always close to, but higher than 0.05). Nevertheless, the two homozygotic classes were significantly different in Esterel x CN106, winter sowing, with the heterozygote in an intermediate position. As the trend shown by the homozygotic allele classes at Esterel x CN16 for this trait was very similar (though not significant in this case), the combined analysis for the two winter sowings also revealed a significant difference of 1.4 days between the two HvBM5A homozygotes (Table 9).

In Esterel x CN106, HvBM5A had a significant effect on the time to stem elongation. The same trend was observed in the other population sown in winter, though the difference was not significant. This was evident in the joint analysis, where there was a significant effect on stem elongation, which resulted mostly from a difference between the two homozygotic classes (Tables 8 and 9) of just above one day.

Exactly the same trend as for time to stem elongation was found for growth habit. Overall, the Esterel allele produced a more prostrate growth habit than the CN106 allele (Table 9). This was observed despite the fact that the parents of this population showed no difference in growth habit.

4.3.3. HvFT1

The second major vernalization gene studied is *Vrn-H3* (marker HvFT1), which segregated only in the Esterel x CN16 F_2 population. HvFT1 had a large effect on flowering time, and also a significant effect on time to stem elongation (Table 8). In these two agronomic traits, plants homozygous for the CN16 allele (AG) were later than those plants carrying the allele from Esterel (TC) with seven days of difference in time of flowering and 1.4 days in the time to stem elongation (Table 9). The heterozygote class for this marker presented averages closer to the “later” allele for these two traits. No effect on growth habit or number of spikes per plant was detected with this marker.

No significant interactions were detected between HvFT3 (*Ppd-H2*) and HvBM5A (*Vrn-H1*), in Esterel x CN106 (Table 8), or between the three markers studied in Esterel x CN16. The only exception was the trait spikes per plant in this last population, which presented a significant triple interaction. Apparently, it was due to a higher number of spikes in classes 650/TC/0, 650/AG/0 and 520/TC/431. Given the overall low genetic component shown by this trait, and the low frequencies of individuals in the classes involved in the interaction (1, 4 and 7), it seems that its origin was merely accidental.

4.4. Estimation of genetic effects for the studied loci

The contrasts between the homozygotic classes, and homozygotes vs. heterozygotes gave rather similar results to the overall analyses of variance presented in Tables 8 and 9. No significant effects for any marker were found for the Esterel x CN106 F_2 population sown in autumn. For the winter sowings, the results were highly dependent on population. HvBM5A showed significant additive effects for time to flowering, time to stem elongation and growth habit in Esterel x CN16, and in the joint analysis for the two populations (Table 10). CN106 and CN16 alleles always conferred more erectness and earliness. No dominance effects were detected for this gene.

HvFT3 is a dominant marker, for this reason we can not estimate separately the additive and dominance effects. Instead, a combined effect (4/3a+2/3d) was calculated. Rather large effects of HvFT3 on flowering time were present in both populations, but just in winter sowings. This marker also presented significant effects on stem elongation and growth habit. In Esterel x CN16, significant additive and dominance effects were detected for HvFT1 in time to flowering and time to stem elongation, with the heterozygote almost as late as the “late” homozygote (AG/AG, Table 9).

	HvBM5A		HvFT3	HvFT1	
Traits	a[1]	d[2]	Difference[3]	a	d
Autumn sown					
Esterel x CN106					
Flowering time (days)	ns[4]	ns	ns	-	-
Stem elongation (days)	ns	ns	ns	-	-
Growth habit (score)	-	-	-	-	-
N° of ear/plant (number)	ns	ns	ns	-	-
Winter sown					
Esterel x CN106					
Flowering time (days)	1.05**	ns	5.2***	-	-
Stem elongation (days)	0.7***	ns	2.2***	-	-
Growth habit (score)	0.2***	ns	0.2**	-	-
N° of ear/plant (number)	ns	ns	ns	-	-
Esterel x CN16					
Flowering time (days)	ns	ns	2.6***	3.7***	1.7***
Stem elongation (days)	ns	ns	ns	0.7***	0.7***
Growth habit (score)	ns	ns	ns	ns	ns
N° of ear/plant (number)	ns	ns	ns	ns	ns
Populations combined					
Flowering time (days)	0.7**	ns	4.0***	-	-
Stem elongation (days)	0.5***	ns	1.2***	-	-
Growth habit (score)	0.1***	ns	0.2***	-	-
N° of ear/plant (number)	ns	ns	ns	-	-

[1] additive effect (homozygote AA – homozygote aa)/2

[2] dominance effect (heterozygote - ((homozygote AA+homozygote aa)/2))

[3] difference between the two classes, estimated to be (4/3)a+(2/3)d

[4] not significant

Table 10. *Estimation of additive and dominance effects for the three loci studied.*

5. Discussion

Phenology is a key factor in the adaptation of crop plants to environment and management practices. Heading (anthesis) or flowering time is a critical determinant of yield potential (Slafer, 2003; Castro *et al.,* 2008).

Selection pressure during domestication and modern breeding of cereals has resulted in a complex flowering mechanism modulated by daylength (Ppd) and vernalization (VRN) genes (Kane *et al.,* 2007). The effect of these genes on phenotypes have been identified via QTL analysis and quantitative approaches (Koti *et al.,* 2006), though there is still much to be researched.

The aim of the present work was to asses the possible effects of *Vrn-H1, Vrn-H3* and *Ppd-H2* in two F2 populations under two contrasting growing conditions in the field.

There were unexpected sources of variation between the F2 plants, attributed to position effects with respect to the shade produced by neighboring trees, and a virotic infection of some plants. The effect due to plant position was only apparent after the jointing stage in the winter sowings (Fig. 3). Therefore, it must not be related to a temperature effect during the winter, but rather to a phenomenon of light intensity, probably under a long photoperiod regime. As plants reached jointing stage about mid-May, the position effect developed during long days. The introduction of the covariates “position” and “virus damage” in the analyses made possible the identification of significant effects, compared to the models which included no covariates (not shown).

In most cases, the distribution of the F2 plants showed apparent transgressive segregation, though the entire range of variation is not attributable to genetic causes, as the inherent error of an experiment like this is high.

The populations sown in winter differed significantly in time to flowering, but not in time to stem elongation. As the development after joint stage occurred all under long days, from mid-May, the two Spanish parents must differ in some genetic factor that might respond to long photoperiod. This factor is probably not *Ppd-H1*, as all parents show the same allele in the polymorphism described for this gene (Table 2). In the overall comparison between populations it was also noticeable that, though CN16 was clearly more erect than CN106, the F2 plants of both populations had an opposite behaviour, being the F2 of Esterel x CN106 more erect than Esterel x CN16. There was also a striking difference between populations in number of spikes per plant. There should be some genetic factor underlying this difference, but either it is not related to the three genes studied (as the variance of the trait they accounted for was negligible), or the error variance of this trait is too large in our experimental setting.

The segregation observed for the markers fitted expectations for HvFT3 at both populations, but differed from expectations for HvFT1 in Esterel x CN16 and for HvBM5A in Esterel x CN106. We have no explanation for the excess of heterozygotes in HvFT1. The low number of homozygotes for the Esterel allele (520) at HvBM5 seems to be the responsible for the distortion observed. Some plants died during sowing and transplant, and also due to virus attack. There may have been a selective disadvantage of the allele coming from the

more "winter" parent (Esterel), either because of the late sowing, or because of linkage of this allele with some putative factor that confers susceptibility to the virus attack suffered.

The effects of two vernalization genes were analyzed. The third one, *Vrn-H2*, whose presence is needed to induce a vernalization requirement in the plant, was fixed in both populations, as Esterel, CN106 andCN16 carry the gene. In the autumn sown plot, we did not detect any influence of *Vrn-H1* in the determination of time to flowering, and in any of the other agronomic traits. The two putative alleles of *Vrn-H1* have the same effects in the autumn sowing and small effects were observed in the winter sowings. This is not surprising, as even in crosses between spring and winter barleys, the region of *Vrn-H1* is not usually identified as possessing flowering time QTL in autumn sowings (Laurie *et al .,* 1995, Cuesta-Marcos *et al .,* 2008). This occurs because the vernalization requirement of the lines with a "winter" genetic constitution (*Vrn-H2* plus a recessive allele at *Vrn-H1*) is usually satisfied for autumn sowings in temperate climates, and other genes are responsible for flowering time variation.

We have found a slight but significant effect of *Vrn-H1* on flowering time, time to stem elongation, and growth habit just in winter sowings, with the allele from CN106 conferring earliness and more erect growth habit, when comparing only the homozygotic plants. The absence of effect of this gene in the autumn sowing suggests that both alleles confer a vernalization requirement, but that the one from the Spanish genotypes fulfilled this requirement earlier in the late sowing, whereas it may have taken longer for the plants with the Esterel allele to fulfill their vernalization needs. The difference between the homozygotic classes for time to flowering (1.4 days, Table 9) can be accounted almost totally for by the difference in time to stem elongation (1.1 days, Table 9). Therefore, differential effect of the two alleles must have occurred before stem elongation, which is consistent with a difference in the amount of vernalization requirement conferred by each allele.

The HvBM5 allele from CN106 and CN16 is the most common among landrace accessions from the highlands of inland Spain, an area with Temperate Mediterranean climate, with real winters, but milder than in most Western and Central Europe. Therefore, it is conceivable that this allele may have been selected for adaptation to intermediate winter harshness.

Regarding type of gene action, although partial dominance of alleles at *Vrn-H1* has been reported in barley and wheat by Koti et al. (2006), we found no evidence of dominance for this gene in our study. Nevertheless, different alleles of *Vrn-H1* were compared in that study, with typical spring and winter types.

Allelic variation at *Ppd-H2* is of great agronomic importance. This locus has significant effects on flowering time in autumn sowings, especially under Mediterranean conditions (Boyd *et al .,* 2003; Cuesta-Marcos *et al.,* 2008). HvFT3 is the candidate gene for the *Ppd-H2* locus (Faure *et al.,* 2007). Under short day conditions, the presence of this gene is associated with early flowering, while the absence of the gene is associated with late flowering. HvFT3 is expressed only under short photoperiod and is not detected under long photoperiod (Faure *et al.,* 2007), which is consistent with its detection as QTL under short photoperiods only in controlled conditions experiments (Cuesta-Marcos *et al.,* 2008). Our results disagree partially with previous findings. In Esterel x CN106 sown in autumn, we expected to find a large effect of this gene, as described in populations with similar polymorphism Beka x Mogador (Cuesta-Marcos *et al . ,* 2008), Igri x Triumph (Laurie *et al.,* 1995), Nure x Tremois (Francia et al., 2004), or Dicktoo x Morex (Pan *et al.,* 1994; Karsai *et al.,* 2008). We can speculate that all those crosses were "winter" or "facultative" by "spring"

and Esterel x CN106 is probably a “winter” by “winter” cross, and thus may have other different genetic factors affecting the expression and effect of *Ppd-H2*.

On the other hand, *Ppd-H2* was a significant determinant of flowering time and other agronomic traits under longer photoperiod, in the winter sowings. HvFT3 is a dominant marker, and all that can be said about it is that plants with active *Ppd-H2* were earlier than the plants without *Ppd-H2.* This effect may be similar to the one found by Cuesta-Marcos *et al.* (2008) in the population Beka x Mogador at a single trial sown in winter, at a similar date than the experiment described here, and under vernalization and long photoperiod controlled conditions. Karsai *et al.* (2008) also found an effect of *Ppd-H2* on flowering time under long photoperiods, as in our winter sowings. Their explanation that “the *PPD-H2* locus is a significant determinant of flowering time under long photoperiods as well, but only in the case of applying synchronous photo and thermo cycles, and when specific allelic configurations are present at the *PPD-H1* and *VRN-H1* loci” also point at an interaction between photoperiod and vernalization response genes as responsible for the variable effect of *Ppd-H2* across populations and growing conditions.

HvFT1, the candidate gene for *Vrn-H3*, accelerates flowering in unvernalized plants under long photoperiod (Yan *et al.,* 2006). The induction of FT requires the *Ppd-H1* gene, and varieties with an inactive version of *Ppd-H1* presented reduced sensitivity to long photoperiod (Trevaskis *et al.,* 2007). Expression of HvFT1 was not detected in plants that carry *Vrn-H2*, which is consistent with previous reports that HvFT1 is expressed at low levels in vernalization requiring varieties (Yan *et al.,* 2006). In another study, HvFT1 expression was also very low in plants null for *Vrn-H2* and carrying inactive *Ppd-H1* (Hemming *et al.,* 2008). In contrast, HvFT1 expression was detected in plants carrying the active *Ppd-H1* allele. This expression was not correlated with *Vrn-H2*, thus suggesting that *Vrn-H2* does not counteract activation of HvFT1 by *Ppd-H1* (Hemming *et al.,* 2008). Therefore, *Vrn-H2* may interact directly with components of long day response pathway to regulate *Vrn-H3*. *Vrn-H2* has a CCT domain, a type of domain known to interact with CCAAT binding factors (Ben- Naim *et al.,* 2006). In Arabidopsis a CCAAT binding factor has been shown to up-regulate FT. *Vrn-H2* might interact negatively with CCAAT site and create a difference in the expression level of HvFT3.

The two parents Esterel and CN16 have an active *Ppd-H1,* also carry *Vrn-H2,* and the population was segregating for *Vrn-H1.* Our results for Esterel x CN16 indicate that *Vrn-H3* had a strong effect in determining flowering time, and on the initiation of stem elongation in a winter sowing, with the CN16 allele (AG, typical of spring cultivars) later than the Esterel one. If the effect found for *Vrn-H3* has occurred as a result of different expression patterns of its two alleles, a necessary condition would have been that *Vrn-H2* expression was not high, as it would have repressed *Vrn-H3* expression.

Vrn-H3 had a large effect in influencing flowering time, and less on time to stem elongation. Contrarily to *Vrn-H1*, most of the effect of this gene on plant development is evident after joint stage, and thus may be responding to different environmental cues than *Vrn-H1,* as suggested by Cockram *et al.* (2007a) and Hemming *et al.* (2008). *Vrn-H3* had significant additive and dominance effects, with the heterozygotes presenting values quite close to the “late” homozygotic class. There is only another study finding an effect of *Vrn-H3* on flowering date. Yan *et al.* (2006) described an acceleration of flowering date conferred by the AG allele of about 40 days for unvernalized plants under long days and high temperature (20-24ºC), in a cross of two winter barleys. Our result in Esterel x CN16, under natural conditions, show a less striking effect (but rather large, anyway), but in the opposite direction.

The particular conditions of the winter sowings at this experiment, a realistic late sowing date, with day length shifting from short to long, and variable temperatures, may have resulted in a combination of environmental conditions causing a significant effect of *Vrn-H3* on flowering date. This effect may be of importance to explain the adaptation role of this gene. At least in late sowings, the TC allele of HvFT1 would confer earliness, which may be convenient for barley plants growing in mid-spring in Mediterranean climates to escape from rapidly rising temperature and risk of drought and heat stress. The polymorphism at this gene may thus provide additional genetic variability to adjust the plant development to environmental conditions.

The variance of the developmental traits not explained by the markers studied may be affected by other genes. A locus that is of great importance in the determination of flowering under Mediterranean conditions is *eps2s*, located near the centromere in 2H (Boyd *et al.,* 2003; Cuesta-Marcos *et al.,* 2008). Flanking markers for this locus were not polymorphic between the three parents of this study. From previous studies, we know that parents Esterel and CN106 probably segregate for earliness per se QTLs localized in 5HL and 7HL (deduced by looking at flanking markers).

6. Conclusions

1. The experimental setup was very simple, and easy to control. We used F2 populations, which are readily available from the breeding program. This kind of experiment has been successful to reveal allelic differences at candidate genes, though the number of plants should be increased in future experiments, and non-desired environmental factors should be controlled better.
2. Vrn-H1, represented by marker HvBM5A, presents a small but significant effect on plant development, mostly before joint stage, coherent with a smaller vernalization requirement conferred by the allele contributed by the Spanish accessions.
3. Vrn-H3 presented a marked effect on flowering time, and less on stem elongation. This is the first report of a phenotypic effect of this gene on plant development under natural conditions.
4. Ppd-H2 presented large effects on flowering time, which depended on sowing time and population. A full explanation of the behaviour of this gene will require the study of materials contrasting for all vernalization and photoperiod genes.

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ANNEXES

Annex 1 : Number of plants belonging to each allelic class for the F2 populations used in this study.

HvFT1	HvFT3	HvBM5A	Esterel x CN106, autumn	Esterel x CN106, winter	Esterel x CN106, total	Esterel x CN16
TC	0	520	7	10	17	4
TC	0	650	13	18	31	1
TC	0	520/650	17	10	27	6
TC	431	520	36	25	61	7
TC	431	650	36	45	81	7
TC	431	520/650	51	65	116	16
AG	0	520				6
AG	0	650				4
AG	0	520/650				10
AG	431	520				4
AG	431	650				12
AG	431	520/650				23
TC/AG	0	520				8
TC/AG	0	650				9
TC/AG	0	520/650				19
TC/AG	431	520				30
TC/AG	431	650				25
TC/AG	431	520/650				50

Annex 2 : Populations combined

The GLM Procedure

Class	Levels	Values
Population	2	Esterel x CN106 Esterel x CN16
HvBM5	3	520 650 650/520
HvFT3	2	0 431

Number of obsevration read 398

Dependent variable: Flowering time

Sources	DF	Sum of squares	Mean square	F-Value	Pr > F
Model	9	6512.53264	723.61474	33.98	<.0001
Error	384	8177.84300	21.29647		
Corrected total	393	14690.37563			

R-square	Coeff Var	Root MSE	Mean
0.443320	3.406210	4.614809	135.4822

Sources	DF	Type I SS	Mean square	F-Value	Pr > F
Damage	1	1644.797868	1644.797868	77.23	<.0001
Position	1	627.422919	627.422919	29.46	<.0001
Populacion	1	3185.019410	3185.019410	149.56	<.0001
HvBM5	2	55.147142	27.573571	1.29	0.2752
HvFT3	1	891.939683	891.939683	41.88	<.0001
Population*HvBM5	2	16.397944	8.198972	0.38	0.6807
Population*HvFT3	1	91.807669	91.807669	4.31	0.0385

Dependent variable: Stem elongation

Sources	DF	Sum of squares	Mean square	F-Valor	Pr > F
Model	9	218.190550	24.243394	4.55	<.0001
Error	321	1711.501293	5.331780		
Corrected total	330	1929.691843			

R-square	Coeff Var	Root MSE	Mean
0.113070	2.037700	2.309065	113.3172

Sources	DF	Type I SS	Mean square	F-Value	Pr > F
Damage	1	17.16457017	17.16457017	3.22	0.0737
Position	1	11.05290962	11.05290962	2.07	0.1509
Population	1	69.34330061	69.34330061	13.01	0.0004
HvBM5	2	34.51228275	17.25614137	3.24	0.0406
HvFT3	1	37.53325632	37.53325632	7.04	0.0084
Population *HvBM5	2	1.38869633	0.69434816	0.13	0.8779
Population *HvFT3	1	47.19553440	47.19553440	8.85	0.0032

Mean

Sources	DF	Type III SS	Mean square	F-Valor	Pr > F
Damage	1	12.47511357	12.47511357	2.34	0.1271
Position	1	14.56045154	14.56045154	2.73	0.0994
Population	1	1.21835899	1.21835899	0.23	0.6330
HvBM5	2	44.83211698	22.41605849	4.20	0.0158
HvFT3	1	67.86062235	67.86062235	12.73	0.0004
Population *HvBM5	2	2.98220353	1.49110176	0.28	0.7562
Population *HvFT3	1	47.19553440	47.19553440	8.85	0.0032

Dependent variable: nº Ear/plant

Sources	DF	Sum of squares	Mean square	F-Valor	Pr > F
Model	9	1485.741996	165.082444	10.18	<.0001
Error	383	6209.301261	16.212275		
Corrected total	392	7695.043257			

R-square	Coeff Var	Root MSE	Mean
0.193078	46.05337	4.026447	8.743003

Sources	DF	Type I SS	Mean square	F-Value	Pr > F
Damage	1	561.4160509	561.4160509	34.63	<.0001
Position	1	84.6198549	84.6198549	5.22	0.0229
Population	1	797.3744914	797.3744914	49.18	<.0001
HvBM5	2	1.2895760	0.6447880	0.04	0.9610
HvFT3	1	0.0407921	0.0407921	0.00	0.9600
Population *HvBM5	2	40.1758870	20.0879435	1.24	0.2908
Population *HvFT3	1	0.8253435	0.8253435	0.05	0.8216

Sources	DF	Type III SS	Mean square	F-Valor	Pr > F
Damage	1	646.1483966	646.1483966	39.86	<.0001
Position	1	122.8903394	122.8903394	7.58	0.0062
Population	1	470.1695070	470.1695070	29.00	<.0001
HvBM5	2	2.6666844	1.3333422	0.08	0.9211
HvFT3	1	0.0435054	0.0435054	0.00	0.9587
Population *HvBM5	2	38.1971626	19.0985813	1.18	0.3090
Population *HvFT3	1	0.8253435	0.8253435	0.05	0.8216

Dependent variable: Growth habit

Sources	DF	Sum of squares	Mean square	F-Valor	Pr > F
Model	9	60.1194215	6.6799357	34.73	<.0001
Error	386	74.2341139	0.1923164		
Corrected total	395	134.3535354			

R-square	Coeff Var	Root MSE	Mean
0.447472	23.03202	0.438539	1.904040

Sources	DF	Type I SS	Mean square	F-Value	Pr > F
Damage	1	16.31023594	16.31023594	84.81	<.0001
Position	1	36.48226990	36.48226990	189.70	<.0001
Population	1	3.76993658	3.76993658	19.60	<.0001
HvBM5	2	1.32640496	0.66320248	3.45	0.0328
HvFT3	1	1.52421842	1.52421842	7.93	0.0051
Population *HvBM5	2	0.57420831	0.28710416	1.49	0.2260
Population *HvFT3	1	0.13214735	0.13214735	0.69	0.4077

Sources	DF	Type III SS	Mean square	F-Valor	Pr > F
Damage	1	10.97734930	10.97734930	57.08	<.0001
Position	1	33.32792806	33.32792806	173.30	<.0001
Population	1	0.94204023	0.94204023	4.90	0.0275
HvBM5	2	1.90360309	0.95180155	4.95	0.0075
HvFT3	1	1.78054037	1.78054037	9.26	0.0025
Population *HvBM5	2	0.61329876	0.30664938	1.59	0.2042
Population *HvFT3	1	0.13214735	0.13214735	0.69	0.4077

Population Esterel X CN16

Class	Levels	Values
HvBM5	3	520 650 650520
HvFT3	2	0 431
HvFT1_Bc	3	hetero invier prima

Number of obsevration read 241

Dependent variable: Flowering time

Sources	DF	Sum of squares	Mean square	F-Valor	Pr > F
Model	7	3095.645930	442.235133	22.04	<.0001
Error	233	4674.130003	20.060644		
Corrected total	240	7769.775934			

R-square	Coeff Var	Root MSE	Mean
0.398422	3.253512	4.478911	137.6639

Sources	DF	Type I SS	Mean square	F-Value	Pr > F
Position	1	166.005952	166.005952	8.28	0.0044
Damage	1	1143.389382	1143.389382	57.00	<.0001
HvBM5	2	71.862266	35.931133	1.79	0.1691
HvFT3	1	371.116835	371.116835	18.50	<.0001
HvFT1_Bc	2	1343.271495	671.635747	33.48	<.0001

Sources	DF	Type III SS	Mean square	F-Valor	Pr > F
Position	1	293.020529	293.020529	14.61	0.0002
Damage	1	1184.540738	1184.540738	59.05	<.0001
HvBM5	2	89.182738	44.591369	2.22	0.1106
HvFT3	1	317.218001	317.218001	15.81	<.0001
HvFT1_Bc	2	1343.271495	671.635747	33.48	<.0001

Dependent variable: Stem elongation

Sources	DF	Sum of squares	mean square	F-Valor	Pr > F
Model	7	75.008684	10.715526	1.88	0.0744
Error	193	1098.971416	5.694152		
Corrected total	200	1173.980100			

R-square	Coeff Var	Root MSE	Mean
0.063893	2.099150	2.386242	113.6766

Sources	DF	Type I SS	Mean square	F-Value	Pr > F
Position	1	1.80599075	1.80599075	0.32	0.5740
Damage	1	3.33275609	3.33275609	0.59	0.4452
HvBM5	2	16.78520505	8.39260252	1.47	0.2316
HvFT3	1	2.27089332	2.27089332	0.40	0.5285
HvFT1_Bc	2	50.81383833	25.40691916	4.46	0.0128

Sources	DF	Type III SS	Mean square	F-Valor	Pr > F
Position	1	0.88731221	0.88731221	0.16	0.6935
Damage	1	2.87052719	2.87052719	0.50	0.4786
HvBM5	2	16.88809072	8.44404536	1.48	0.2295
HvFT3	1	1.34621673	1.34621673	0.24	0.6274
HvFT1_Bc	2	50.81383833	25.40691916	4.46	0.0128

Dependent variable: Growth habit

Sources	DF	Sum of squares	mean square	F-Valor	Pr > F
Model	7	33.59476089	4.79925156	22.09	<.0001
Error	232	50.40419744	0.21725947		
Corrected total	239	83.99895833			

R-square	Coeff Var	Root MSE	Mean
0.399943	25.68694	0.466111	1.814583

Sources	DF	Type I SS	Mean square	F-Value	Pr > F
Position	1	27.92005952	27.92005952	128.56	<.0001
Damage	1	4.19994025	4.19994025	19.33	<.0001
HvBM5	2	0.29018455	0.14509227	0.67	0.5138
HvFT3	1	0.71608798	0.71608798	3.30	0.0707
HvFT1_Bc	2	0.45848860	0.22924430	1.06	0.3498

Sources	DF	Type III SS	Mean square	F-Valor	Pr > F
Position	1	22.80037203	22.80037203	104.95	<.0001
Damage	1	4.22007074	4.22007074	19.42	<.0001
HvBM5	2	0.28431722	0.14215861	0.65	0.5207
HvFT3	1	0.65972292	0.65972292	3.04	0.0827
HvFT1_Bc	2	0.45848860	0.22924430	1.06	0.3498

Dependent variable: n° Ear/plant

Sources	DF	Sum of squares	mean square	F-Valor	Pr > F
Model	19	624.108153	32.847798	2.80	0.0002
Error	220	2577.354347	11.715247		
Corrected total	239	3201.462500			

R-square	Coeff Var	Root MSE	Mean
0.194945	44.81511	3.422754	7.637500

Sources	DF	Type I SS	Mean square	F-Value	Pr > F
Position	1	5.9937508	5.9937508	0.51	0.4752
Damage	1	357.5444904	357.5444904	30.52	<.0001
HvBM5	2	19.3105037	9.6552518	0.82	0.4400
HvFT3	1	1.8260128	1.8260128	0.16	0.6934
HvFT1_Bc	2	9.2102294	4.6051647	0.39	0.6754
HvBM5*HvFT3	2	22.6063896	11.3031948	0.96	0.3827
HvBM5*HvFT1_Bc	4	46.6882971	11.6720743	1.00	0.4104
HvFT3*HvFT1_Bc	2	7.1445430	3.5722715	0.30	0.7375
HvBM5*HvFT3*HvFT1_Bc	4	153.7838366	38.4459592	3.28	0.0123

Sources	DF	Type III SS	Mean square	F-Valor	Pr > F
Position	1	1.7545539	1.7545539	0.15	0.6991
Damage	1	329.7087074	329.7087074	28.14	<.0001
HvBM5	2	19.0685648	9.5342824	0.81	0.4445
HvFT3	1	0.0695414	0.0695414	0.01	0.9387
HvFT1_Bc	2	27.7674321	13.8837160	1.19	0.3077
HvBM5*HvFT3	2	54.9586923	27.4793461	2.35	0.0982
HvBM5*HvFT1_Bc	4	52.7099945	13.1774986	1.12	0.3456
HvFT3*HvFT1_Bc	2	18.3427609	9.1713804	0.78	0.4584
HvBM5*HvFT3*HvFT1_Bc	4	153.7838366	38.4459592	3.28	0.0123

Population Esterel X CN106

A- *Autumn sown*

The GLM Procedure

Class	Levels	Values
HvBM5	3	520 650 650520
HvFT3	2	0 431

Number of obsevration read 160

Dependent variable: Flowering time

Sources	DF	Sum of squares	mean square	F-Valor	Pr > F
Model	5	592.900165	118.580033	17.47	<.0001
Error	154	1045.074835	6.786200		
Corrected total	159	1637.975000			

R-square	Coeff Var	Root MSE	Mean
0.361971	2.282864	2.605034	114.1125

Sources	DF	Type I SS	Mean square	F-Value	Pr > F
Position	1	465.8632555	465.8632555	68.65	<.0001
Damage	1	106.5651646	106.5651646	15.70	0.0001
HvBM5	2	4.2984048	2.1492024	0.32	0.7290
HvFT3	1	16.1733404	16.1733404	2.38	0.1247

Sources	DF	Type III SS	Mean square	F-Valor	Pr > F
Position	1	541.9156562	541.9156562	79.86	<.0001
Damage	1	115.5514432	115.5514432	17.03	<.0001
HvBM5	2	5.7204964	2.8602482	0.42	0.6568
HvFT3	1	16.1733404	16.1733404	2.38	0.1247

Dependent variable: nº Ear/plant

Sources	DF	Sum of squares	mean square	F-Valor	Pr > F
Model	5	771.323696	154.264739	8.03	<.0001
Error	154	2958.576304	19.211534		
Corrected total	159	3729.900000			

R-square	Coeff Var	Root MSE	Mean
0.206795	41.84240	4.383096	10.47500

Sources	DF	Type I SS	Mean square	F-Value	Pr > F
Position	1	643.6087872	643.6087872	33.50	<.0001
Damage	1	83.5506967	83.5506967	4.35	0.0387
HvBM5	2	26.4591453	13.2295726	0.69	0.5038
HvFT3	1	17.7050672	17.7050672	0.92	0.3386

Sources	DF	Type III SS	Mean square	F-Valor	Pr > F
Position	1	705.1761715	705.1761715	36.71	<.0001
Damage	1	92.0337588	92.0337588	4.79	0.0301
HvBM5	2	23.9808737	11.9904369	0.62	0.5371
HvFT3	1	17.7050672	17.7050672	0.92	0.3386

Dependent variable: Stem elongation

Sources	DF	Sum of squares	mean square	F-Valor	Pr > F
Model	5	160.191420	32.038284	3.62	0.0041
Error	149	1320.285999	8.860980		
Corrected total	154	1480.477419			

R-square	Coeff Var	Root MSE	Mean
0.108203	3.563168	2.976740	83.54194

Sources	DF	Type I SS	Mean square	F-Value	Pr > F
Position	1	22.9408894	22.9408894	2.59	0.1097
Damage	1	120.1713230	120.1713230	13.56	0.0003
HvBM5	2	14.2789554	7.1394777	0.81	0.4487
HvFT3	1	2.8002526	2.8002526	0.32	0.5749

Sources	DF	Type III SS	Mean square	F-Valor	Pr > F
Position	1	38.4050711	38.4050711	4.33	0.0391
Damage	1	108.5269072	108.5269072	12.25	0.0006
HvBM5	2	12.7209592	6.3604796	0.72	0.4895
HvFT3	1	2.8002526	2.8002526	0.32	0.5749

A- *Winter sown*

The GLM Procedure

Class	Levels	Values
HvBM5	3	520 650 650520
HvFT3	2	0 431

Number of observation read 157

Dependent variable: Flowering time

Sources	DF	Sum of squares	mean square	F-Valor	Pr > F
Model	5	1831.623451	366.324690	25.22	<.0001
Error	147	2135.056287	14.524192		
Corrected total	152	3966.679739			

R-square	Coeff Var	Root MSE	Mean
0.461752	2.886168	3.811062	132.0458

Sources	DF	Type I SS	Mean square	F-Value	Pr > F
Position	1	349.5880215	349.5880215	24.07	<.0001
Damage	1	866.8898338	866.8898338	59.69	<.0001
HvBM5	2	19.0439743	9.5219871	0.66	0.5206
HvFT3	1	596.1016218	596.1016218	41.04	<.0001

Sources	DF	Type III SS	Mean square	F-Valor	Pr > F
Position	1	430.4889528	430.4889528	29.64	<.0001
Damage	1	810.7096441	810.7096441	55.82	<.0001
HvBM5	2	78.2460590	39.1230295	2.69	0.0710
HvFT3	1	596.1016218	596.1016218	41.04	<.0001

Dependent variable: Stem elongation

Sources	DF	Sum of squares	mean square	F-Valor	Pr > F
Model	5	159.9686894	31.9937379	7.49	<.0001
Error	124	529.6390030	4.2712823		
Corrected total	129	689.6076923			

R-square	Coeff Var	Root MSE	Mean
0.231971	1.832813	2.066708	112.7615

Sources	DF	Type I SS	Mean square	F-Value	Pr > F
Position	1	41.57709047	41.57709047	9.73	0.0022
Damage	1	19.01177664	19.01177664	4.45	0.0369
HvBM5	2	18.98211766	9.49105883	2.22	0.1127
HvFT3	1	80.39770458	80.39770458	18.82	<.0001

Sources	DF	Type III SS	Mean square	F-Valor	Pr > F
Position	1	45.82618302	45.82618302	10.73	0.0014
Damage	1	7.17785338	7.17785338	1.68	0.1973
HvBM5	2	31.33664347	15.66832174	3.67	0.0283
HvFT3	1	80.39770458	80.39770458	18.82	<.0001

Dependent variable: nº Ear/plant

Sources	DF	Sum of squares	mean square	F-Valor	Pr > F
Model	5	513.489108	102.697822	4.68	0.0005
Error	147	3226.680826	21.950210		
Corrected total	152	3740.169935			

R-square	Coeff Var	Root MSE	Mean
0.137290	44.71747	4.685105	10.47712

Sources	DF	Type I SS	Mean square	F-Value	Pr > F
Position	1	249.1708017	249.1708017	11.35	0.0010
Damage	1	217.4947814	217.4947814	9.91	0.0020
HvBM5	2	46.3625234	23.1812617	1.06	0.3504
HvFT3	1	0.4610018	0.4610018	0.02	0.8850

Sources	DF	Type III SS	Mean square	F-Valor	Pr > F
Position	1	288.0183424	288.0183424	13.12	0.0004
Damage	1	233.3918235	233.3918235	10.63	0.0014
HvBM5	2	42.0587239	21.0293619	0.96	0.3860
HvFT3	1	0.4610018	0.4610018	0.02	0.8850

Dependent variable: Growth habit

Sources	DF	Sum of squares	mean square	F-Valor	Pr > F
Model	5	22.94927911	4.58985582	30.56	<.0001

Error	150	22.52988755	0.15019925
Corrected total	155	45.47916667	

R-square	Coeff Var	Root MSE	Mean
0.504611	18.98231	0.387555	2.041667

Sources	DF	Type I SS	Mean square	F-Value	Pr > F
Position	1	12.86259662	12.86259662	85.64	<.0001
Damage	1	7.55131003	7.55131003	50.28	<.0001
HvBM5	2	1.68074026	0.84037013	5.60	0.0045
HvFT3	1	0.85463220	0.85463220	5.69	0.0183

Sources	DF	Type III SS	Mean square	F-Valor	Pr > F
Position	1	11.30980825	11.30980825	75.30	<.0001
Damage	1	7.25786372	7.25786372	48.32	<.0001
HvBM5	2	2.00153300	1.00076650	6.66	0.0017
HvFT3	1	0.85463220	0.85463220	5.69	0.0183