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Environment-dependent regulation of flowering time in a barley (*Hordeum vulgare* L.) MAGIC population

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Abstract

Barley (*Hordeum vulgare* L.) is used as a source for food and feed and is the fourth widely cultivated cereal world-wide. Flowering time is a complex trait controlled by endogenous and environmental factors that marks switching of plant life cycle from vegetative to reproductive development. Flowering time mechanism has important role in crop adaptation to environment and abiotic stressors including heat and drought and has an impact on crop yield. Flowering time in barley, as a model for small grain cereals, is extensively studied. Nevertheless, what is known about its flowering time mechanism in response to environment is under-represented compared to model plants such as *Arabidopsis thaliana*. Therefore, the present thesis aims to improve understanding of environment-dependent regulation of flowering time in barley and provide insights into novel flowering time regulators. For this purpose a series of approaches were employed aiming to 1) better understand the interconnected dynamics of epistasis and environment using a MAGIC population and look for novel regulators; 2) identify candidate gene(s) underlying novel barley flowering time QTL; 3) investigate effect of flowering time genetic regions on yield-related traits under different environments. Spring barley MAGIC population was used in three detailed studies as plant material. This population can provide sufficient diversity and mapping power to study a complex trait such as flowering time and is constructed of an eight-way cross of barley landraces known as "founders of German barley breeding" and one elite cultivar Barke. The first study focused on QTL, epistasis and environment interaction regulation in flowering time pathway of barley under different environments including field and semi-controlled conditions. A set of 534 spring barley MAGIC DH lines were used for analyzing of quantitative trait loci (QTLs), epistatic interactions, QTL × environment interactions, and epistasis × environment interactions effects with single SNP and haplotype approaches. Results of this study, revealed overall 18 QTLs and 2,420 epistatic interactions which included regions for major genes such as *Ppd-H1*, *Vrn-H1*, *Vrn-H3*, and *denso/sdw1*. Distinguishable epistatic interactions were detected in field and semi-controlled conditions and findings from analysis of QTL × environment interactions and epistasis × environment interactions suggested the influence of temperature on regulators of flowering time pathway. Additionally, the results revealed a novel QTL harboring a flowering-delaying allele on chromosome 1H, engaged in epistatic and environment interactions, which we named "*HvHeading*". The findings showed that this region was involved in epistasis and epistasis × environment interactions with regions harboring *Ppd-H1*, *Vrn-H3*, and *Vrn-H1* and *denso/sdw1*

suggesting that it might have an important role in environment-dependent regulation of flowering time in barley. The second study aimed to investigate the newly-detected QTL *HvHeading* to identify the underlying candidate gene(s) by a targeted background effect elimination approach based on epistasis. The spring barley MAGIC DH lines were screened to select flowering-time-specific-near-isogenic pairs of DH lines that have the same background regarding the genes, *Ppd-H1*, *Vrn-H1* and *Vrn-H3* which are involved in major epistasis with *HvHeading*. One DH line in the pair had the haplotype harboring the flowering-delaying allele underlying *HvHeading* which originated from parental line Danubia. The apex and inflorescence development was investigated using microscopic phenotyping. Differential gene expression analysis by RNA-sequencing and RT-qPCR in apex and leaf tissue was conducted. Phenotypic effect of *HvHeading* was detected as early as after vegetative-to-reproductive transition. Analyzing transcripts from RNA-sequencing identified differentially expressed genes in *HvHeading* region in flowering-time-specific-near-isogenic pair of DH lines. These findings led to refining *HvHeading* interval to <8.50 Mbp and identifying up-regulation of *Spt6* gene in delayed-flowering DH line. Differential gene expression analysis using RT-qPCR validated up-regulation of *Spt6* starting before double-ridge stage and showed down-regulation of *Ppd-H1*, *Vrn-H1* for the delayed-flowering DH line. Most of the promoter region of *Spt6* gene in flowering-time-specific-near-isogenic pair of DH lines was sequenced and showed many mutations. Additionally, comparing the sequenced transcripts of *Spt6* gene with published *Spt6* isoforms in barley showed that DH line with Danubia haplotype might produce a novel isoform. The third study was conducted to evaluate effect of flowering time genetic regions on yield-related traits including grain yield components under different environments. This study used the same set of 534 MAGIC DH lines and evaluated seven traits under well-watered and terminal drought treatments. The analysis of QTL for each treatment, maker by treatment interaction (M×T) and QTL for drought tolerance was conducted. Results revealed all traits were affected by treatment apart from days to heading. In total 69, 64 and 29 QTL were found under well-watered and terminal drought treatment and drought tolerance, respectively. The M×T analysis revealed total 25 loci for four traits. The identified QTL included loci that co-located with known genes/QTL as well as novel regions. The results revealed genetic regions for various traits which coincided with flowering time loci under well-watered and terminal drought, hinting to pleiotropic effect of flowering time genes such as *Ppd-H1*, *Vrn-H1*, *Vrn-H3* and *denso/sdw1* on grain yield and plant development. Some detected QTL showed favorable effect on grain number, ear number and grain weight under terminal drought treatment including QTL corresponding to

major flowering time gene *Ppd-H1* for grain weight. Spring barley MAGIC population is a valuable asset which offered powerful mapping of flowering time QTL and epistasis under different environments including novel regulators; these outcomes can be transferred to more complex crops such as wheat. Validating effect of epistatic QTL *HvHeading* and identifying *Spt6* gene as a candidate gene by a targeted elimination of background effect approach based on epistasis showed that mapping epistasis interactions can help compose strategies to facilitate gene identification. The role of candidate gene *Spt6* in flowering time pathway of barley should be validated and further studied. Detecting favorable pleiotropic effect of flowering time loci on grain weight components under terminal drought hinted to possibility of usefulness of flowering time genes for improving these traits under extreme environments through timing of flowering or their contribution to developmental pathways. The findings showed the importance of exploring epistasis and environment interaction in addition to more common approaches such as QTL analysis, to explore adaptation of flowering time to different environment in barley. The series of studies presented in this dissertation gives new insights into environment-dependent regulation of flowering time in barley through a multidisciplinary approach which highlights importance of employing approaches that better explain complex traits in future research and breeding programs.

Zusammenfassung

Gerste (*Hordeum vulgare* L.) wird als Nahrungs- und Futtermittelquelle genutzt und ist weltweit die viertmeist verbreitete Getreideart. Die Blütezeit ist ein komplexes Merkmal, das durch endogene und Umweltfaktoren gesteuert wird und den Wechsel des pflanzlichen Lebenszyklus von der vegetativen zur reproduktiven Entwicklung markiert. Der Mechanismus der Blütezeit spielt eine wichtige Rolle bei der Anpassung der Pflanzen an die Umwelt und an abiotische Stressfaktoren wie Hitze und Dürre und wirkt sich auf den Ernteertrag aus. Die Blütezeit bei Gerste, als Modell für kleinkörnige Getreidearten, wird umfassend untersucht. Dennoch ist, was über den Mechanismus der Blütezeit als Reaktion auf die Umwelt bekannt ist, im Vergleich zu Modellpflanzen wie *Arabidopsis thaliana*, unterrepräsentiert. Daher zielt die vorliegende Arbeit darauf ab, das Verständnis der umweltabhängigen Regulation der Blütezeit bei Gerste zu verbessern und Einblicke in neuartige Blütezeitregulatoren zu gewinnen. Zu diesem Zweck wurde eine Reihe von Ansätzen angewandt, die darauf abzielen, 1) die zusammenhängende der dynamischen von Epistase und Umwelt mit einer MAGIC-population besser zu verstehen und nach neuen Regulatoren zu suchen; 2) Kandidatengen(e) zu identifizieren, die der neue Gerstenblütezeit QTL zugrunde liegen; 3) die Auswirkungen der Blütezeit genetischer Regionen auf ertragsbezogene Merkmale unter verschiedenen Umweltbedingungen zu untersuchen. Die MAGIC-Population der Sommergerste wurde in drei detaillierten Studien als Pflanzenmaterial verwendet. Diese Population kann eine ausreichende Diversität und Kartierungskraft bieten, um ein komplexes Merkmal wie die Blütezeit zu untersuchen, und besteht aus einer Acht-Wege-Kreuzung von Gerstenlandrassen, die als "Begründer der deutschen Gerstenzüchtung" bekannt sind, und einer Elite-Sorte Barke. Die erste Studie konzentrierte sich auf QTL, Epistase und die Regulation der Umweltinteraktion im Blütezeitverlauf von Gerste unter verschiedenen Umweltbedingungen, einschließlich Freiland- und halbkontrollierten Bedingungen. Ein Satz von 534 MAGIC DH-Linien aus Sommergerste wurde für die Analyse der quantitativen Merkmalloci (QTLs), epistatischen Interaktionen, QTL × Umweltinteraktionen und Epistasis × Umweltinteraktionen mit einzelnen SNP- und Haplotypenansätzen verwendet. Die Ergebnisse dieser Studie ergaben insgesamt 18 QTLs und 2.420 epistatische Interaktionen, die Regionen für wichtige Gene wie *Ppd-H1*, *Vrn-H1*, *Vrn-H3* und *denso/sdw1* einschlossen. Unterscheidbare epistatische Interaktionen wurden unter Freiland- und halbkontrollierten Bedingungen festgestellt, und die Ergebnisse der QTL × Umweltinteraktionen und Epistasis × Umweltinteraktionen legten

den Einfluss der Temperatur auf die Regulatoren des Blütezeitverlaufs nahe. Zusätzlich enthüllten die Ergebnisse ein neues QTL, das ein blühverzögerndes Allel auf Chromosom 1H beherbergt und an epistatischen und Umweltinteraktionen beteiligt ist, die wir "*HvHeading*" nannten. Die Ergebnisse zeigten, dass diese Region an Epistase und Epistase × Umweltinteraktionen beteiligt war, mit Regionen, die *Ppd-H1*, *Vrn-H3* und *Vrn-H1* und *denso/sdw1* beherbergen, was darauf hindeutet, dass sie eine wichtige Rolle bei der umweltabhängigen Regulation der Blütezeit in Gerste spielen könnte. Die zweite Studie zielte darauf ab, den neu entdeckten QTL *HvHeading* zu untersuchen, um das/die zugrundeliegende(n) Kandidatengen(e) durch einen gezielten Ansatz zur Eliminierung von Hintergrundeffekten auf der Basis von Epistase zu identifizieren. Die MAGIC DH-Linien der Sommergerste wurden gescreent, um Blütezeit-spezifisch-nah-isogenen Paare von DH-Linien zu selektieren, die den gleichen Hintergrund bezüglich der Gene *Ppd-H1*, *Vrn-H1* und *Vrn-H3* haben, die an der Hauptepistase mit *HvHeading* beteiligt sind. Bei einer DH-Linie des Paares beherbergte der Haplotyp das verzögert blühende Allel, das *HvHeading* zugrunde liegt und von der Elternlinie Danubia abstammt. Die Entwicklung von Apex und Infloreszenz wurde mittels mikroskopischer Phänotypisierung untersucht. Es wurde eine differentielle Genexpressionsanalyse mittels RNA-Sequenzierung und RT-qPCR mit Apex- und Blattgewebe durchgeführt. Der phänotypische Effekt von *HvHeading* wurde bereits nach dem vegetativ-reproduktiven Übergang festgestellt. Bei der Analyse von Transkripten aus der RNA-Sequenzierung wurden unterschiedlich exprimierte Gene in der *HvHeading*-Region in einem Blütezeit-spezifisch-nah-isogenen Paar von DH-Linien identifiziert. Diese Ergebnisse führten zur Verfeinerung des *HvHeading*-Intervalls auf <8,50 Mbp und zur Identifizierung der Hochregulation des *Spt6*-Gens in der verzögert blühenden DH-Linie. Eine differentielle Genexpressionsanalyse mittels RT-qPCR validierte die Hochregulation des *Spt6*-Gens, die vor dem Doppelkammstadium begann, und zeigte eine Herunterregulation von *Ppd-H1*, *Vrn-H1* für die verzögert blühende DH-Linie. Der grösste Teil der Promotorregion des *Spt6*-Gens in einem Blütezeit-spezifisch-nah-isogenen Paar von DH-Linien war sequenziert und zeigte viele Mutationen. Zusätzlich zeigte der Vergleich der sequenzierten Transkriptionen des *Spt6*-Gens mit veröffentlichten *Spt6*-Isoformen in Gerste, dass die DH-Linie mit dem Danubien-Haplotyp eine neue Isoform produzieren könnte. Die dritte Studie wurde durchgeführt, um die Wirkung der Blütezeit genetischer Regionen auf ertragsbezogene Merkmale, einschließlich der Kornertragskomponenten, unter verschiedenen Umweltbedingungen zu bewerten. In dieser Studie wurde der gleiche Satz von 534 MAGIC DH-Linien verwendet und sieben Merkmale unter gut bewässerten und endständigen Trockenheitsbedingungen bewertet. Die

Analyse der QTL für jede Behandlung, der maker by treatment interaction (M×T) und der QTL für die Dürretoleranz wurde durchgeführt. Die Ergebnisse zeigten, dass alle Merkmale bis auf die Tage bis zur Überschrift durch die Behandlung beeinflusst wurden. Insgesamt wurden 69, 64 und 29 QTL unter gut bewässerter und terminaler Dürrebehandlung bzw. Dürretoleranz gefunden. Die M×T-Analyse ergab insgesamt 25 Loci für vier Merkmale. Die identifizierten QTL umfassten Loci, die mit bekannten Genen/QTL ko-loziert waren, sowie neue Regionen. Die Ergebnisse deckten genetische Regionen für verschiedene Merkmale auf, die mit Blütezeitloci unter gut bewässerter und terminaler Dürre zusammenfielen, was auf einen pleiotropen Effekt von Blütezeitgenen wie *Ppd-H1*, *Vrn-H1*, *Vrn-H3* und *denso/sdw1* auf Kornertrag und Pflanzenentwicklung hindeutet. Einige entdeckte QTL zeigten eine günstige Wirkung auf Kornzahl, Ährenzahl und Korngewicht unter der Behandlung von terminaler Trockenheit, einschließlich QTL, das dem Hauptblütezeitgen Ppd-H1 für das Korngewicht entspricht. Die MAGIC-Population der Sommergerste ist ein wertvoller Aktivposten, der eine aussagekräftige Kartierung der Blütezeit, der QTL und der Epistase unter verschiedenen Umweltbedingungen einschließlich neuartiger Regulatoren ermöglichte; diese Ergebnisse können auf komplexere Kulturpflanzen wie Weizen übertragen werden. Die Validierung der Wirkung von epistatischem QTL *HvHeading* und die Identifizierung des *Spt6*-Gens als Kandidatengen durch einen gezielten Ansatz zur Eliminierung von Hintergrundeffekten auf der Basis von Epistase zeigte, dass die Kartierung von Epistaseinteraktionen bei der Zusammenstellung von Strategien zur Erleichterung der Genidentifizierung helfen kann. Die Rolle des Kandidatengens *Spt6* im Blütezeitverlauf von Gerste sollte validiert und weiter untersucht werden. Der Nachweis eines günstigen pleiotropen Effekts der Blütezeitloci auf die Korngewichtskomponenten unter terminaler Dürre deutete auf die Möglichkeit hin, dass die Blütezeitgene zur Verbesserung dieser Merkmale unter extremen Umweltbedingungen durch den Zeitpunkt der Blüte oder oder ihren Beitrag zu den Entwicklungswegen nützlich sein könnten. Die Ergebnisse zeigten, wie wichtig es ist, die Epistase und die Interaktion mit der Umwelt zu untersuchen, zusätzlich zu gemeinsame Ansätze wie der QTL-Analyse zu erforschen, um die Anpassung der Blütezeit an verschiedene Umgebungen bei Gerste zu untersuchen. Die in dieser Dissertation vorgestellte Reihe von Studien gibt neue Einblicke in die umweltabhängige Regulierung der Blütezeit bei Gerste durch einen multidisziplinären Ansatz, der die Bedeutung der Anwendung von Ansätzen unterstreicht, die komplexe Merkmale in zukünftigen Forschungs- und Züchtungsprogrammen besser erklären.

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Chapter 1

General introduction

General introduction

Flowering time plays a critical role in adaptation of crops to different environments and is a result of the coordination between gene networks and environmental cues (Blümel *et al.*, 2015). This trait has major importance for spread of crops into new environments and has been frequently targeted during breeding process to adapt the wild ancestors of modern cultivars (Ross-Ibarra *et al.*, 2007). Timing of flowering is one of the key determining factors of yield production.

Cereals are among the major sources for food and feed world-wide. Barley is the fourth most abundant cereal with a yearly production of ca. 170 Mt in 2018/2019 (FAO 2019 http://fao.org/faostat; Figure 1.1). From global production, approximately 75% is consumed as animal livestock feed, 20% is malted and 5% is used for food products (Blake *et al.*, 2011). Barley (*Hordeum vulgare* L.) belongs to the tribe of Triticeae in the family of grass, Poaceae (von Bothmer *et al.*, 2003) and as one of the earliest domesticated crops, had prominent role in the development of early agriculture. It was domesticated 10,000 years ago from its wild relative *H. vulgare* L. ssp. *spontaneum* (C. Koch) Thell. during the Neolithic revolution (Harlan and Zohary, 1966; Wang *et al.*, 2015). Barley is established as an experimental model for small grain cereals, especially temperate cereals such as wheat, to understand genetic pathways and the mechanism of increasing yield (Mayer *et al.*, 2012). Flowering time in barley is extensively studies, however its mechanism in response to environment is still not well understood; particularly compared to other model crops

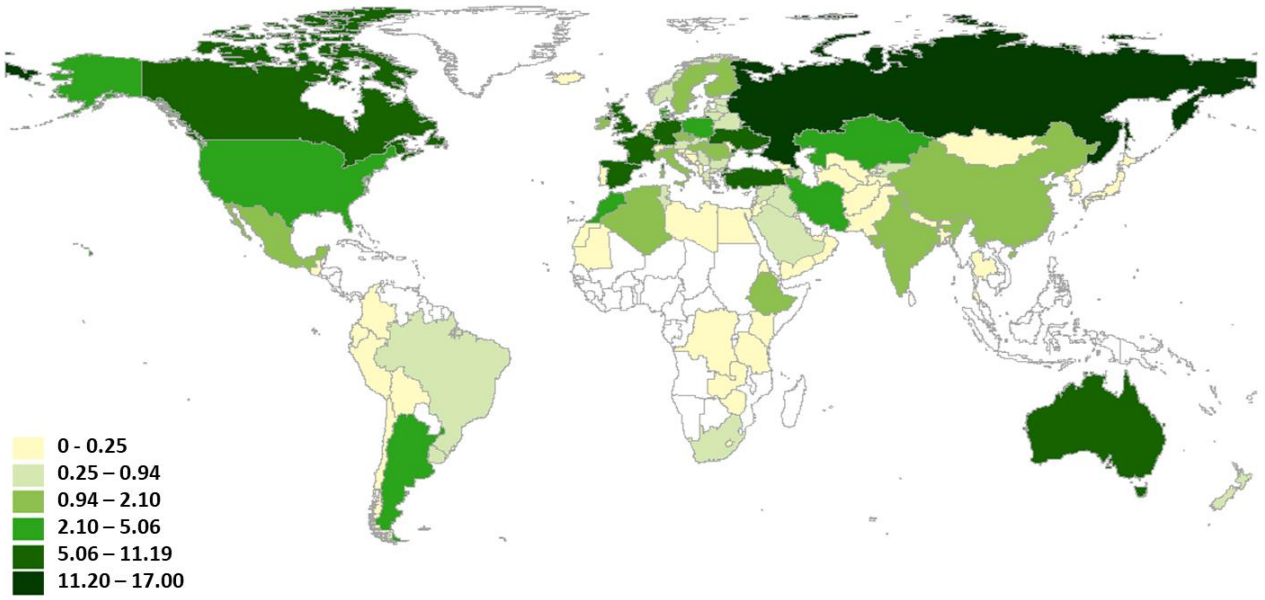

Fig. 1.1. World map showing countries according to their barley production (million tonnes) in 2018/2019 based on data from the Food and Agriculture Organization Corporate Statistical Database (www.fao.org/faostat/).

such as *Arabidopsis thaliana*. Flowering time mechanism has crucial role in barley adaptation to different environment and impacts yield (Blümel *et al.*, 2015). Due to ability of barley to adapt to various environmental conditions, it is more stress tolerant to cold, drought, alkalinity and salinity compared to wheat and can survive better under extreme environmental condition (Colmer *et al.*, 2006; Kosová *et al.*, 2014).

1.1 Barley adaptation and timing of flowering

The successful expansion of barley cultivation from its origin, the Fertile Crescent in middle east (Figure 1.2), to other regions around the world is due to its strong capability for adaptation to different environments including marginal conditions (Wallace *et al.*, 2019). It has adapted to cold

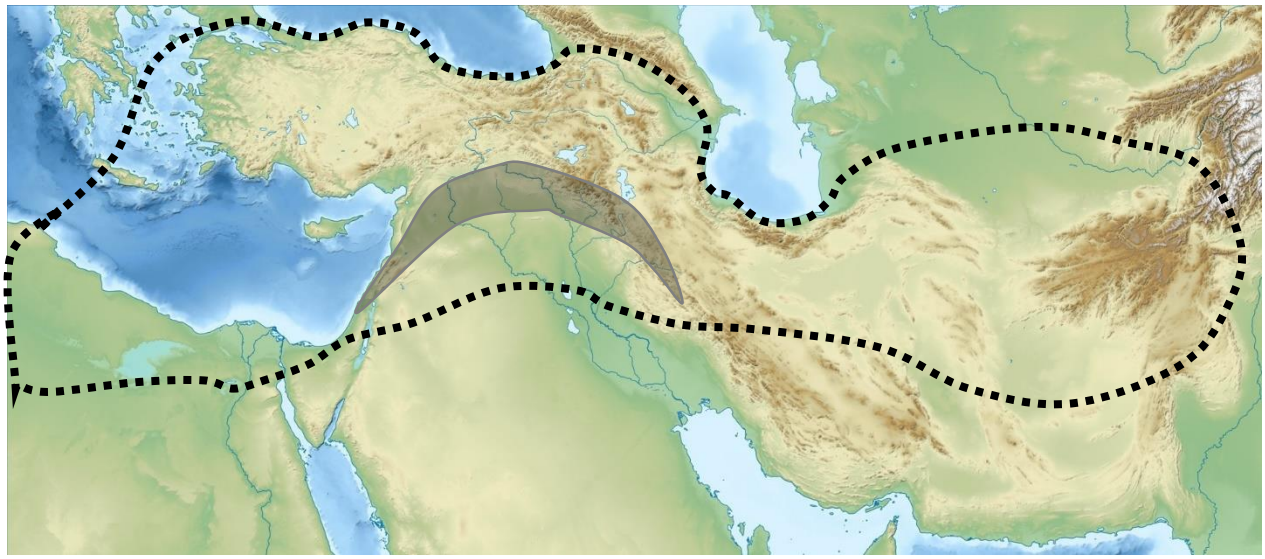

Fig. 1.2. Early domestication of cultivated barley (*Hordeum vulgare* ssp. *vulgare*) is originated from the Fertile Crescent in the Middle East, marked here as the grey area. The distribution of the wild progenitor of barley (*Hordeum vulgare* ssp. *spontaneum*) is indicated within the black line. The Figure is adapted from von Bothmer *et al.*, (2003).

temperatures by vernalization requirement which prevents flowering in winter. After completion of vernalization, response of plants to photoperiod allows flowering in response to long days and therefore barley can take advantage of longer vegetative phase in long growth seasons (Putterill *et al.*, 2004; Trevaskis *et al.*, 2007). A gradation is observed from extreme winter to typical spring type in barley which is due to genetic variation in the vernalization and photoperiod pathways which played an important role in barley adaptation to temperate climates (Casao *et al.*, 2011).

Barley has developed three major growth habits due to requirement of vernalization and photoperiod; including winter, spring and facultative. Wild barley (*Hordeum vulgare* ssp. *spontaneum*) has winter growth habit and sensitivity to long day (LD) photoperiod. The distribution

of wild barley extends over the Eastern Mediterranean basin and Western Asiatic countries (Schmid *et al.*, 2018). Flowering in plants with winter growth habit depends on fulfillment of vernalization requirement by prolonged exposure to cold temperature. Spring barley does not require vernalization and the facultative type has varying sensitivity to vernalization (Von Zitzewitz *et al.*, 2005). The distribution of winter and spring type genotypes in the Fertile Crescent coincides with the continental weather patterns from west and south to direction of east (Casao *et al.*, 2011; Nitcher *et al.*, 2013; Dawson *et al.*, 2015). Winter type genotypes have been selected and improved for cold resistance for cultivation in northern latitudes (Cockram *et al.*, 2007). Spring type genotypes have been selected and bred for sowing in spring and have a reduced photoperiod response to take advantage of the long growing season and late flowering which allows barley crops to exploit an extended vegetative period (Dawson *et al.*, 2015). This type is more common in regions with mild winters such as coastal areas and southern parts of the Fertile Crescent (Casao *et al.*, 2011; Nitcher *et al.*, 2013; Dawson *et al.*, 2015).

1.2 Flowering time and its role as determinant of yield in barley

The timing of flowering is the concluding step of an elaborate genetic and molecular network composed of endogenous epi-genetic factors and their interaction with environmental cues. It divides plant's development to pre-anthesis and post-anthesis period and marks transition of plant's life cycle from vegetative to reproductive phase (Blümel *et al.*, 2015).

Barley is a long-day (LD) plant and its flowering time is controlled by different regulatory mechanisms such as photoperiod and vernalization that correspond to environmental cues and earliness *per se* (*Eps*) that works independently from environment (Cockram *et al.*, 2007). Temperature, day length (photoperiod) and sensitivity to cold temperatures (vernalization) are known as the main controllers of barley phenology (Ibrahim *et al.*, 2016; Gol *et al.*, 2017) and have a direct influence on development, adaptation and geographic distribution of cultivars (Boyd, 1996). Plants response to environment is controlled genetically and determines the duration and timing of different developmental phases independent from each other (González *et al.*, 2005; Whitechurch *et al.*, 2007).

Timing of flowering determines the duration of pre-anthesis and post-anthesis development and therefore, can critically effect major yield components such as grain number and grain yield (Ibrahim *et al.*, 2018). Pre-anthesis includes vegetative and early reproductive sub-phases and post-

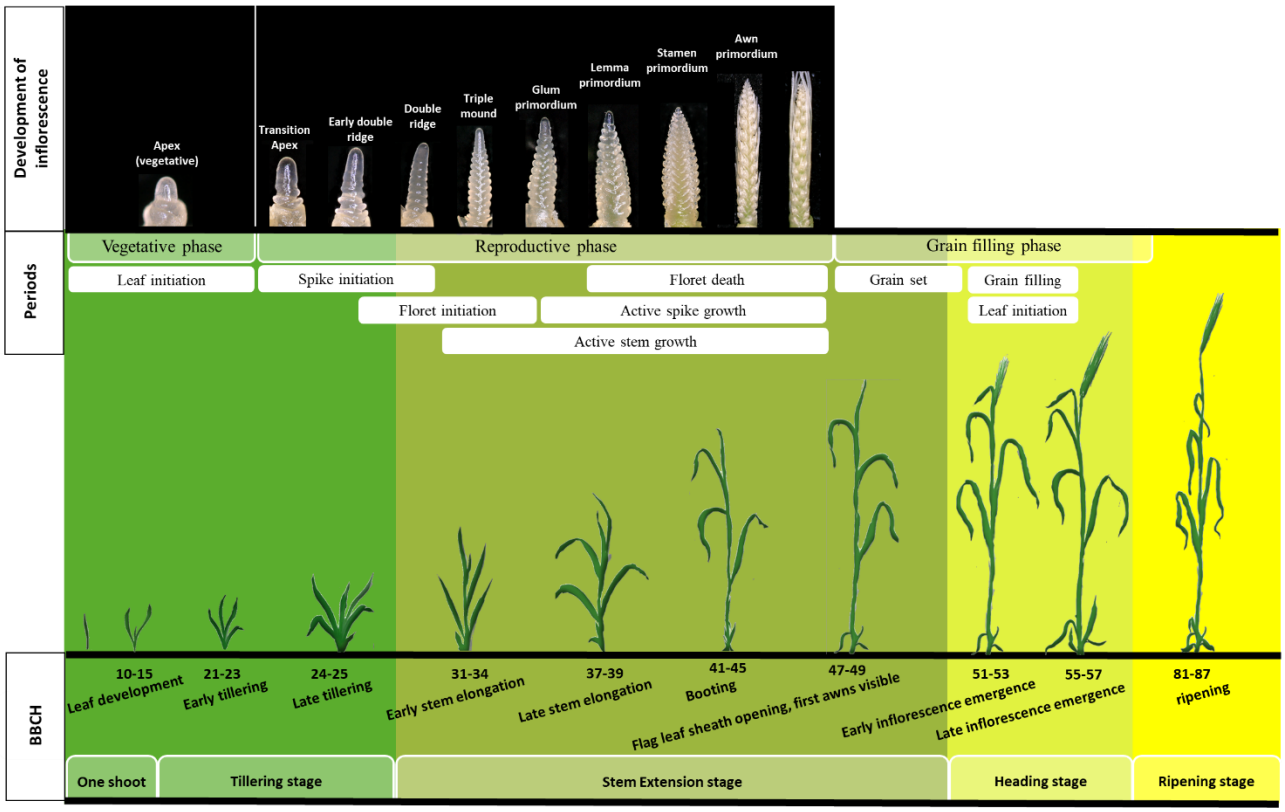

Fig. 1.3. Developmental stages of barley and barley floral meristem. The scale used for describing the development of barley and barley floral meristem are according to BBCH scale (Hack *et al.*, 1992) and Waddington scale (Waddington *et al.*, 1983) respectively.

anthesis is composed of late reproductive or grain-filling sub-phases (Sreenivasulu and Schnurbusch, 2012) (Figure 1.3). Grain number and single grain weight are determined during early reproductive stage and late reproductive stage, respectively (Borràs *et al.*, 2009; Sreenivasulu and Schnurbusch, 2012; Alqudah and Schnurbusch, 2014). Number of leaves, tillers and ears are determined during vegetative phase (del Moral and del Moral, 1995). Vernalization majorly affects timing of flowering by determining the duration of the vegetative phase (Roberts *et al.*, 1988; González *et al.*, 2002; Griffiths *et al.*, 2003). Long days shorten the vegetative phase in spring barley and vernalized winter barley, and speed up the late reproductive phase of stem elongation and inflorescence development (Roberts *et al.*, 1988; Miralles and Richards, 2000; Digel *et al.*, 2015*a*, 2016). Flowering time genes that function in photoperiod and vernalization pathways indirectly determine production and partitioning of dry matter as well as yield components by influencing the duration of crop developmental phases (Boyd, 1996).

In water limiting conditions, duration of stem elongation period can decrease and competition between spike and stem for limited assimilates during the maximum stem and spike growth phase

might lead to abortion of floret primordia in barley and wheat (Miralles *et al.*, 2000; González *et al.*, 2002, 2011; Ghiglione *et al.*, 2008). The number of fertile floret is correlated to the spike dry weight at anthesis and sets the final grain number and eventually grain yield (Miralles *et al.*, 2000; Reynolds *et al.*, 2009). Therefore, phenological adjustment is one of the key factors for development of adaptation strategies to extreme events such as heat and drought and contributes to genotype × environment interactions in barley (Ibrahim *et al.*, 2016). The post-anthesis phase can mostly affect yield by determining the duration of grain-filling period (Evans, 1976; Egli, 2004), which determines grain weight, as a major yield determining factor (Distelfeld *et al.*, 2014). Therefore environmental factors that control timing of flowering can influence different developmental stages that affect yield in barley (Miralles *et al.*, 2001; McMaster and Wilhelm, 2003; Hossain *et al.*, 2012); and the sensitivity to environmental factors could vary among stages and genotypes (Voltas Velasco, 1998). BBCH-scale is commonly used to identify phenological development stages of cereals including barley (Hack *et al.*, 1992). Shoot apex and inflorescence

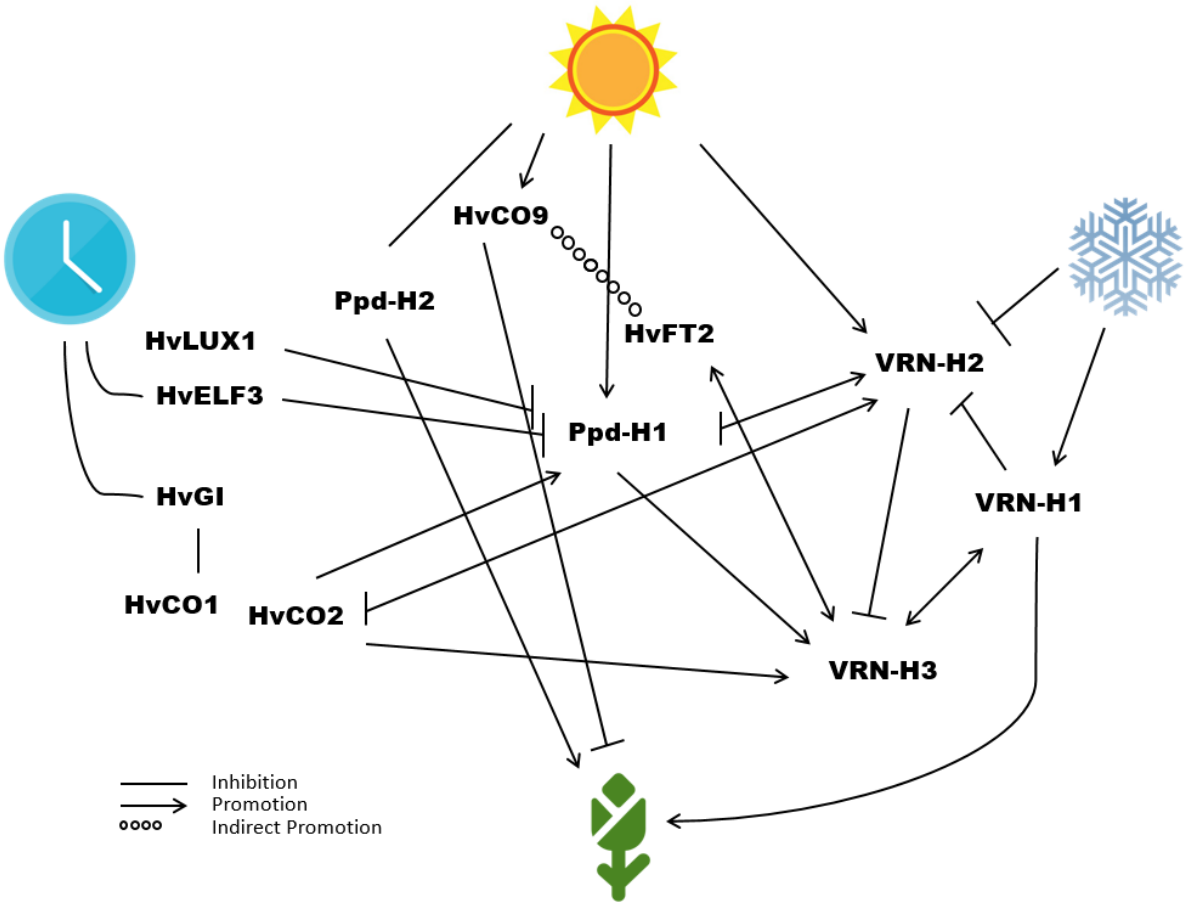

Fig. 1.4. An overview of major barley flowering time genes and their role in the flowering time pathway (Dubcovsky *et al.* 2005; Turner *et al.* 2005; Campoli *et al.* 2012; Wang *et al.* 2010; Zakhrabekova *et al.* 2012).

growth in barley is quantified according to Waddington scale (Waddington *et al.*, 1983) (Figure 1.3).

1.3 Flowering time genes and floral pathways in barley

Genetic pathways such as photoperiod, vernalization and circadian clock regulate timing of flowering in response to environment in barley. There are other genetic pathways that are reported to control this trait independent of environment such as earliness *per se* (*Eps*) (Cockram *et al.*, 2007). Adjustment of flowering time to environmental cues is a result of complex interaction of environment with flowering time genetic pathway as well as interaction among genes within the pathway.

1.3.1 Photoperiod pathway

Photoperiod pathway is one of the major regulators of flowering time in barley which is responsible for plant's response to photoperiod (Figure 1.4). A major gene that have been detected to control this pathway is *Ppd-H1 (PHOTOPERIOD RESPONSE LOCUS1)*, which is a pseudo-response regulator gene (*HvPRR37*) located on short arm of chromosome 2H and promotes flowering under long day (LD) condition. *Ppd-H1* is orthologous to the circadian clock gene *PRR7* in *Arabidopsis* (Turner *et al.*, 2005). *Ppd-H1* interacts with *CONSTANS* (*CO*) in the circadian clock oscillator and up-regulates expression of *Vrn-H3 (HvFT1)* (Turner *et al.*, 2005). The dominant *Ppd-H1* allele is associated with early flowering under LD in winter and wild barley which are mostly adapted to Middle East and Mediterranean regions and have winter growth habit. Mutations in the conserved CCT-domain or the sixth exon of the gene were linked with reduced sensitivity to LD and are present in spring barley genotypes which are adapted to Northern European regions (Turner *et al.*, 2005; Jones *et al.*, 2008). The other major photoperiod gene is *Ppd-H2* (*HvFT3*), which is detected on 1H chromosome and promotes flowering under short day condition. In genotypes that have not fulfilled vernalization requirement, role of *Ppd-H2* in controlling flowering time, is an adaptation mechanism to mild winters (Casao *et al.*, 2011). The dominant functional allele of *Ppd-H2* is found in Southern European barley germplasm and promotes faster flowering under short days and independent of vernalization requirement (Cuesta-Marcos *et al.*, 2008; Casao *et al.*, 2011). The recessive allele is common in winter barley, carries a partial deletion and has a loss of function (Faure *et al.*, 2007; Kikuchi *et al.*, 2009). *Ppd-H2* interacts with vernalization pathway and its expression is repressed by *Vrn-H2* (Yan *et al.*, 2006; Casao *et al.*, 2011). In addition to *HvFT1* and *HvFT3*, other *FT*-like genes detected in barley are *HvFT2*, *HvFT4* and *HvFT5* (Faure *et al.*, 2007).

1.3.2 Vernalization pathway

Vernalization pathway is majorly controlled by epistasis (gene-gene interaction) among three major vernalization genes *Vrn-H1*, *Vrn-H2* and *Vrn-H3* (Figure 1.4). The gene that promotes flowering after fulfillment of vernalization requirement is *Vrn-H1* which is a positive regulator in response to temperature (Distelfeld *et al.*, 2009). This gene is located on the long arm of chromosome 5H and encodes an *APETALA1* family MADS-box transcription factor, highly similar to the *Arabidopsis* meristem identity genes *APETALA1*, *CAULIFLOWER*, and *FRUITFUL* (Yan *et al.*, 2003). Barley genotypes with winter growth habit require vernalization to promote flowering and carry the recessive winter *Vrn-H1* allele that is expressed after exposure to cold. The dominant *Vrn-H1 allele* in spring barley carries deletions in its first intron and promotes flowering time independent of vernalization (Hemming *et al.*, 2009). The other major vernalization gene *Vrn-H3*, a homolog of *Arabidopsis thaliana FLOWERING LOCUS T* (*FT*), is up-regulated by *Vrn-H1* and is a promoter of flowering time. *Vrn-H3* locus is on the short arm of chromosome 7H in barley. SNP polymorphisms in *Vrn-H3* gene have been associated with differentiating the alleles as recessive or dominant (Yan *et al.*, 2006). *Arabidopsis FT* gene produces florigen hormone in leaves and moves to the shoot apical meristem to promote apex transition from vegetative to reproductive growth (Corbesier and Coupland, 2006). In rice *Hd3a* gene is orthologous to *FT* gene which also moves from the leaf to the shoot apical meristem (Tamaki *et al.*, 2007). *Vrn-H2* is the vernalization gene up-stream of *Vrn-H3* and has a repressing effect on flowering. It is encoded by a *ZCCT1* and its predicted protein contains a zinc-finger and CCT domain which is found in *CO*, *CO*-like and *TOC1* genes and has no clear orthologues in *Arabidopsis* (Cockram *et al.*, 2015*a*). *Vrn-H2* is located on long arm of barley chromosome 4H (Yan *et al.*, 2004).

Vernalization response in barley is predominantly controlled by genetic variation at *Vrn-H1* and *Vrn-H2* genes (Trevaskis *et al.*, 2007*b*). In photoperiod-sensitive winter barley, up-regulation of *Vrn-H3* by *Ppd-H1* is suppressed by *Vrn-H2* before winter; to stop the plants from flowering in cold season and protect sensitive organs. As the plants are exposed to cold and vernalization is fulfilled, the expression of *Vrn-H2 is* down-regulated by *Vrn-H1*. Therefore it promotes inflorescence meristem identity at the shoot apex and accelerates inflorescence initiation by up-regulating *Vrn-H3* expression under LDs (Yan *et al.*, 2006; Hemming *et al.*, 2008; Campoli *et al.*, 2012*b*). Deletion of *Vrn-H2* gene results in decrease or elimination of vernalization requirement in spring barley (Cockram *et al.*, 2007).

1.3.3 Circadian clock

Circadian clocks act as endogenous time-keeping mechanisms which synchronize plant biological processes to day length using molecular mechanisms (Dodd *et al.*, 2005). The major role of *Ppd-H1* in interaction with *CONSTANS* (*CO*) to promote flowering suggests that the circadian clock plays an important role in the control of flowering time in barley (Faure *et al.*, 2012). *Ppd-H1* and *HvCO1* can both promote flowering by up-regulating *Vrn-H3* (*HvFT1*) (Turner *et al.*, 2005; Campoli *et al.*, 2012*a*) (Figure 1.4).

Circadian clock genes of barley and their functions are mainly conserved in cereals compared to *Arabidopsis thaliana* (Campoli *et al.*, 2012*b*). However, clock genes such as *PRR* genes have independent duplications or deletions in eudicots and monocots. In monocots one homolog of the two paralogous *Arabidopsis* clock genes *LHY/CCA1* is detected (Takata *et al.*, 2010; Campoli *et al.*, 2012*b*). Also, *Ppd-H1,* the orthologue of *Arabidopsis PRR* genes, is a major mediator of plant response to long day photoperiod based on natural variation (Turner *et al.*, 2005). However natural variation in *Arabidopsis PRR* genes did not have a strong effect on flowering time (Ehrenreich *et al.*, 2009). Nine orthologues of the *Arabidopsis CO* gene (*AtCO)* were reported in barley including *HvCO1* and *HvCO2* that show strongest similarity to *AtCO*. *HvCO1* gene is the positional orthologue of *Hd1*, which is a major gene responsible for photoperiod sensitivity in rice (Griffiths *et al.*, 2003; Higgins *et al.*, 2010). In barley, *HvGI* is the single orthologue of clock protein *GIGANTEA* (*GI*) which controls *CO* transcription in *Arabidopsis*; however, whether they have the same function still is not confirmed (Fowler *et al.*, 1999; Dunford *et al.*, 2005).

The *EARLY FLOWERING3* (*ELF3*) gene is located on barley 1H chromosome and regulates flowering under the influence of photoperiod (Boden *et al.*, 2014). The recessive allele of this gene (*elf3*,*eam8, mat-a*) promotes early flowering independent of photoperiod (Faure *et al.*, 2012; Boden *et al.*, 2014). Another circadian clock locus, QTL *EAM5*, is located on chromosome 5H and a photoreceptor family gene, *HvPHYTOCHROME C* (*HvPHYC*), is reported as the candidate gene (Pankin *et al.*, 2014). A mutation at *HvPHYC* is reported to effect heading day under long days (Nishida *et al.*, 2013).

1.3.4 **Earliness *per se***

Earliness in intrinsic or per se pathway is reported to fine tune flowering time independent of environment or independent of vernalization and photoperiod pathways (Bullrich *et al.*, 2002; Lewis *et al.*, 2008; Zikhali and Griffiths, 2015). When the requirements of vernalization and

photoperiod are fulfilled, this pathway contributes in determining the time and duration of reproductive phases (Hoogendoorn, 1985; Worland and Law, 1986; Slafer, 1996; Appendino and Slafer, 2003; Zikhali *et al.*, 2014). Therefore, it has an important role in optimizing flowering time and reproductive developmental stages in barley and wheat (Slafer, 2003; Lewis *et al.*, 2008; Griffiths *et al.*, 2009) and is mainly driven by temperature (Slafer and Rawson, 1994, 1995*a*,*b*). Several regions have been associated with *Eps* pathway in barley and wheat (Scarth and Law, 1984; Hoogendoorn, 1985; Miura and Worland, 1994; Laurie DA, Pratchett N, Snape JW, 1995; Worland, 1996; Miura *et al.*, 1999; Bullrich *et al.*, 2002; Griffiths *et al.*, 2009; Gawroński and Schnurbusch, 2012; Zikhali *et al.*, 2014; Zikhali and Griffiths, 2015). Recently few genes underlying *Eps* QTL have been identified (Comadran *et al.*, 2012; Faure *et al.*, 2012; Zakhrabekova *et al.*, 2012; Gawroński *et al.*, 2014) including *HvCEN*, the candidate gene for *Eps2* QTL on chromosome 2H of barley (Laurie *et al.*, 1995). This gene is a homolog of the *Antirrhinum* gene *CENRORADIALIS* (*CEN*) (Comadran *et al.*, 2012). In *Antirrhinum*, mutations promote wild type indeterminate inflorescence to terminate into flowering (Bradley *et al.*, 1996).

1.4 Pleiotropic effects of flowering time genes on yield components

Genes involved in flowering time mechanism can contribute to genetic pathways that control basic developmental mechanisms and grain yield components (Drosse *et al.*, 2014). Major flowering time genes such as photoperiod gene *Ppd-H1* and vernalization genes, *Vrn-H1* and *Vrn-H3* are reported to have pleiotropic effects on plant development and grain yield and have a favorable effect on yield and yield components under harsh environment (Wang *et al.*, 2010; Wiegmann *et al.*, 2019). For instance, a genetic region associated with stem elongation and inflorescence development in barley was detected near *Vrn-H1* in a barley mapping population (Chen *et al.*, 2009). On the other hand, variation in *Ppd-H1* gene and over-expression of *HvCO1* are also reported to be linked with mediating the stem elongation phase and inflorescence development (Campoli *et al.*, 2012). *Eps* QTL are reported to have an impact on grain yield in wheat by affecting timing and duration of reproductive phases as well as spikelet number (Lewis *et al.*, 2008; Griffiths *et al.*, 2009) and grain protein (Herndl *et al.*, 2008).

1.5 Molecular markers

Molecular markers play an important role in genetics and are essential to study and understand genomes and complex traits. They are very popular tools due to their stability, cost-effectiveness and ease of use for applications such as genome mapping, gene cloning and marker assisted plant

breeding, genome fingerprinting and investigating genetic relatedness. The genotyping techniques are different regarding costs, work labor, range of use and repeatability as well as the population and aim of application. Genetic markers function based on restriction enzymes or two priming sites created by DNA polymorphisms in the nucleotide sequences of genomic regions (Bernardo, 2008). Since their introduction, different types of molecular markers were developed and implemented; such as RFLP (co-dominant restriction fragment length polymorphism) (Botstein *et al.*, 1980), RAPD (random amplified polymorphic DNA) (Williams *et al.*, 1990), AFLP (amplified fragment length polymorphism) (Vos *et al.*, 1995), SSR (single sequence repeat) (Morgante and Olivieri, 1993), DArT (diversity array technology) (Jaccoud *et al.*, 2001) and SNP (single nucleotide polymorphism) (Sachidanandam *et al.*, 2001). For mapping purposes type of markers, the coverage and density of markers on map should be considered (Liu, 1997).

SNP is one nucleotide base difference in two DNA sequences (Collins *et al.*, 1997). SNP markers (SNPs) are highly stable due to low mutation rate and their abundance in plants largely offers highest genetic resolution, which makes them very suitable for studying genomes and deciphering complex traits (Syvänen, 2001; Ching *et al.*, 2002; Schmid *et al.*, 2003; Yu *et al.*, 2005). Regarding barley, currently methods based on SNPs such as genotyping by sequencing (GBS) (Elshire *et al.*, 2011), 9K SNP array (Comadran *et al.*, 2012) and recently 50K SNP array (Bayer *et al.*, 2017) are considered mainstream genotyping platforms. Both GBS and SNP array approaches produce positively correlated data from the same population but they might cover different information from genome (Darrier *et al.*, 2019). Barley 9K SNP array and its successor 50K SNP array contain 7,864 SNPs (Comadran *et al.*, 2012) and 49,267 SNPs (Bayer *et al.*, 2017), respectively. As mainstream tool for genotyping barley in recent years, they have robust SNP calling and offer comparability of data between different populations and studies which is essential for QTL mapping (Comadran *et al.*, 2012; Bayer *et al.*, 2017; Darrier *et al.*, 2019). SNP arrays have been used for QTL mapping of flowering time genes in different types of barley population (Alqudah *et al.*, 2014, 2016; Maurer *et al.*, 2015, 2016; Sannemann *et al.*, 2015) and the comparable results of these studies show that a moderate number of SNP is still informative which is essential to confirm the well-known QTL as the proof of concept. The robust SNP calling procedures in SNP array provides solid datasets and allows a straight-forward data processing (Comadran *et al.*, 2012; Bayer *et al.*, 2017; Darrier *et al.*, 2019). GBS is technically demanding (Soto *et al.*, 2015) and creates a big amount of data that is time-consuming to process; but at the end only a small portion of it might turn out to be informative. In addition, the SNPs selected for the array are well characterized, with

a good genome wide distribution and well-defined map locations (Comadran *et al.*, 2012) which is not the case for GBS derived markers (Beissinger *et al.*, 2013; Darrier *et al.*, 2019).

1.6 QTL mapping

Quantitative trait locus (QTL) analysis is a statistical method that aims to bridge the trait phenotype (phenotypic data) with underlying genes (with genotypic data) in order to explain the role of genetic factors in phenotypic variance in complex traits (Falconer and Mackay, 1996; Kearsey, 1998; Lynch and Walsh, 1998). A quantitative trait is a measurable phenotype that is a result of cumulative influences of many genes and environmental factors (Abiola *et al.*, 2003). Most traits of interest in crops such as flowering time and yield are quantitative and polygenetically controlled, showing continuous variation within or between species (Falconer and Mackay, 1996) which makes their inheritance complex. A region of DNA that is associated with a quantitative trait is called "quantitative trait loci" (QTL) (Geldermann, 1975). The aim of QTL mapping (or gene mapping) is finding the regions on genome that are associated with the trait of interest. In plant breeding, a population is used to construct a linkage map and then associate phenotypic traits with genomic regions (McCough and Doerge, 1995). Classically, the first step for QTL mapping technique includes providing a mapping population which could be developed out of parents segregating for the trait of interest (QTL mapping) (Sannemann *et al.*, 2015) or by a collection of genotypes that show diversity regarding the trait of interest (association mapping) (Alqudah *et al.*, 2014). Second step is genotyping the population with polymorphic genetic markers to constructing a linkage map that shows the position of genetic markers relative to each other or their genetic or physical position. Next, correct and precise phenotyping of the traits of interest is performed. QTL can be mapped by linking phenotypic values (trait measurements) and genotypic data (usually molecular markers) (Mir *et al.*, 2012). Many studies have successfully performed QTL mapping or association mapping in barley using SNPs (Burris *et al.*, 1998; Cockram *et al.*, 2010; Comadran *et al.*, 2011*b*,*a*; Wang *et al.*, 2012; Alqudah *et al.*, 2014, 2016; Sannemann *et al.*, 2015; Wehner *et al.*, 2015; Maurer *et al.*, 2015, 2016; Pham *et al.*, 2019; Afsharyan *et al.*, 2020).

1.7 Statistical approaches for QTL mapping

Statistical approaches used for QTL mapping play an important role in the power of QTL detection including precise QTL mapping and reduction of false positive QTL (Akond *et al.*, 2019). The three popular methods are single-marker analysis (marker regression method; MR) (Broman *et al.*,

2003), simple interval mapping (SIM) (Lander and Botstein, 1989) and composite interval mapping (CIM) (Lander and Botstein, 1989; Li *et al.*, 2007).

Single-marker analysis uses methods including t-test, analysis of variance (ANOVA) and regression (Broman *et al.*, 2003). Its major advantage is not requiring a linkage map and is compatible with epistatic effects, different mapping populations, different experimental designs that include further factors such as environments, treatments (Tanksley, 1993; Collard *et al.*, 2005). However, it required a high marker density and its power decreases when markers are widely spaced (Broman *et al.*, 2003).

Simple Interval Mapping (SIM) is based on Maximum likelihood (ML) and multiple regressions which are the most widely used interval mapping approaches (Lander and Botstein, 1989). It is considered statistically more powerful compared to the single-marker method especially since the later needs high marker density. This method requires presence of a linkage map, assumes the presence of a single QTL, each locus is considered one at a time and analyses intervals between adjacent pairs of linked markers simultaneously. Within the interval, two adjacent markers at every locus are used and a likelihood ratio test (LRT) is conducted to examine the presence of a QTL (Lander and Botstein, 1989). The disadvantage to this method can be interfering effect of QTLs located elsewhere on the genome. This can influence the estimation of locations and effects of QTLs which might lead to detecting false positives (Lander and Botstein, 1989; Haley and Knott, 1992; Martinez and Curnow, 1992).

Composite Interval Mapping (CIM) is based on iterative genome scans to find QTL and adding them to the regression model as they are detected (Lander and Botstein, 1989; Jansen, 1993; Zeng, 1994; Li *et al.*, 2007). The advantage of this method to single-QTL approaches is that it estimates QTL position and effects size more precisely, particularly in case of small mapping populations and when linked markers are involved (Lander and Botstein, 1989; Li *et al.*, 2007). This method has limitations such as inability to calculate epistatic effects and genotype × environment interactions (Collard *et al.*, 2005).

1.8 Epistasis mapping

Epistasis is a term used to describe the interaction of alleles, or variants, at two or more loci (Phillips, 2008). Early studies mostly reported qualitative epistasis as loci masking the effect of other loci (Bateson and Mendel, 1909); afterwards quantitative epistasis was defined termed as "epistasy". In statistics, epistasis is the deviation from additive expectations of two or more loci

(Fisher, 1919). It is reported that quantitative epistasis explains a major part of genetic variance of the population of model plants such as *Arabidopsis* (Malmberg *et al.*, 2005; Kusterer *et al.*, 2007) as well as crop species including maize (Stuber and Moll, 1971; Melchinger *et al.*, 1986; Lamkey *et al.*, 1995; Wolf and Hallauer, 1997; Lukens and Doebley, 1999), rice (Yu *et al.*, 1997; Li *et al.*, 2008; Shen *et al.*, 2014) and barley (Maurer *et al.*, 2015; Mathew *et al.*, 2018; Afsharyan *et al.*, 2020) as well as making major contribution to evolution (Doebley *et al.*, 1995; Lukens and Doebley, 1999; Carlborg *et al.*, 2006; Phillips, 2008; Hansen, 2013; Doust *et al.*, 2014).

Difficulty of statistical detection of epistatic interactions is mainly due to large number of pairwise tests needed between markers; for instance, 100 markers would require 4,950 tests for pairwise epistasis (Santantonio *et al.*, 2019). Several approaches have been suggested to minimize the multiple testing problem and increase the epistasis detection efficiency such as performing genome-wide scans for variants with a significant additive effect and performing pairwise test only for them (Carlson *et al.*, 2004; Hill *et al.*, 2008). Other factors that can reduce the efficacy of pairwise marker epistasis detection are low allelic frequency, low linkage disequilibrium between markers and lower allele frequency as a result of more number of alleles (or haplotypes) at a given locus (Carlson *et al.*, 2004). Using linked SNP blocks as haplotypes can help to reduce the effect of these factors by increasing the power detection of additive and interaction effects by providing the possibility of accurately tracking larger segments of DNA composed of markers that are high linkage disequilibrium (LD), and determining multiple alleles at each position (Lin and Zeng, 2006; Zhang *et al.*, 2012; Jiang *et al.*, 2018).

1.9 Next generation sequencing approach: RNA-sequencing

Transcriptomes are functional element of genome and include a wide variety of RNA molecules including mRNA, miRNA, ncRNA, rRNA, and tRNA. Gaining insights into transcriptome regulation is very important to understand the functional genome and how RNA molecules control the physiological responses of crops to environmental factors (Shabalina and Spiridonov, 2004; Elgar and Vavouri, 2008; Blignaut, 2012; Mattick and Dinger, 2013).

Invention of Next Generation Sequencing (NGS) technology enabled parallel sequencing of millions of nucleotide fragments (Mardis, 2011). RNA Sequencing (RNA-seq) is an NGS approach that was introduced as a revolutionary tool for transcriptomics compared to other popular methods such as tiling microarrays (Yamada *et al.*, 2003; Bertone *et al.*, 2004; Cheng *et al.*, 2005; David *et al.*, 2006) and cDNA or EST sequencing (based on hybridization and sanger sequencing

respectively) (Boguski *et al.*, 1994; Gerhard *et al.*, 2004). It offered a powerful method to study genome-wide transcriptome characterization and profiling by high-throughput sequencing (Wang *et al.*, 2009). Regarding size of the produced data, RNA-seq data is much smaller than datasets produced by whole genome sequencing and its handling and interpretation is less complicated (Sánchez-Martín *et al.*, 2016). RNA-seq can provide data on rare transcripts, novel transcripts and gene expression; which is wider range of information compared to other NGS approaches which are limited to gene space in reference genomes such as exome capture sequencing (ExomeSeq) (Fu *et al.*, 2009; Mascher *et al.*, 2013*b*). Compared to array-based techniques, this method offers in-depth and more accurate data on transcriptome and does not require pre-designed complement sequence detection probe (Zhao *et al.*, 2014). The assembly of transcriptome can be performed de novo or based on available reference genome. After this step, downstream analysis to understand gene regulation can be conducted which includes expression profiling, gene ontology, and pathway enrichment analyses. Ensuring high productivity and reproducibility of RNA-seq data requires collecting samples from proper tissue and time-point, suitable sequencing depth and regulation of target genes at transcript level (Sánchez-Martín *et al.*, 2016).

1.10 Gene identification: Strategies to identify genes underlying QTL

One of the most challenging processes in plant genetics is identifying the gene(s) underlying a QTL and revealing the molecular function. This process traditionally includes fine-mapping the QTL and then positional cloning of the candidate gene(s) to reveal the gene underlying the QTL (Kumar *et al.*, 2017). In barley, a number of genes were cloned with success (Francia *et al.*, 2007; Sutton *et al.*, 2007; Furukawa *et al.*, 2007); however execution of this strategy remains time-consuming and labor-some particularly in large genomes such as barley and wheat. Recently, whole genome sequencing of a number of plant species (Chen *et al.*, 2002; Cao *et al.*, 2011) including barley was completed (International Barley Genome Sequencing Consortium, 2012). Availability of information regarding the physical gene space, has facilitated gene identification process by providing the possibility of searching QTL interval directly for all possible positional candidate genes also known as candidate gene approach (Monclus *et al.*, 2012; Bargsten *et al.*, 2014; Correa *et al.*, 2014; Yano *et al.*, 2016). However depending on the size of the QTL interval, the number of genes located in it could reach to tens to hundreds of genes from which one or few would be related to the trait of interest. Prioritizing the genes within the QTL interval for the trait could be performed based on sequence information, overrepresentation regarding the corresponding biological

processes or functional annotation of genes based on their orthologues in related or model species (such as gene ontology annotations approach) and differential expression analysis in transcriptome and/or proteome levels (Monclus *et al.*, 2012; Bargsten *et al.*, 2014; Kumar *et al.*, 2017; Gudys *et*

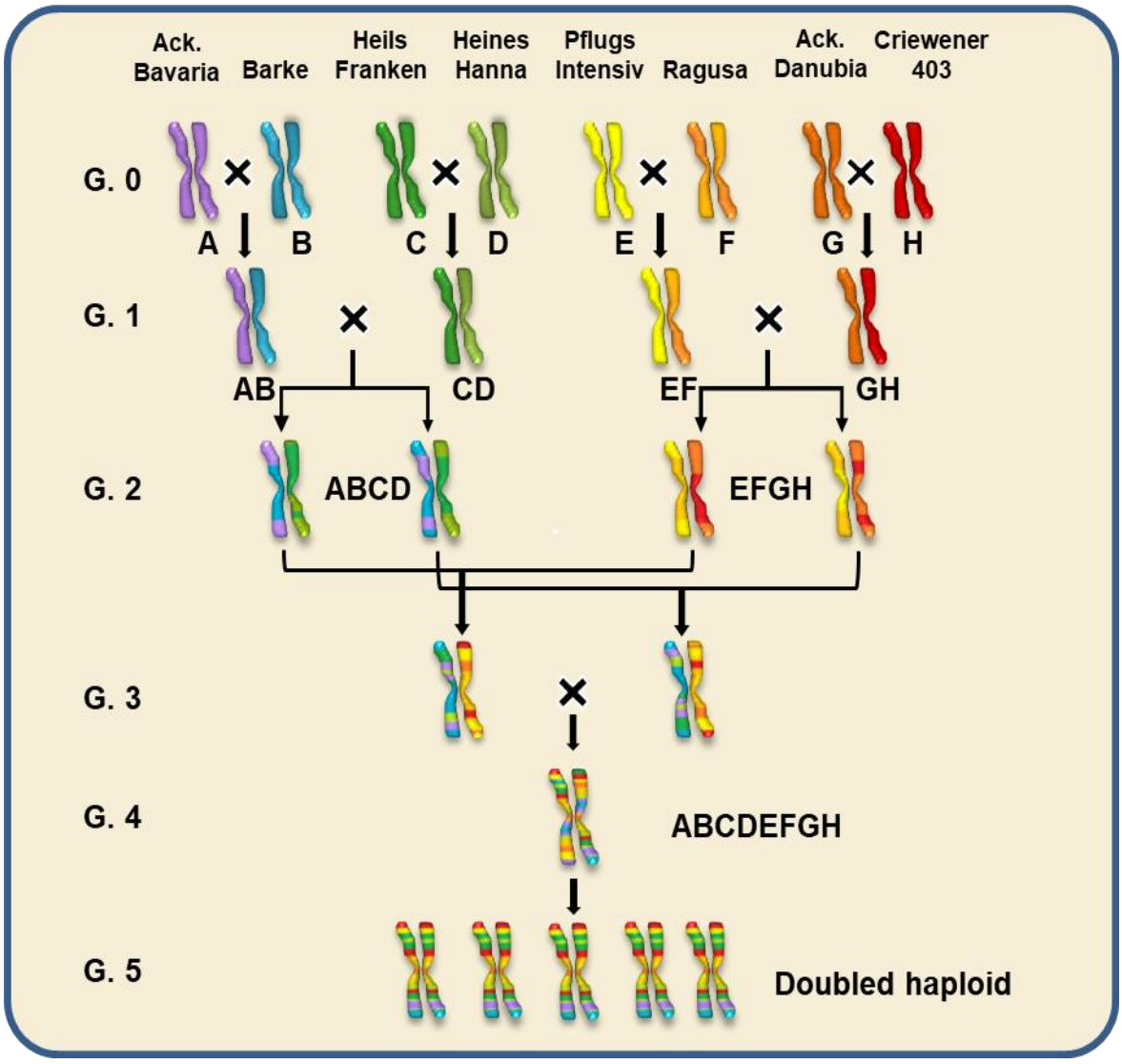

Fig. 1.5. Crossing scheme for spring barley Multiparent Advanced Generation InterCross (MAGIC) population developed by INRES plant breeding (Prof. Dr. Jens Léon), University of Bonn as described by Sannemann *et al.*, (2015).

al., 2018). When the candidate genes are selected, their effects on the phenotype of the trait of interest can be studied by approaches such as using TILLING knockout mutants (Talamè *et al.*, 2008) or by CRISPR-Cas gene editing (Kumar and Jain, 2015; Hamada *et al.*, 2018).

1.11 Multiparent mapping populations

Population derived from two parents known as bi-parental populations are traditionally used by plant molecular geneticists and plant breeders for variety development and QTL mapping for traits

of interest. However this approach provides limited genetic variation and the efforts for increasing the variations by performing multiple crosses is labor-intensive and large population sizes are needed to detect recombinants for traits of interest (Allard, 1960; Jensen, 1970).

Common populations used for QTL mapping are from bi-parental crosses such as F2, backcross (BC) or recombinant inbred (RI) populations. The limitations of using these populations are the presence of only two alleles in each locus and low genetic resolution for QTL mapping due to limited genetic recombination especially in F2 or BC populations (Huang *et al.*, 2009).

Multiparent Advanced Generation InterCross (MAGIC) strategy in plants were first developed in *Arabidopsis* as described by Cavanagh *et al.* (2008) to improve power and precision in QTL mapping by allowing interrogation of multiple alleles (Cavanagh *et al.*, 2008; Bandillo *et al.*, 2013). This approach aims to increase mapping power and resolution by taking advantage of linkage-based design and shuffling the genome by multiple generations of recombination (King *et al.*, 2012). Multiparental populations offer material that segregate for more traits based on parents involved in the cross; and increased level for recombination which improves the power of QTL mapping by elevating the precision and resolution (Cavanagh *et al.*, 2008). Development of high-throughput genotyping platforms and advancements in statistical methods to analysis multiparent populations has attracted more interest in them (Bandillo *et al.*, 2013).

Multiparental populations were successfully used to study complex traits in *Arabidopsis thaliana* (Kover *et al.*, 2009), spring wheat (Huang *et al.*, 2012), rice (Bandillo *et al.*, 2013), winter wheat (Mackay *et al.*, 2014) and barley (Sannemann *et al.*, 2015; Maurer *et al.*, 2016; Maurer *et al.*, 2015; Mathew *et al.*, 2018).

1.12 Research hypothesis and objectives

Response to environmental factors has an essential role in survival of barley. Timing of flowering can have major influence on yield and is controlled by endogenous genetic pathways as well as environmental factors, including day length, temperature, and stress. Environmental cues have major role in initiating the switch from vegetative to reproductive development and also influence later stages of inflorescence development towards flowering time (Blümel *et al.*, 2015; Cho *et al.*, 2017; Ibrahim *et al.*, 2018). Previous studies majorly investigated flowering time by focusing on QTL (Bezant *et al.*, 1996; Pillen *et al.*, 2003; Wang *et al.*, 2010; Alqudah *et al.*, 2014; Sannemann *et al.*, 2015; Maurer *et al.*, 2016) and there were few attempts to describe epistatic interactions in flowering time pathway of barley (Maurer *et al.*, 2016; Mathew *et al.*, 2018). Therefore, the effects

of epistasis and environment and their collective contribution to flowering time are not well understood. Investigating environment-dependent regulation of flowering time pathway can improve our understanding regarding its timing and the role of flowering time genes in plant development in response to environment and ultimately shed more light on its contribution to mechanisms that improve crop yield in barley. Furthermore, it can provide insights to uncover novel regulators that interact with the genetic pathway and environment. A spring barley MAGIC population (Figure 1.5) from an eight-way cross was constructed by Chair of Plant Breeding (Prof. Dr. Jens Léon), Institute of Crop Science and Resource Conservation, University of Bonn. This population was used in recent studies on flowering time which led to successful detection of QTL (Sannemann *et al.*, 2015) and epistatic effects (Mathew *et al.*, 2018) involved in barley flowering time control. Therefore, the aim of this thesis is to improve the understanding of the environment-dependent regulation of flowering time in barley. The following research hypothesis were addressed in this thesis:

1. MAGIC population is a tool to investigate the regulation of flowering time.
2. Timing of flowering is endogenously controlled by genes and their interaction (epistasis) and is influenced by environmental factors.
3. Influence of environment on gene as well as epistatic effects can be controlled at transcriptome level.
4. Flowering time genes have pleiotropic effects on yield-related traits.

Three major objectives of the present thesis were as follows:

1. To investigate if the MAGIC can be useful to analyze epistatic interaction and environment interaction.
2. To identify novel regulators involved with epistasis as well as environment influence.
3. To identify candidate gene(s) underlying novel flowering time QTL.

Chapter 2

Effect of epistasis and environment on flowering time in barley reveals a novel flowering-delaying QTL allele

Based on:

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Abstract

Flowering time is a complex trait and has a key role in crop yield and adaptation to environmental stressors such as heat and drought. This study aimed to better understand the interconnected dynamics of epistasis and environment and look for novel regulators. We investigated 534 spring barley MAGIC DH lines for flowering time at various environments. Analysis of quantitative trait loci (QTLs), epistatic interactions, QTL × environment (Q×E) interactions, and epistasis × environment (E×E) interactions were performed with single SNP and haplotype approaches. In total, 18 QTLs and 2420 epistatic interactions were detected, including intervals harboring major genes such as *Ppd-H1*, *Vrn-H1*, *Vrn-H3*, and *denso/sdw1*. Epistatic interactions found in field and semi-controlled conditions were distinctive. Q×E and E×E interactions revealed that temperature influenced flowering time by triggering different interactions between known and newly detected regulators. A novel flowering-delaying QTL allele was identified on chromosome 1H (named "*HvHeading*") and was shown to be engaged in epistatic and environment interactions. Results suggest that investigating epistasis, environment, and their interactions, rather than only single QTLs, is an effective approach for detecting novel regulators. We assume that barley can adapt flowering time to the environment via alternative routes within the pathway.

Keywords: Barley, environmental effect, epistasis, flowering time, MAGIC population, novel QTL, QTL analysis

Introduction

Flowering time indicates the transition from the vegetative to the reproductive phase in plants (Mouradov *et al.*, 2002), and as a major determinant for biomass accumulation and grain filling period length, affects grain yield. It was targeted during crop domestication and breeding to adapt wild ancestors of modern cultivars (Ross-Ibarra *et al.*, 2007). Barley (*Hordeum vulgare* ssp. *vulgare* L.) is the fourth most cultivated cereal worldwide and is consumed for food, feed, and the malting process (Schulte *et al.*, 2009). The flowering time regulatory network of barley, as a model for small-grain cereals, is relatively well described (International Barley Genome Sequencing Consortium, 2012). However, there is currently relatively little available information about the genes involved in this network, and their epistatic and environmental interactions, compared with the dicot model plant *Arabidopsis thaliana* (Blümel *et al.*, 2015). Flowering time regulators belong to a complex network of genes that interact with environmental cues (Putterill *et al.*, 2004). Focusing on the whole system of interacting factors, which includes genes and environment, could offer more informative solutions that provide new insights to identify novel regulators (Blümel *et al.*, 2015; Valentim *et al.*, 2015).

The multiparent advanced generation inter-cross (MAGIC) strategy was designed to improve power and precision in quantitative trait locus (QTL) mapping and overcome limitations of populations derived from biparental crossing systems by providing extensive genetic variance (Cavanagh *et al.*, 2008). MAGIC populations are constructed by linkage-based design and multiple generations of recombinations, which provide high genetic mapping power and resolution (King *et al.*, 2012). Common statistical methods for QTL and epistatic interactions using molecular markers such as single nucleotide polymorphisms (SNPs) have been used successfully with MAGIC populations in A. thaliana (Kover *et al.*, 2009), wheat (Huang *et al.*, 2012), rice (Bandillo *et al.*, 2013) and winter wheat (Mackay *et al.*, 2014). However, the involvement of more than two parents and their possible genetic similarities pose statistical challenges for analyzing the effects of parental alleles (Sannemann *et al.*, 2015). SNP data from progenies can be transformed to a recognizable parental pattern of content, also known as the "haplotype phase", for mapping purposes (Browning and Browning, 2011). Recent efforts have been made to use both single SNP and haplotype-phase analysis to provide sufficient mapping power (Sannemann *et al.*, 2015; N'Diaye *et al.*, 2017; Ogawa *et al.*, 2018). A spring barley MAGIC population was constructed using an eight-way cross of seven barley landraces and one elite cultivar. This population was used to study flowering time

QTL (Sannemann *et al.*, 2015) and epistatic interactions (Mathew *et al.*, 2018) in pot experiments, which detected regions harboring major flowering time genes such as *Vrn-H1* and *Vrn-H3*.

Flowering time in barley is regulated by photoperiod and vernalization as well as environment-independent pathways such as earliness *per se* (*Eps*) (Cockram *et al.*, 2007). Under long-day conditions, one of the key regulators that responds to photoperiod is *PSEUDO-RESPONSE REGULATOR* (*HvPRR37*), also known as *PHOTOPERIOD RESPONSE LOCUS1* (*Ppd-H1*). It functions in the circadian clock oscillator and is orthologous to clock gene *PRR7* in *Arabidopsis* and *osPRR37* in rice (Turner *et al.*, 2005). *Ppd-H1* interacts with *CONSTANS* (*CO*) and promotes flowering by initiating expression of *Vrn-H3*, a homolog of *A. thaliana FLOWERING LOCUS T* (*FT*) (Yan *et al.*, 2006). *Vrn-H3* functions at the intersection of three main pathways - vernalization, photoperiod, and circadian clock (Campoli *et al.*, 2012) - and is involved in the development of the reproductive apex and inflorescence (Faure *et al.*, 2007; Digel *et al.*, 2015*b*). Another major gene upstream of *Vrn-H3*, encoding an *APETALA1* family MADS-box transcription factor, *Vrn-H1*, is a positive regulator of flowering time in response to temperature (Distelfeld *et al.*, 2009) that induces flowering time by promoting the transition from the vegetative to the reproductive phase (Hemming *et al.*, 2008). Epistasis between *Vrn-H1* and *Vrn-H3*, and the effect of lower temperature on promoting *Vrn-H1*, is an example of flowering time control beyond the effect of single genes (Hemming *et al.*, 2008; Cockram *et al.*, 2015*b*). Epistasis is the term used to describe different levels of interactions among genes, including the functional interaction (protein level), allelic variation affecting the pathway (gene level), and deviations from additivity detected by statistical models (Phillips, 2008). Most mapping studies have successfully introduced novel regulators by focusing on single QTL (Bezant *et al.*, 1996; Pillen *et al.*, 2003; Wang *et al.*, 2010; Alqudah *et al.*, 2014; Sannemann *et al.*, 2015; Maurer *et al.*, 2016). Few reports have described epistatic interactions in the flowering time pathway of barley (Maurer *et al.*, 2015; Mathew *et al.*, 2018). Thus, the effects of epistasis and environment and their collective contribution to flowering time are not well understood. To provide more detailed insights into the flowering time regulation network in barley, using strategies beyond single QTL analysis is crucial.

The main aim of this study was to investigate the effect of epistasis and environment on flowering time in barley to detect novel mediators. Objectives of the study were (i) to investigate epistatic interactions under different environments in field and semi-controlled conditions, (ii) to investigate the effect of environment on the timing of flowering, by analyzing QTL × environment and

epistasis × environment interactions, and (3) to shed light on novel flowering time regulator(s) involved in epistatic and/or environment interactions.

Materials and methods

Plant material

The MAGIC population was constructed by inter-crossing eight barley (*Hordeum vulgare* ssp. *vulgare*) genotypes, including one plant from each of seven landraces, Ackermanns Bavaria IPK No. HOR 100 (Ack. Bavaria), Ackermanns Danubia IPK No. BCC 1427 (Ack. Danubia), Criewener 403 IPK No. HOR 62, Heils Franken IPK No. BCC 1433, Heines Hanna IPK No. HOR 59, Pflugs Intensiv IPK No. BCC 1441, and Ragusa IPK No. BCC 1359, and the elite cultivar Barke, in an eight-way cross. Then, double haploid (DH) lines were produced as described in Sannemann *et al.* (2015). Ragusa represents a facultative and the others are spring barley ecotypes. The landraces used in this cross have contributed as founders of German barley cultivars.

Experimental setup and phenotypic data

Data for days to heading were collected under field and semi-controlled conditions. Field trials were conducted in 2016 and 2017 at Campus Klein-Altendorf (50°36′46.6″N, 6°59′39.7″E) of the University of Bonn. The lines were sown on 11 April 2016 (mean temperature 12.14 °C), and 3 April 2017 (mean temperature 8.63 °C). An unreplicated experimental design with a check every third plot (Mangelsdorf, 1953; Warner, 1953; Federer, 1956*a*) was employed, which is the standard design used for early-generation field trials in breeding programs (Federer, 1956*b*, 1993). The DH lines were completely randomized. The eight parents and cultivar scarlet were also randomized as controls. Each DH line was sown in one row containing 10 plants. Each plot was 1 m × 1.5 m in size and contained six DH lines, and the space between plots was 1 m. Field trials were subjected to fertilization and pest management following local practices. All DH lines reached the heading stage and the flowered plants for each line were counted; 99% had at least four plants that headed and were considered for data collection. The number of heading plants per DH line was on average six or seven for both years. The setup for semi-controlled conditions for the experiments conducted in 2011 and 2012, which were done under a foil tunnel at Campus Poppelsdorf (50°43′34.1″N, 7°05′14.6″E) of the University of Bonn, is detailed in Sannemann *et al.* (2015). The sowing dates were 4 April 2011 (mean temperature 11.99 °C), and 3 April 2012 (mean temperature 12.51 °C). Days to heading (BBCH 49; Hack *et al.*, 1992) was scored as the number of days after sowing when at least 50% of plants of each DH line showed 3 cm of awns. The data for each year were

analyzed separately. Phenotypic data are provided in Supplementary Dataset 2.1 available at Dryad Digital Repository (https://doi.org/10.5061/dryad.g25cm28; Afsharyan *et al.*, 2020). To evaluate the effect of environment, the daily average temperature for 100 days after sowing was measured for the 4 sowing years (2011, 2012, 2016, and 2017) to calculate growing degree-days (GDD), using base temperature 3 °C (Schelling *et al.*, 2003), for each growing season (Supplementary Dataset 2.2).

DNA extraction and genotyping

DNA was isolated from each barley MAGIC DH line according to the protocol for Diversity Arrays Technology marker analysis (https://www.diversityarrays.com) and prepared as described by Sannemann *et al.* (2015). Then, DNA samples were genotyped using the 9k iSelect SNP array (Comadran *et al.* 2012) at TraitGenetics GmbH (Stadt Seeland OT, Gatersleben, Germany) (Supplementary Dataset 2.3). The processing of raw genotypic data was done as described by Sannemann *et al.* (2015). Genotypes with less than 10% missing values were included and missing data were imputed according to the mean imputation approach (Rutkoski *et al.*, 2013). Then, the genotyping dataset was constructed by eliminating markers with a minor allele frequency (MAF) of less than 1%. Finally, 5199 SNP markers were used for further analysis. Due to the inclusion of more SNPs in this study than in the study of Sannemann *et al.* (2015), the SNPs were haplotyped in two groups and then collected in one dataset. Construction of haplotype data using SNPs with MAF ≥5% was performed as described by Sannemann *et al.* (2015). The remaining SNPs (5%>MAF≥1%) were haplotyped by the K-means clustering method using Proc Fastclus in SAS 9.4 (SAS Institute Inc., Cary, NC, USA). The SAS script and sample data are provided in Supplementary Method 2.1. Then, manual corrections were made by comparing the phase patterns of parents and DH lines to improve data accuracy and resolution of the haplotype data. Finally, 4557 SNP markers with missing haplotype-phase data ≤15.5% were used for further analysis. In silico analysis was performed using the IPK barley BLAST server (https://webblast.ipk-gatersleben.de/barley_ibsc/) (Colmsee *et al.*, 2015).

Statistical analysis

Analysis of phenotypic data

Descriptive statistics was performed in R software (R core team, 2015) by using the following core generic functions in R: summary to calculate minimum, maximum, and mean; std.error, sd, and var to calculate standard error, standard deviation (SD), and coefficient of variation (CV),

respectively. Analysis of row and column effect was conducted in R with the lm function from the package stats by considering non-replicated MAGIC DH lines, row, column, and controls as fixed effects. The same R function was used to perform analysis of variance (ANOVA) for days to heading by taking DH lines and year as fixed effect. The GDD accumulation in the 100 days after sowing was compared among years by a paired Student's t-test. In addition, the relationship between days to heading and the respective GDD (flowering time GDD) of genotypes was tested by using Pearson's correlation coefficient (r). Variance components were estimated using the PROC VARCOMP procedure (all effects as random) in SAS 9.4. Heritability (H^2) was calculated as:

$$H^2 = \frac{V_G}{V_G + \frac{V_{GY}}{y} + \frac{V_E}{y}}$$

where V_G: variance of genotype, V_{GY}: variance of genotype × year, V_E: variance of experimental error, and y: number of years.

QTL mapping and QTL × environment interaction models

QTL analysis and QTL × environment interaction through single SNP analysis (single SNP approach; SA) and haplotype analysis (haplotype approach; HA) was conducted, using the PROC MIXED procedure in SAS 9.4, by the following linear model:

$$Y_{ij} = \mu + M_i + C_j + M_i \times C_j + \varepsilon_{ij}$$

where Y_{ij}: response variable; μ: general mean; M_i: the fixed effect of the ith marker genotype; C_j: the fixed effect of the jth calendar year; $M_i \times C_j$: the fixed interaction effect of the ith marker genotype with the jth calendar year; and ε_{ij}: the residual. To reduce the number of detected false positives, the multilocus procedure, as an efficient selection strategy, was implemented within the model (Sillanpää and Corander, 2002; Kilpikari and Sillanpää, 2003; Bauer *et al.*, 2009). This process is composed of a forward selection procedure that inserts the most informative SNP inside the model in each iterative cycle, then uses it to re-analyze the remaining SNPs. The results of each round are considered as the basis for the next round of the forward selection process, and iteration of the multilocus QTL model continues until no other SNP is detected. Additionally, the control of QTL false discovery rate (FDR) was incorporated inside the model, which was conducted by using the PROC MULTTEST procedure in SAS 9.4. A threshold of P-value ≤0.001 with 1000 permutations and FDR value ≤0.05 was determined (Doerge and Churchill, 1996). Due to strong decay in linkage disequilibrium, a confidence interval of 3.5 cM and 15 cM was defined on both

sides of the most significant SNP marker in SA and HA, respectively. The model defined QTL intervals by clustering SNPs based on their significance in the first iteration of the multilocus procedure. To test the significance of QTLs, a "leave20%out" cross-validation procedure that randomly left out 20% of the genotypes from the original dataset and re-analyzed the remaining genotypes was performed. This process was executed 20 times and a new *P*-value was calculated using the mean of all. For analysis of QTL × environment interactions across 4 years, five times cross-validation was used. The SAS script employed for QTL and QTL × environment interaction analysis, including implementation of FDR and cross-validation procedures, is provided in Supplementary Method 2.2. Genetic variance explained by a single SNP marker (R_M^2) was calculated as:

$$R_M^2 = SQ_M / SQ_g$$

where SQ_M is the sum of squares of M_i and SQ_g was calculated as the type I sum of squares of the DH lines in an ANOVA model (von Korff *et al.* 2006). Finally, the total proportion of explained genetic variance was estimated.

Epistatic interaction and epistasis × environment interaction models

A two-way epistatic interaction multilocus approach and epistasis × environment interaction was performed through SA and HA in SAS 9.4. A threshold of *P*-value ≤0.001 and FDR value ≤0.05 was set followed by cross-validation using a hierarchical model:

$$Y_{ijk} = \mu + M1_i + M2_j + M1_i \times M2_j + C_k + M1_i \times M2_j \times C_k + \varepsilon_{ijk}$$

where Y_{ijk}: response variable; μ: general mean; $M1_i$ and $M2_j$: fixed effects of the *i*th marker genotype and the *j*th marker genotype, respectively; $M1_i \times M2_j$: the fixed interaction effect of the *i*th *M1* marker genotype with the *j*th *M2* marker genotype; C_k: the fixed effect of the *k*th calendar year; $M1_i \times M2_j \times C_k$: the fixed interaction of the ith *M1* marker genotype with the *j*th *M2* marker genotype and the *kth* calendar year; and ε_{ijk}: the residual. The SAS script for epistatic interaction and epistasis × environment interaction analysis, including implementation of FDR and cross-validation procedures, is provided in Supplementary Method 2.2. Genetic variance explained by a single interaction was analyzed by fitting the model same as genetic variance by a single QTL. Subsequently, the total proportion of genetic variance explained by interactions was calculated.

Results

Flowering time under various environmental conditions

To study flowering time and the effect of environment on it, days to heading was scored in the spring barley MAGIC DH lines grown under field and semi-controlled conditions. Flowering time in the field showed a large phenotypic variation of more than 30 days each year, which was more diverse than that of the parents. Under semi-controlled conditions, the range of flowering time was shorter than under field conditions for both the DH lines and the parents, resulting in a higher CV (%) under field conditions (Table 2.1). Row and column effects were not significant in all experiments. ANOVA for years revealed highly significant differences (P<0.01) (see Supplementary Table 2.1). Evaluation of GDD accumulation for 100 days after sowing by a paired Student's t-test showed a significant difference between years. GDD accumulated faster in the foil tunnel conditions during the growing seasons, corresponding to an earlier flowering time (Fig. 2.1A; Supplementary Table 2.2). Daily average temperature for the first 2 weeks after sowing was measured in the 4 sowing years to investigate the effect of lack of vernalization (which requires temperatures in the range 4-12 °C), which can delay flowering in some genotypes (Trione and Metzger, 1970). Flowering time was not accelerated in the years in which there was a lower number of days with mean temperature higher than 12 °C. Furthermore, Pearson's correlation coefficient between days to heading and GDD was >0.99 for each year. The DH lines were subjected to the same day length each year as a result of very similar sowing dates (Figure 2.1B).

Table 2.1. Descriptive statistics and heritability for days to heading (DHE) for parental lines and spring barley MAGIC DH lines under foil tunnel (2011-2012) and field (2016-2017) conditions

		2011	**2012**	**2016**	**2017**
Parents	Min	50.00	55.00	65.00	61.00
	Max	55.00	60.00	74.00	71.00
	Mean	52.00	57.63	68.00	64.63
MAGIC population	Min	44.00	48.00	57.00	55.00
	Max	73.00	75.00	95.00	88.00
	Mean	53.73	59.91	68.00	69.00
	SE	0.16	0.17	0.22	0.20
	SD	3.85	3.96	5.02	4.69
	CV	14.82	15.64	25.19	21.98
	H^2	0.47		0.63	

CV, Coefficient of variation; H^2, heritability (for years 2011-2012 and 2016-2017); Max, maximum; Min, minimum; SD, standard deviation (%); SE, standard error.

Identification of QTL for flowering time under field conditions

To identify genetic regions that control flowering time, the association between phenotypic and genotypic data was evaluated. The results revealed 11 QTLs by SA (Figure 2.2A) and 7 QTLs by HA (Figure 2.2B). All seven chromosomal regions found by HA were also detected by SA. One QTL on chromosome 2H was associated with earlier flowering by 8.79 days (Tables 2.2 and 2.3). QTLs detected with SA and HA explained in total 48.43% and 52.92% of the genetic variance, respectively. One single parent was the source of five loci mapped by both SA and HA on chromosomes 1H, 2H, 3H, 5H, and 7H (Tables 2.2 and 2.3). According to SA, the strongest association with flowering time was detected on chromosome 7H at 34.35 cM (BOPA2-12-30895), and explained 9.96% of the genetic variance (Table 2.2). For HA, the most significant QTL was mapped to chromosome 2H at 27.69 cM (SCRI_RS_140819), and explained 14.96% of the genetic variance (Table 2.3). The alleles for both loci originated from the parental line Ragusa.

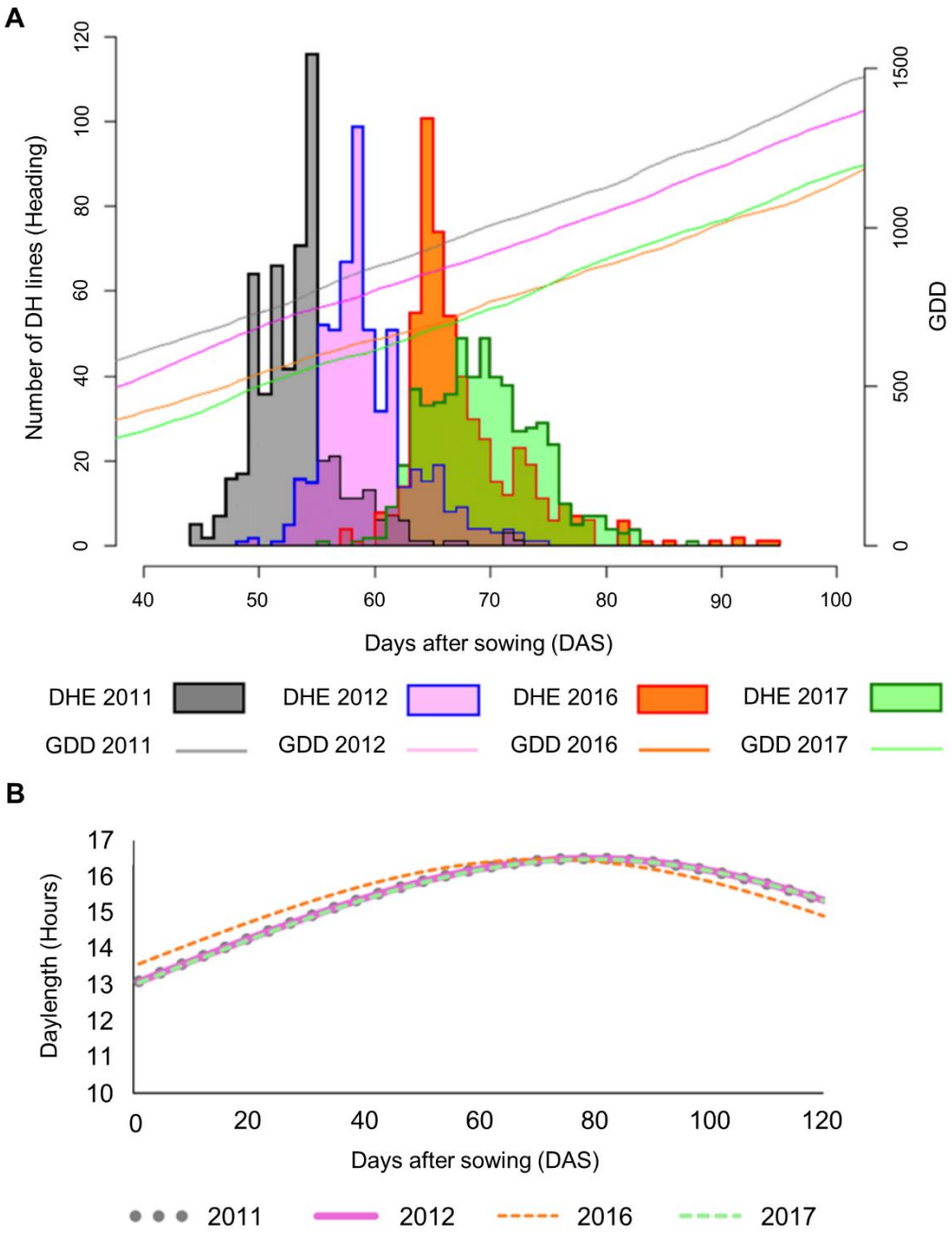

Fig. 2.1. (A) Comparison of flowering time (days to heading) distribution and GDD values for the years 2011, 2012, 2016 and 2017. (B) Comparison of day length in the same 4 years.

Table 2.2. Significant QTL associated with flowering time in single SNP approach under field condition

QTL	Peak marker[a]	Chr[b]	cM[c]	mbp[d]	Flanking region (cM)[e]	*F*-value	*P*-value	FDR value	Var (%)[f]	Parent [g]	MAF[h]	SNP1/effect[i]	SNP2/effect[j]	Gene[k]
HvHeading-1H-SA	BOPA1_1016_376	1H	71.03	480.40	70.89 - 71.030	35.76	2.33E-07	3.89E-05	6.37	2	0.08	G/3.82	A/-0.38	
HvHeading-2H-SA	SCRI_RS_233272	2H	18.91	27.30	18.91 - 23.80	41.19	1.43E-07	3.05E-05	7.22	8	0.02	T/-8.79	G/0.36	Ppd-H1
HvHeading-2H.2-SA	BOPA1_ABC14531_1_2_91	2H	91.21	687.20	91.21	15.56	9.31E-04	1.00E-02	2.88	1, 8	0.22	A/1.52	G/-0.38	
HvHeading-3H-SA	SCRI_RS_103215	3H	109.21	634.07	108.85 - 109.21	24.73	5.8E-05	3.00E-03	4.48	3	0.1	A/-0.02	G/3.17	denso/sdw1
HvHeading-5H-SA	SCRI_RS_152347	5H	69.31	522.50	69.31 - 74.93	18.91	1.62E-04	6.00E-03	3.4	8	0.14	A/2.06	G/-0.11	
HvHeading-5H.2-SA	SCRI_RS_204275	5H	80.21	543.30	77.52 - 85.56	22.06	6.96E-05	4.00E-03	3.91	1, 3, 8	0.49	C/-0.67	A/1.04	
HvHeading-5H.3-SA	BOPA2_12_21471	5H	122.43	595.20	118.89 - 128.54	53.97	1.78E-09	1.39E-06	9.22	8	0.17	G/2.73	A/-0.63	Vrn-H1
HvHeading-6H-SA	SCRI_RS_9648	6H	118.98	577.00	118.56 - 118.98	23.85	4.00E-05	3.00E-03	4.23	2, 5	0.31	A/-1.29	C/0.73	
HvHeading-7H-SA	BOPA2_12_30895	7H	34.35	39.70	34.35	60.33	3.14E-10	7.32E-07	9.96	8	0.04	C/5.79	G/-0.17	Vrn-H3
HvHeading-7H.2-SA	BOPA1_1107_392	7H	65.44	109.70	61.33 - 70.96	42.66	1.73E-07	3.36E-05	7.34	8	0.13	A/3.14	C/-0.35	HvCO1
HvHeading-7H.3-SA	BOPA1_ABC10040_1_1_238	7H	76.47	501.80	70.33 - 76.47	26.15	5.12E-05	3.00E-03	4.58	8	0.10	G/2.99	A/-0.26	
Total									48.43					

[a]Most significant marker associated with the QTL.

[b]The chromosome that the QTL was located.

[c]Genetic position of the most significant QTL according to Comadran *et al.* (2012)

[d]Physical position of the most significant QTL according to barley pseudomolecules genome assembly (2012).

[e]The range of QTL interval according to Comadran *et al.* (2012)

[f]Cross-validated proportion of explained genetic variance of QTL.

[g]The parent(s) carrying the allele with less frequency: Ack. Bavaria (1), Ack. Danubia (2), Barke (3), Criewener (4) , Heils Franken (5), Heines Hanna (6), Pflugs Intensiv(7), Ragusa (8)

[h]Minor allele frequency

[i]Flowering effect of allele with lower frequency (days).

[j]Flowering effect of allele with higher frequency (days).

[k]Candidate gene corresponding to QTL.

Table 2.3. Significant QTL associated with flowering time in haplotype approach under field conditions

QTL	Peak marker[a]	Chr[b]	cM[c]	Flanking region (cM)[d]	*F*-value	*P*-value	FDR value	Var (%)[e]	Parent 1[f]	Parent 2[f]	Parent 3[f]	Parent 4[f]	Parent 5[f]	Parent 6[f]	Parent 7[f]	Gene[g]
HvHeading-1H-HA	BOPA2_12_30147	1H	66.86	60.84 - 86.47	6.72	8.27E-05	6.72E-04	7.79	0.59	3.82	-0.44	-0.73	-0.38	-0.05	0.11	
HvHeading-2H-HA	SCRI_RS_140819	2H	27.69	3.82 - 53.75	13.61	1.42E-09	9.77E-07	14.96	-0.22	2.31	-0.39	-0.58	0.19	0.40	-8.79	Ppd-1
HvHeading-2H.2-HA	SCRI_RS_160958	2H	92.78	91.15 - 99.26	5.25	4.54E-04	2.24E-03	6.25	0.66	-3.37	-1.04	-3.33	-0.17	-0.05	2.45	
HvHeading-3H-HA	BOPA1_ABC13753-1-2-167	3H	105.31	100.71 - 109.84	6.37	1.24E-04	9.14E-04	7.10	-1.00	-0.08	2.42	-0.29	0.45	-0.91	0.36	denso/sdw1
HvHeading-5H-HA	BOPA2_12_30377	5H	125.76	95.90 - 131.94	10.79	1.63E-08	1.43E-06	11.34	-1.15	-0.49	-0.37	-0.98	-0.05	0.05	3.00	Vrn-H1
HvHeading-6H-HA	SCRI_RS_144034	6H	119.33	116.15 - 119.33	6.07	5.96E-05	5.21E-04	7.67	1.48	-1.29	0.19	0.99	-1.14	-1.14	2.34	
HvHeading-7H-HA	BOPA1_ConsensusGBS0356-1	7H	37.61	23.80 - 67.42	12.63	1.87E-09	9.77E-07	12.65	-0.73	-1.07	-2.31	0.63	0.22	0.70	3.91	Vrn-H3
Total								52.92								

[a]Most significant marker associated with the QTL.

[b]The chromosome that the QTL was located.

[c]Genetic position of the most significant QTL according to Comadran *et al.* (2012)

[d]The range of QTL interval according to Comadran *et al.* (2012)

[e]Cross-validated proportion of explained genetic variance of QTL.

[f]Effect of allele for each parent (days) in reference to flowering time mean of the population: Ack. Bavaria (1), Ack. Danubia (2), Barke (3), Criewener/Pflugs Intensiv (4) , Heils Franken (5), Heines Hanna (6), Ragusa (7)

[g]Candidate gene corresponding to QTL.

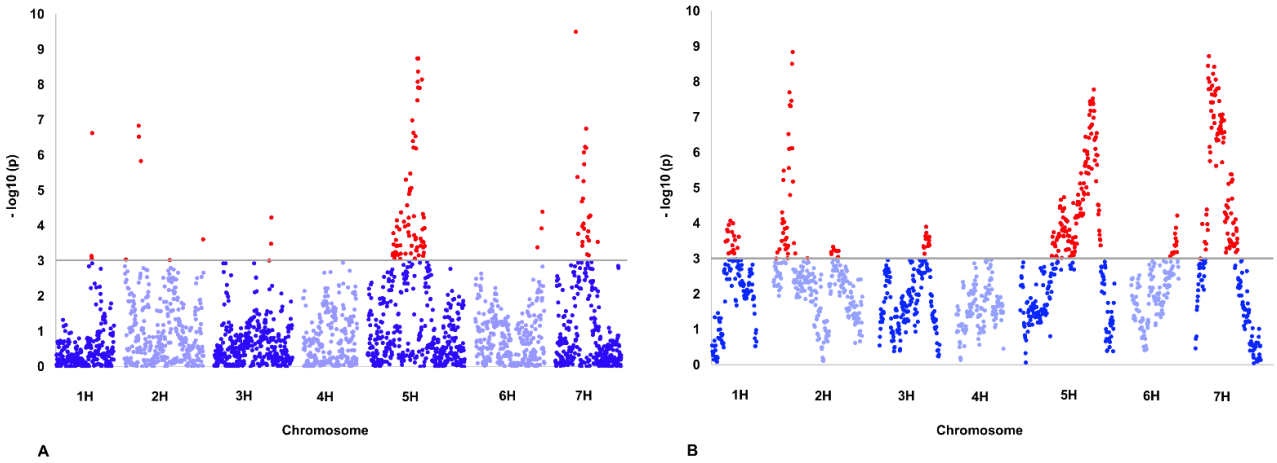

Fig. 2.2. Manhattan plots for (A) the single SNP approach and (B) haplotype approach for the spring barley MAGIC population grown under field conditions. The y axes denote the significance of SNP markers as -log10 (*P*) for flowering time (days to heading) in the barley population; the chromosomes are denoted on the x axes. The highlighted SNP markers above the cut-off line are significant by a threshold of $P \leq 0.001$ with 1000 permutations plus 20 times cross-validation.

Identification of epistatic interactions under field and semi-controlled conditions

To evaluate how the interaction among genetic loci affects flowering time, genome-wide epistatic interaction analysis was performed. In total, 55 and 27 epistatic interactions were detected ($P \leq 0.1E-15$) under field conditions by SA (Figure 2.3A; Supplementary Table 2.3) and HA (Figure 2.3B; Supplementary Table 2.4), respectively. The most significant epistatic interaction was between two regions located on chromosomes 7H (34.35 cM, SA; 37.61 cM, HA) and 2H (18.91 cM, SA; 27.69 cM, HA). This interaction affected flowering time by more than 20 days and explained 28.13% and 38.02% of the genetic variation by SA and HA, respectively. Both of the alleles causing the polymorphism originated from the parental line Ragusa; the one on chromosome 2H induced earlier flowering, whereas the one on chromosome 7H delayed flowering time (see Supplementary Table 2.4).

Analysis of epistatic interactions under foil tunnel conditions revealed 1139 and 1199 interactions ($P \leq 0.1E-15$) using SA (Figure 2.3C; Supplementary Table 2.5) and HA (Figure 2.3D; Supplementary Table 2.6), respectively. Both analyses identified the same interacting regions on chromosomes 7H (32.79 cM, SA; 32.79 cM, HA) and 5H (125.49 cM, SA; 118.75 cM, HA) as the most significant ones. This interaction influenced flowering time by more than 4 days in both approaches, and explained 26.94% and 42.01% of the genetic variance by SA and HA, respectively (Supplementary Tables 2.5 and 2.6). The allele on chromosome 5H contributed to earlier flowering whereas the one on chromosome 7H delayed flowering time (Supplementary Table 2.6); both originated from the parental line Ragusa.

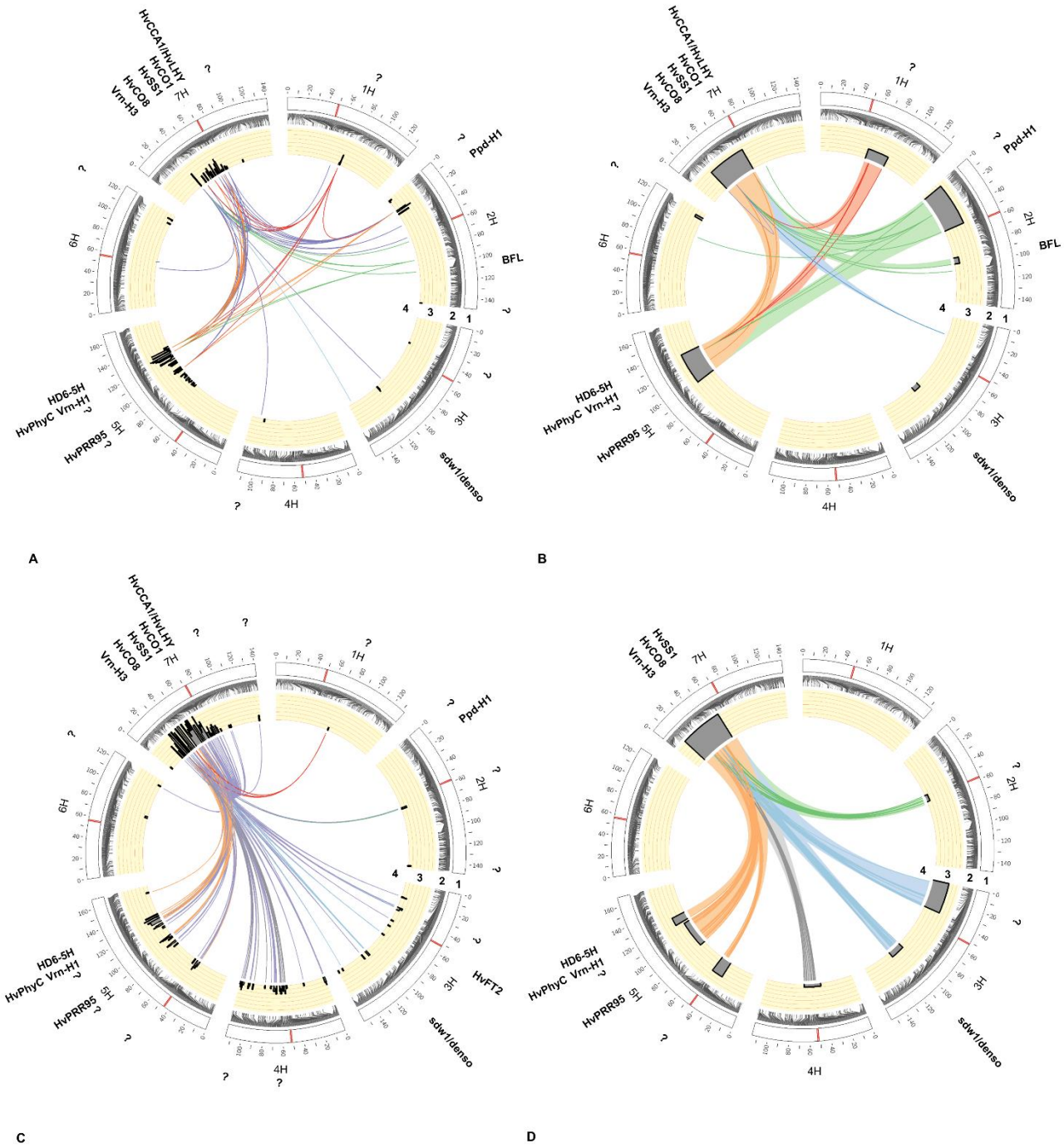

Fig. 2.3. Genetic composition of flowering time in a spring barley MAGIC population under field conditions, (A) single SNP approach and (B) haplotype approach, and under foil tunnel conditions, (C) single SNP approach and (D) haplotype approach. The candidate genes that might correspond to QTLs and digenic interactions are indicated outside the plots. 1, Barley chromosomes are shown as white bars and centromeres are highlighted within these bars. 2, Genetic position of SNPs on the chromosomes. 3, Probability of QTLs detected with $P \leq 0.001$ and 1000 permutations plus cross-validation via multilocus QTL analysis are shown as peak SNPs in SA and peak SNPs/interval (blocks) in HA. 4, Bridges in the center of each circle represent detected digenic interactions between SNP markers with $P \leq 0.1E\text{-}15$ via cross-validated multilocus epistatic interaction analysis. Question marks indicate loci where no genes for flowering time have been reported so far. Plots were drawn by Circos (Krzywinski *et al.*, 2009).

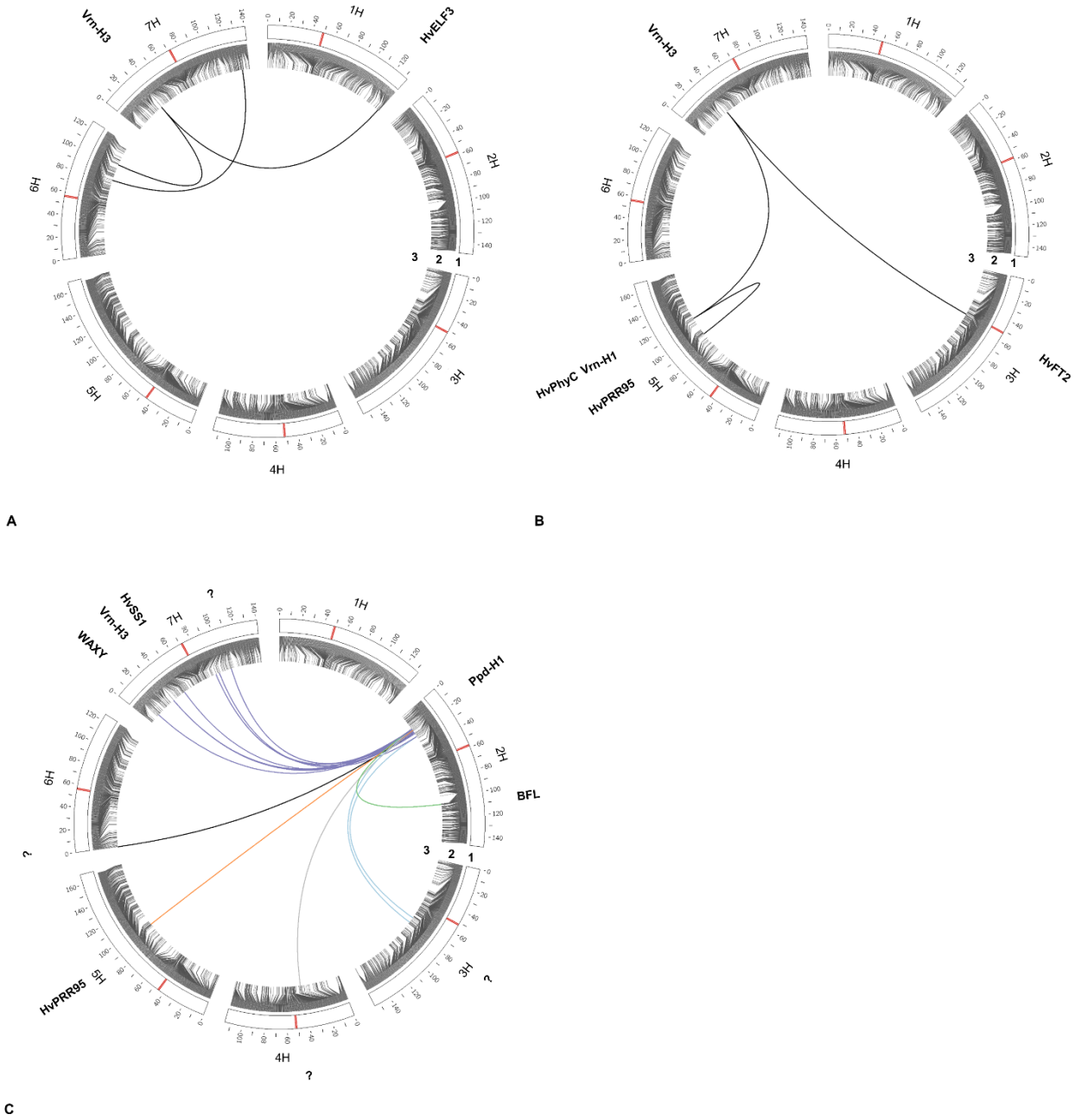

Fig. 2.4. Epistasis × environment interactions for flowering time in a spring barley MAGIC population by SA in (A) field conditions, (B) foil tunnel conditions, and (C) both field and foil tunnel conditions (4 years). Candidate genes that might correspond to QTLs and digenic interactions are indicated outside the plots. 1, Barley chromosomes are shown as white bars and centromeres are highlighted within these bars. 2. Genetic position of SNPs on the chromosomes. 3, Bridges in the center of each circle represent detected digenic interactions between SNP markers (A and B, $P \leq 0.1E\text{-}6$; C, $P \leq 0.1E\text{-}27$) via cross-validated multilocus epistatic interaction analysis. Question marks indicate loci where no genes for flowering time have been reported so far. Plots were drawn by Circos (Krzywinski *et al.*, 2009).

Detection of HvHeading, a novel flowering-delaying QTL allele

To distinguish the novel QTL, the detected loci in this study were compared with previously reported regions. SA and HA consistently revealed a novel QTL allele with a flowering-delaying effect on chromosome 1H, which explained 6.37% (Table 2.2) and 7.79% (Table 2.3) of the genetic variation, respectively. We named this QTL *HvHeading*. The QTL allele segregated from the parent Danubia and was located within interval 70.89–71.03 cM (BOPA1-1016–376, 71.03 cM) by SA (Table 2.2) and 60.84–86.47 cM (BOPA2_12_30147, 66.86 cM) by HA (Table 2.3). Analysis of allelic effect showed that as a single QTL it delayed flowering time by up to 3.82 days compared with the population average.

Several loci were involved in epistatic interactions with *HvHeading* (Figure 2.3). SA revealed that in field conditions, an interaction of *HvHeading* with one locus on chromosome 7H, 34.35 cM (BOPA2_12_30895) could postpone flowering time by 12.38 days, while its interaction with a locus on chromosome 2H, 18.91 cM (SCRI_RS_233272) could accelerate flowering time by up to 10 days (Supplementary Table 2.3). HA showed that interaction of the allele from Danubia with locus 7H, 37.61 cM (SCRI_RS_155061, interval 27.79–67.42 cM) from Ragusa could delay flowering time by 10.83 days, while its interaction with locus 2H, 27.69 cM (SCRI_RS_140819, interval 3.82–53.75 cM) from Ragusa could advance flowering by 10 days (Supplementary Table 2.4). According to SA, under foil tunnel conditions *HvHeading* strongly interacted with several loci (Supplementary Table 2.5). The most significant interaction was with the locus at 7H, 32.79 cM (BOPA1_12701_485), which could influence flowering time by 3.36 days.

To perform *in silico* analysis, the overlapping region between the QTL intervals from SA (7 cM) and HA (30 cM) was determined. The *in silico* approach revealed 160 annotated genes with predicted function in this region with different Gene Ontology annotations.

Influence of environment on flowering time

To evaluate the effect of environment on flowering time, the interaction of single and epistatic QTLs with the four environments was analyzed. The QTL × environment interaction analysis showed no significant effects under field conditions for both years. However, under foil tunnel conditions one QTL on chromosome 2H was detected by SA (19.90 cM) and HA (23.02 cM) (Supplementary Tables 2.7 and 2.8). Considering all 4 years, SA and HA identified the most prominent region that interacted with environment on chromosome 2H (19.90 cM) and 7H (31.37 cM), respectively (Supplementary Tables 2.9 and 2.10).

Analysis of epistasis × environment interactions using SA revealed 86 interactions with environment under field conditions (Supplementary Table 2.11). Position 34.35 cM on

chromosome 7H was involved in highly significant interactions (Figure 2.4A). Analysis of epistasis × environment interactions in foil tunnel conditions by SA showed that 527 epistatic interactions were affected by environment, including *HvHeading*. The prominent regions involved in the 10 strongest interactions were located on chromosomes 5H (84.38–122.01 cM) and 7H (28.98–62.18 cM) (Supplementary Figure 2.4B; Table 2.12). No significant interactions were found when evaluating epistasis × environment interactions via HA. Across all 4 years, strong epistasis × environment interactions were detected (Supplementary Table 2.13), which involved the interval located on chromosome 2H at 18.9–23.8 cM (Figure 2.4C). Interactions of *HvHeading* with loci on chromosomes 2H, (SCRI_RS_233272, 18.9 cM), 7H (BOPA1_12701_485, 32.79 cM), and 5H (BOPA2_12_21471, 122.43 cM) were highly affected by environment (Supplementary Table 2.14).

Discussion

Power of QTL and epistatic interaction analysis in a MAGIC population

Genetic mapping showed that the single SNP approach precisely pinpointed loci that represent prominent flowering time genes. The peak marker BOPA2-12-30895 on chromosome 7H is located in the Vrn-H3 gene (Colmsee *et al.*, 2015). This finding, as well as the detection by both SA and HA of QTLs and epistatic interactions that corresponded to previously described flowering time genes, is a proof of concept for the power of the spring barley MAGIC population and the mixed linear model approach used in this study.

SA has been predominantly performed in QTL studies that used biparental populations or association panels (Xu *et al.*, 2017). However, in multiparental populations, if more than two parents are involved, SA is not able to unambiguously identify the parental origin of a given allele (i.e. SNP). This drawback can be overcome by using HA, which has offered a more informative evaluation of associated loci (Sannemann *et al.*, 2015; Ogawa *et al.*, 2018; Huang and George, 2011). In the present study, HA provided an estimation of allelic effect of each parent and, due to the potential contribution of all SNP information in one haplotype block, it produced smoother *P*-value plots (Figure 2.2B) and created larger QTL intervals (Figure 2.5) compared with SA. The presence of more markers in these QTL intervals allowed for the higher explanatory power of HA. This was in agreement with previous reports stating that marker–trait associations based on haplotype-phase data detected more SNPs associated with the trait (Sannemann *et al.*, 2015; N'Diaye *et al.*, 2017; Ogawa *et al.*, 2018).

Higher resolution of "haplotype-phasing" data is mandatory for greater precision in mapping and depends on the linkage disequilibrium of the population, which is affected by the population size, diversity among the parents, population structure, marker density, and recombination frequency (Wang *et al.*, 2002). Considering the nature of the MAGIC population, including the presence of similarity among the founders, more cross-over rounds will not guarantee a greater number of assigned genetic regions (Stadlmeier *et al.*, 2018*a*). One of the challenges during the current research was the construction of high-resolution haplotype data. Performing manual corrections clarified part of the unassigned regions, which shows that the existing algorithms for haplotype-phasing need to be improved to avoid wasting available data. Therefore, besides

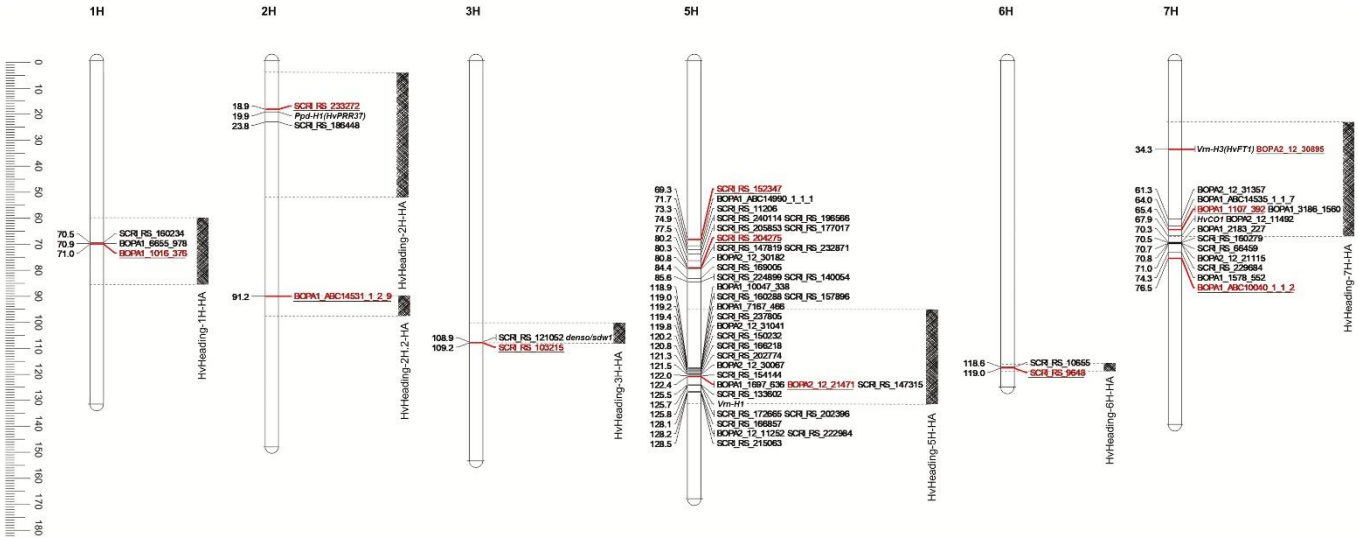

Fig. 2.5. Genetic map of QTLs for flowering time in spring barley MAGIC DH lines. Barley chromosomes are represented by white bars. The most significant SNP marker for each QTL according to SA is highlighted and underlined. The position of the haplotype that is associated with the QTL according to HA is shown with grey hatched blocks accompanied by the name of the corresponding QTL. Italicized gene names indicate the position of major flowering time genes as described for the Barke × Morex RILs by Mascher *et al.* (2013). The ruler on the left shows the chromosome length.

attempts to increase the number of cross-overs in the analyzed populations, developing an efficient haplotype-phase algorithm to maximize the usability of existing data seems to have a high priority in future studies.

The spring barley MAGIC DH lines supplied unique and diverse genetic material. The lower or higher than expected frequency (12.5%) for some SNP alleles might be due to the limited number of viable seeds obtained from the newly produced DH lines in the first round of cultivation.

Flowering time QTLs in the spring barley MAGIC population

Collectively, 18 QTLs were detected by SA and HA, including the QTL on chromosome 1H, which we named *HvHeading*, and nine QTLs that correspond to known flowering time genes.

Due to the different data resolution of the two analyses, SA and HA found the same reported key genes for flowering time regulation at slightly varying genetic positions, suggesting that the novel region detected by both approaches on chromosome 1H has the same underlying gene. A region was mapped on chromosome 2H (18.91 cM, SA; 27.69 cM, HA) corresponding to the position of Ppd-H1 (HvPRR37) (Alqudah *et al.*, 2014; Maurer *et al.*, 2015). The QTL detected on chromosome 3H (109.21 cM, SA; 105.31 cM, HA) was located in the region harboring the semi-dwarf gene denso/sdw1 (Wang *et al.*, 2010; Maurer *et al.*, 2015, 2016; Sannemann *et al.*, 2015; Alqudah *et al.*, 2016). Furthermore, an interval on chromosome 5H (122.43 cM, SA; 125.76 cM, HA) was mapped to the position of the vernalization-response gene *Vrn-H1* (Wang *et al.*, 2010; Alqudah *et al.*, 2014; Maurer *et al.*, 2015; Sannemann *et al.*, 2015), and the detected region on chromosome 7H (34.35 cM, SA; 37.61 cM, HA) matched the position of another major vernalization-response gene, *Vrn-H3* (*HvFT1*) (Wang *et al.*, 2010; Alqudah *et al.*, 2014; Maurer *et al.*, 2015; Sannemann *et al.*, 2015).

Epistatic interactions in field and semi-controlled conditions

Different epistatic interactions were detected in field and semi-controlled conditions. The locus corresponding to *Vrn-H3* had the strongest epistatic interaction in both environments irrespective of the genetic approach used for analysis.

In field conditions, regions corresponding to *Vrn-H3* and *Ppd-H1* had the strongest epistatic interaction. It has been reported that *Ppd-H1* advances flowering time under long-day conditions by promoting *Vrn-H3* (Yan *et al.*, 2006). The epistatic interactions that involved the *Ppd-H1* region from Ragusa accelerated flowering time remarkably, showing an early-flowering haplotype-specific effect. The most significant epistatic interaction in foil tunnel conditions was among regions that corresponded to *Vrn-H3* and *Vrn-H1*. *Vrn-H1* seems to respond to low and high temperatures and is known to up-regulate *Vrn-H3* (Fu *et al.*, 2005; Von Zitzewitz *et al.*, 2005; Gol *et al.*, 2017).

These results support previous descriptions of complex genetic networks in the flowering time pathway in barley (Maurer *et al.*, 2015). Epistatic interaction analysis for the different environments suggested that the flowering time of the MAGIC population was shaped by distinctive digenic interactions that adapt the DH lines to various environments.

HvHeading and participation in epistatic interactions

HvHeading, a novel flowering-delaying QTL allele, originated from the parental line Danubia. *HvHeading* was involved in significant epistatic interactions with loci that correspond to positions of major genes such as *Ppd-H1*, *Vrn-H1*, *Vrn-H3*, *sdw1*/*denso*, *HvPRR95*, *HvPhyC*,

HvCO8, and *HvSS1*, indicating that it might have a major role in controlling flowering time in barley.

The closest known candidate gene to this QTL is *Ppd-H2* (*HvFT3*) at 93.l cM (Halliwell *et al.*, 2016), which is involved in the photoperiod response under short-day conditions (Casao *et al.* 2011). Nevertheless, no association was detected between the region and flowering time in the spring barley MAGIC DH lines.

In silico analysis revealed several genes within the QTL interval that were annotated for families involved in flowering time, such as the MADS-box transcription factor protein (Trevaskis *et al.*, 2003), basic-leucine zipper (bZIP) transcription factor protein (Abe, 2005), *FAR1* (Hudson *et al.*, 1999) and *OVATE* (Wang *et al.*, 2016) families. Further analysis is needed to identify and characterize the gene underlying *HvHeading* and its role in the flowering time pathway.

Epistasis × environment interactions and involvement of HvHeading

The different times of flowering in field and semi-controlled conditions were linked to environmental factors. The plants in the foil tunnel condition were exposed to higher temperatures compared with those in the field, resulting in a faster accumulation of GDD, which accelerated flowering time.

Analyzing QTL × environment interactions across the four environments (i.e. all 4 years) revealed a strong interaction of QTL regions harboring *Ppd-H1* and *Vrn-H3*. The results of epistasis × environment interaction analysis for the four environments also showed that most of the interactions had *Ppd-H1* region in common. *Ppd-H1* and *Vrn-H3* are both promoted by temperature (Turner *et al.*, 2005; Yan *et al.*, 2006), and recently it was reported that higher ambient temperature triggers *Ppd-H1* (Ejaz and von Korff, 2017). The detection of an interaction of *Vrn-H3* with the environment suggests that the outstanding effect of *Vrn-H3* in epistatic interactions under foil tunnel conditions could be due to the warmer environment. Epistasis × environment interactions showed that under foil tunnel conditions, loci corresponding to *Vrn-H1*, *HvPRR95*, and *Vrn-H3* were prominent, which supports previous reports that higher temperature triggers *Vrn-H1* and the downstream gene *Vrn-H3* (Karsai *et al.*, 1997; Yan *et al.*, 2003; Von Zitzewitz *et al.*, 2005; Ejaz and von Korff, 2017; Gol *et al.*, 2017), which engage in a positive feedback loop that leads to early flowering under long-day conditions (Distelfeld *et al.*, 2009).

HvHeading showed strong effects in epistasis × environment interactions in all four environments. It was involved in interactions with regions harboring *Ppd-H1*, *Vrn-H3*, and *Vrn-*

H1, suggesting that *HvHeading* might have an effect on flowering time via an interaction with temperature.

Conclusion

Flowering time in barley is ultimately controlled by interactions among genes that can take different routes depending on environmental cues to adapt and fulfill timely flowering. The spring barley MAGIC population provided a genetic depth and richness that was required to study the effect of epistasis and environment interactions on complex traits such as flowering time. The results highlighted flowering time modulators as well as one novel QTL allele, *HvHeading* that strongly interacted with regions corresponding to the *Vrn-H3*, *Vrn-H1*, and *Ppd-H1* genes that are at the intersection of other genetic competitors. Further studies are needed to elaborate the underlying gene (or genes) and decipher its role and function in the pathway by shedding light on its interaction with other genetic and environmental factors.

Data availability

The following supplementary data are available at Dryad Digital Repository: https://doi.org/10.5061/dryad.g25cm28.

Dataset 2.1. Phenotypic data for days to heading (4 years) using MAGIC DH lines.

Dataset 2.2. Growing degree-days (GDD) during 100 days after sowing (2011, 2012, 2016, and 2017).

Dataset 2.3. Genotyping data by barley 9k iSelect SNP array using MAGIC DH lines.

Methods 2.1. SAS script and sample data for haplotype construction by the K-means clustering method.

Methods 2.2. SAS script for QTL and epistasis mapping process as well as environment interaction analysis including implementation of FDR and cross-validation procedures.

Table 2.1. Analysis of variance for 534 MAGIC DH lines.

Table 2.2. Descriptive statistics for growing degree-days (GDD) during 100 days after sowing.

Table 2.3. Epistatic interactions by single SNP approach under field conditions.

Table 2.4. Epistatic interactions by haplotype approach under field conditions.

Table 2.5. Epistatic interactions by single SNP approach under foil tunnel conditions.

Table 2.6. Epistatic interactions by haplotype approach under foil tunnel conditions.

Table 2.7. QTL × environment interactions by single SNP approach under foil tunnel conditions.

Table 2.8. QTL × environment interactions by haplotype approach under foil tunnel conditions.

Table 2.9. QTL × environment interactions by single SNP approach for 4 years (field and foil tunnel conditions).

Table 2.10. QTL × environment interactions by haplotype approach for 4 years (field and foil tunnel conditions).

Table 2.11. Epistasis × environment interactions by single SNP approach under field conditions.

Table 2.12. Epistasis × environment interactions by single SNP approach under foil tunnel conditions.

Table 2.13. Epistasis × environment interactions by single SNP approach for 4 years.

Table 2.14. Epistasis × environment interactions that engaged peak marker for *HvHeading* (BOPA1_1016_376) by single SNP approach for 4 years.

Acknowledgements

We thank the German Research Foundation (DFG) for funding this research under priority program 1530, “Flowering time control: from natural variation to crop improvement”.

Chapter 3

A systemic approach for targeted background effect elimination based on epistasis identifies transcription elongation factor *Spt6* as candidate gene underlying "*HvHeading"* QTL in barley

Based on:

Afsharyan N. P., Léon J., Ballvora A. (in preparation) A systemic approach for targeted background effect elimination based on epistasis identifies transcription elongation factor *Spt6* as candidate gene underlying "*HvHeading"* QTL in barley

To be submitted for publication in a peer-reviewed journal.

Abstract

Identification of genes that control flowering time can assist in understanding the mechanisms that improve crop yield due to its high correlation with final grain yield. The aim of this study is to identify candidate gene(s) underlying barley (*Hordeum vulgare* L.) flowering time QTL "*HvHeading*" by targeted background effect elimination approach based on epistasis. For this purpose, we selected flowering-time-specific-near-isogenic pairs of spring barley MAGIC DH lines; so that both DH lines have the same alleles for major genes in epistasis with *HvHeading* including *Ppd-H1*, *Vrn-H1* and *Vrn-H3* and one DH line carries the haplotype harboring the flowering-delaying *HvHeading* allele. Microscopic phenotyping of apex development and differential gene expression analysis by RNA-seq and RT-qPCR was performed using tissue from apex and leaf. Effect of *HvHeading* was detected as early as after vegetative-to-reproductive transition. RNA-seq results showed differentially expressed genes in *HvHeading* region in flowering-time-specific-near-isogenic pair of DH lines and refined *HvHeading* region to a <8.50 Mbp interval. Differential transcript expression analysis revealed up-regulation of *Spt6* gene, a transcription elongation factor, in delayed-flowering DH line. RT-qPCR results showed up-regulation of *Spt6* starting before double-ridge stage and down-regulation of *Ppd-H1*, *Vrn-H1* for delayed-flowering DH line. Sequencing majority of promoter region of *Spt6* gene in flowering-time-specific-near-isogenic pair of DH lines showed mutations that influence transcription factor binding sites. Also, comparing the transcripts of *Spt6* gene with known *Spt6* isoforms in barley showed that this DH line might carry a novel isoform of this gene. The findings revealed new insights into flowering time of barley by narrowing down *HvHeading* QTL interval and identifying *Spt6* gene as a candidate gene. It showed that the approach for targeted background effect elimination based on epistasis can be useful to facilitate gene identification for crops with complex genomes in post-genomic era.

Keywords: Barley, RNA-sequencing, flowering time, *HvHeading*, *Spt6*, novel QTL, MAGIC population, gene identification, epistasis

Introduction

The flowering time is one of the major traits that affects final grain yield; therefore identification of genes that control its genetic pathway can assist in understanding the mechanisms that improve yield (Ross-Ibarra *et al.*, 2007). Barley (*Hordeum vulgare* L.), a long day (LD) crop, is the fourth most cultivated cereal used for food and feed world-wide and a model for small grain cereals (Consortium *et al.*, 2012). Barley genome is characterized by its large 5.5 Gb size, complexity and extensive regions of reduced recombination which makes gene identification based on traditional map-based methods very time-consuming, costly and not as successful compared to plants with smaller genome such as *Arabidopsis thaliana* and rice (Künzel *et al.*, 2000; Schneeberger *et al.*, 2009; Takagi *et al.*, 2015). Emergence and application of next generation sequencing (NGS) approaches as well as recent availability of barley genome sequence reference has greatly enhanced gene identification in this crop in post-genome era (Consortium *et al.*, 2012; Beier *et al.*, 2017; Mascher *et al.*, 2017).

Common traditional method for gene identification is fine mapping the QTL region and search for recombinations segregating with the phenotype in a population; then, performing positional cloning of the candidate gene(s) to reveal the gene underlying the QTL (Kumar *et al.*, 2017). There are a few number of genes that were successfully cloned in barley (Francia *et al.*, 2007; Sutton *et al.*, 2007; Furukawa *et al.*, 2007) which is an indication of resource-intensive nature of this approach, particularly for complex genomes (Bettgenhaeuser and Krattinger, 2019; Jaganathan *et al.*, 2020). Due to currently available knowledge regarding barley physical gene space, the possible positional candidate genes can be directly searched within the QTL interval, also known as the candidate gene approach (Monclus *et al.*, 2012; Bargsten *et al.*, 2014; Correa *et al.*, 2014). The candidate genes can be prioritized based on sequence information, functional annotation and differential expression analysis (transcriptome and/or proteome levels) (Monclus *et al.*, 2012; Bargsten *et al.*, 2014; Kumar *et al.*, 2017; Gudys *et al.*, 2018). Various NGS approaches such as transcriptome sequencing (RNA-seq) has been used in gene identification process in barley; that reduce its genome sequence complexity with little or no requirement for recombination (Schneeberger, 2014; van Esse *et al.*, 2017). RNA-seq provides data regarding rare transcripts, novel transcripts and gene expression as opposed to other NGS approaches such as exome capture sequencing (ExomeSeq) that are limited to gene space present in reference genomes (Fu *et al.*, 2009; Mascher *et al.*, 2013*b*). Data produced by RNA-seq is easier to handle and interpret compared to very large datasets produced by whole genome sequencing (Wang *et al.*, 2009). RNA-Seq results reveal high levels of productivity and reproducibility if the target gene is regulated at transcript level, samples are collected from

proper tissue and time-point as well as presence of enough sequencing depth (Sánchez-Martín *et al.*, 2016). RNA-seq has been successfully utilized to identify genes involved in tissue-specific albinism using near-isogenic lines (NILs) (Shmakov *et al.*, 2016), to narrow down the candidate gene underlying a QTL for awn length using NILs (Liller *et al.*, 2017), and to isolate six-rows spike3 (*VRS3*) gene using allelic mutants (van Esse *et al.*, 2017).

Barley flowering time pathway is regulated by a complex network of interacting genes, however few major genes are reported to have a strong influence on determining the time of flowering (Cockram *et al.*, 2007; Maurer *et al.*, 2015; Afsharyan *et al.*, 2020). Flowering time under long-day (LD) is controlled by *PSEUDO-RESPONSE REGULATOR* (*HvPRR37*), a major photoperiod response gene also known as *PHOTOPERIOD RESPONSE LOCUS1* (*Ppd-H1*) (Turner *et al.*, 2005). A natural mutation in the conserved CCT (*CONSTANS*, *CONSTANS-LIKE*, and *TOC1*) domain of *Ppd-H1* causes a reduced response to LDs (Turner *et al.*, 2005). *Ppd-H1* promotes flowering by initiating expression of *Vrn-H3* gene which is a homolog of *Arabidopsis thaliana FLOWERING LOCUS T* (*FT*) gene. Mutations in the first intron of the *Vrn-H3* gene, are completely associated with the dominant or recessive *Vrn-H3* allele that promotes and delays flowering respectively (Yan *et al.*, 2006). *Vrn-H1* gene is an *APETALA1* family MADS-box transcription factor which is another major gene that can positively regulates flowering time by interacting with its downstream gene, *Vrn-H3* (Distelfeld *et al.*, 2009). Deletions in a regulatory region of *Vrn-H1* eliminates the vernalization requirement for flowering in spring barley (Hemming *et al.*, 2009; Rollins *et al.*, 2013).

Gene identification studies usually require eliminating the background effect that obscure the effect of target gene. The common strategy is to develop NILs or mutants to produce genotypes that have nearly the same genomic content, which requires investing lots of time and labor (Mascher *et al.*, 2014; Schneeberger, 2014). An alternative approach can be proposed as focusing only on the regions that control the trait of interest, particularly the ones in epistatic interaction with the targeted QTL interval that can have an impact on its phenotypic effect regardless of the remaining genomic background. To date, there are no studies that attempted to facilitate the gene identification process by performing targeted-elimination of background effect and thus reduce the requirement for construction of NILs or mutant populations.

Populations based on multiparent advanced generation intercross (MAGIC) strategy are designed to provide increased level of recombination and more genetic variance (Cavanagh *et al.*, 2008; King *et al.*, 2012). A Spring barley MAGIC population was constructed using an eight-way cross of seven barley landraces known as “founders of German barley” and one elite cultivar that was used to successfully map QTL and epistatic interactions that control flowering

time of barley (Sannemann *et al.*, 2015; Mathew *et al.*, 2018; Afsharyan *et al.*, 2020). Afsharyan *et al.* (2020) detected "*HvHeading*", a novel epistatic QTL on barley 1H chromosome using this population. The causative flowering-delaying allele for *HvHeading* descended from parental line Danubia. This QTL was involved in strong epistatic interaction with *Ppd-H1*, *Vrn-H1* and *Vrn-H3* which suggested that the gene underlying *HvHeading* might have an important role in timing of flowering (Afsharyan *et al.*, 2020).

The aim of this study was to better understand the mechanism controlling flowering time of barley by identifying the candidate gene underlying the novel epistatic QTL "*HvHeading*". Objectives of this research were to (1) investigate the phenotypic effect of *HvHeading* QTL on apex development by a targeted elimination of background effect approach based on epistasis using MAGIC DH lines, (2) analyze the transcripts detected in *HvHeading* interval among flowering-time-specific-near-isogenic DH lines using apex and leaf tissues, and (3) narrow down the QTL interval to identify candidate gene(s) underlying *HvHeading* QTL. Summary of the workflow is described in Figure 3.1.

Material and Methods

Plant material and growth condition

In total 910 DH lines of the spring barley MAGIC population were used in this study. The construction of the population is detailed by Sannemann *et al.* (2015). For all experiments the plants were sown in 96-cell growing trays (100 mL/cell). To equalize germination, moist seeds were kept in the dark at 4°C for 3 days. Subsequent to germination, they were transferred to short-day (SD) conditions (8 h, 22°C day; 16 h, 18°C night; PAR 270 µM (m2 s)) in growth chamber. The plants were kept in SD for one week then switched to LD conditions (16 h, 22°C day; 8 h, 18°C night; PAR 270 µM (m2 s)). Separate plant cultivations were carried out for phenotyping, sample collection for RNA-seq and sample collection for real-time quantitative PCR (RT-qPCR) experiments.

DNA extraction and genotyping

Total genomic DNA of leaves tissues was isolated from 910 spring barley MAGIC DH lines and genotyping *HvHeading* QTL interval was performed in two groups. First, DNA extraction and genotyping was performed for 534 DH lines using Barley 9k iSelect array (Comadran *et al.*, 2012) as described by Sannemann *et al.* (2015). From this set, 12 SNPs were selected that covered 9.98 cM interval in *HvHeading* region. Then DNA of additional 376 DH lines was isolated using the protocol of Virginia Tech Small Grains Breeding (www.cropgenetics.cses.vt.edu) and SNP-genotyped by KASPar technique using four

polymorphic markers in *HvHeading* interval designed based on SNPs from barley 9k iSelect array: SCRI_RS_138527, BOPA1_6655-978, BOPA1_1016-376 and SCRI_RS_181239. Developing of the KASPar markers and genotyping procedures were conducted by

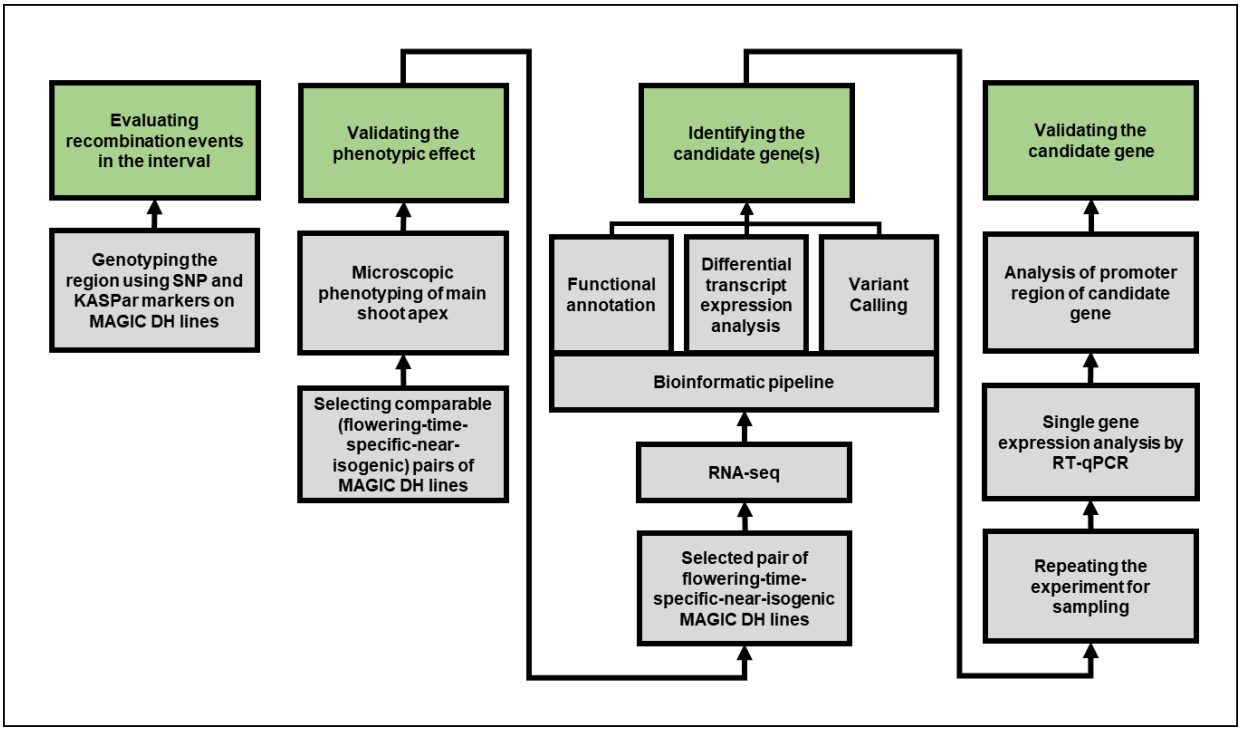

Fig. 3.1. Overview of the workflow to identify the candidate gene(s) underlying *HvHeading* QTL interval

TraitGenetics GmbH (Stadt Seeland OT, Gatersleben, Germany). The QTL interval was determined based on population LD (linkage disequilibrium) and QTL analysis according to Afsharyan *et al.* (2020). *In silico* analysis was performed using the IPK barley BLAST server (https://webblast.ipk-gatersleben.de/barley_ibsc/) (Colmsee *et al.*, 2015) and Ensembl Plants (http://plants.ensembl.org) (Bolser *et al.*, 2016). The major flowering time genes *Ppd-H1*, *Vrn-H3* and *Vrn-H1* were genotyped for each parent using gene specific primers listed in Supplementary Table 3.1.

Selecting comparable DH lines

To investigating the effect of *HvHeading* QTL allele on flowering time, 534 DH lines were subjected to a rigorous selection process to find comparable (flowering-time-specific-near-isogenic) pair based on similarity in flowering time loci/alleles. The DH lines were first separated in two groups based on the haplotype (linked SNP block) in *HvHeading* region. The QTL and epistatic interactions that control flowering time in spring barley MAGIC population including *HvHeading* were described by Afsharyan *et al.* (2020). One group was build up from DH lines that carried the haplotype from parental line Danubia in *HvHeading* interval, the donor

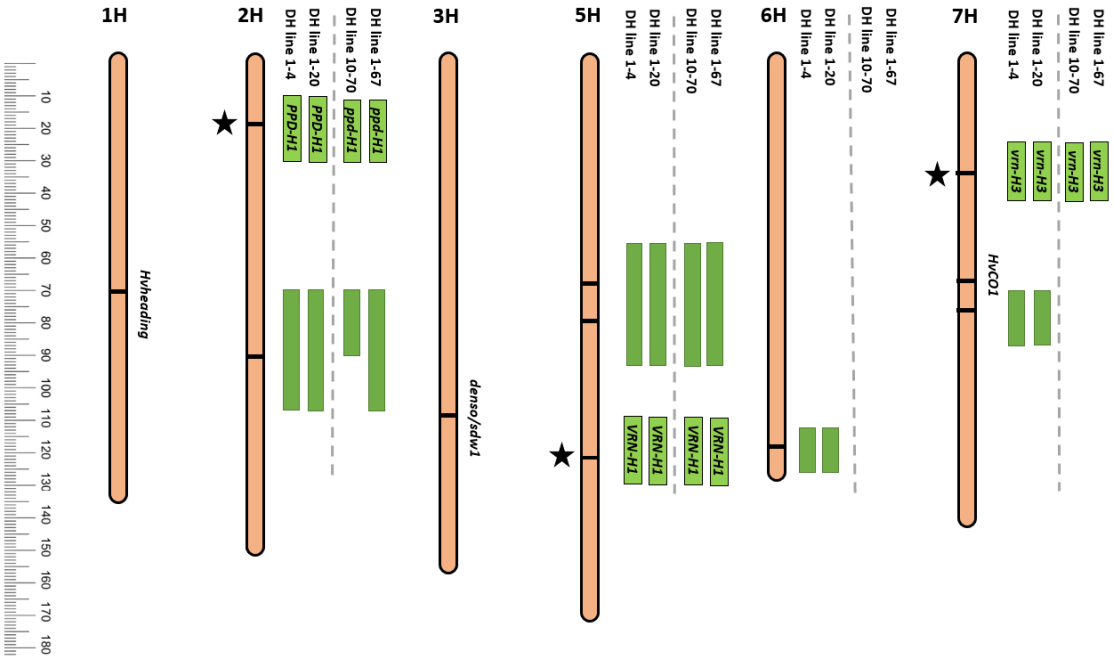

Fig. 3.2. An overview of flowering time regions in each pair of comparable (flowering-time-specific-near-isogenic) DH lines; 1-4 and 1-20, 10-70 and 1-67. Chromosomes are shown as bars. The position of QTL for flowering time in MAGIC population are shown in black marks on each chromosome. The regions/genes involved in major epistatic interaction with *HvHeading* are indicated with a star. The QTL and epistatic interactions are according to Afsharyan *et al. 2020*. For each pair: the blank green block indicated the region that is descended from the same parent; the bordered block with italianized gene name shows the same allele from a gene. The ruler on the left side shows the length of chromosomes.

parent for flowering-delaying allele; and the other group was composed of the rest. Then, these two groups were compared to find DH lines that had the same allele of major flowering time genes *Ppd-H1*, *Vrn-H3* and *Vrn-H1* that are engaged in epistasis with *HvHeading*. The selected DH lines were further narrowed down to find the pair with the most similarity in parental origin for haplotype blocks in the other seven QTL that associated with flowering time in MAGIC population. Finally, two pairs of comparable DH lines, 1-4 and 1-20 as well as 10-70 and 1-67 were chosen for further analysis (Figure 3.2). According to SNP genotyping, haplotype carrying the *HvHeading* interval in DH lines 1-20 and 1-67 corresponded to parental line Danubia and DH lines 1-4 and 10-70 to Barke and Criewener40, respectively.

Phenotyping of main shoot apex development

For phenotyping main shoot apex (MSA) development, two separate experiments were conducted to validate the developmental stages. Three plants per genotype were dissected every two days from germination to heading. At each time-point, the developmental stage of MSA was determined and quantified according to the quantitative scale (Waddington stage; W) described by Waddington *et al.*, (1983). In addition, data for days to heading were collected for 10 plants per genotype. Images of apices were obtained using Digital microscope KEYENCE model VHX-900F (KEYENCE Corporation, Osaka, Japan). The development of apex and inflorescence among each pair of DH lines were compared by paired student's t-test. Broken-line regressions were calculated using the "SiZer" package in R software (R core team, 2015).

Sample preparation for RNA-sequencing and RT-qPCR

For RNA-sequencing (RNA-seq), the apex and leaf tissues were harvested from the main shoots. Samples were collected at six time-points which was composed of two times a day, after lightening and darkening of climate chamber, at 11 days, 19 days and 33 days after germination. Sample collection for each time point was performed within 3 hours in average. Before each sampling time, three plants per genotype were dissected, and the developmental stage was confirmed according to Waddington stage. For sampling the apex, the leaves surrounding the MSA were removed manually, and the apex was cut with a Paragon Nr.10 microsurgical disposable stab knife under a stereo microscope. From each tissue, minimum three pooled biological replicates were taken per time-point for each DH line. The MSA samples collected during the vegetative phase W1.0 consisted of 30 pooled apices. During reproductive stage, at W1.5-2.25 stage and W2.25-7.0 stage, 15 and 11 shoot apices were pooled, respectively. The leaf samples were harvested from a subset of 10 plants, from which apex tissue was collected. The harvested leaf tissue was restricted to the distal part of the leaf, at 2 to 4 cm before the leaf tip. For each day the two apex pools from light and dark time-points of each genotype were mixed. Sample collection for RT-qPCR was performed the same as described for RNA-seq with the exception that the samples were collected once a day, after lightening. The harvested samples were immediately frozen in liquid nitrogen, then stored at -80°C.

RNA Sequencing, quality control and mapping

Total RNA extraction, RNA-sequencing and initial quality control was performed commercially at Novogene Co. Ltd. (HK, China). The Illumina PE150 platform was applied for RNA-seq analysis. A set of 36 libraries were constructed and 1,970,926,721 paired-end reads were generated for further bioinformatics analyses. The sequencing data quality was

verified using FastQC software (version 0.11.8; https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Further Quality check was performed using the Trimmomatic program version 0.39 (Bolger *et al.*, 2014) to ensure the adaptors and short reads were trimmed using the following criteria: phred 33, leading and trailing 10, sliding window 30:15, headcrop 15 and a minimum read length of 70. Finally, 1,840,628,787 reads were allowed for further analysis. For Mapping, the reference genome sequence of barley cultivar Morex and the annotation data (Consortium *et al.*, 2012) were used as reference. Quality controlled and trimmed reads of each library were mapped against the reference sequence using BWA-MEM (version 0.7.15; Li, 2013). The aligned reads were filtered to eliminate non proper-paired reads and low quality (<30) alignments, and then sorted using SAMtools (Li *et al.*, 2009).

Differential gene expression analysis

For analyzing the differentially expressed transcripts (DET), counts of the mapped proper-paired reads were extracted by featureCounts from Subread package (Liao *et al.*, 2014) in R software (R Core Team, 2015) and used for downstream analyses. Transcripts with expression levels greater than three counts in three libraries were retained. Normalization and differential expression analysis was performed with the R/Bioconductor package "edgeR" version 3.26.4 (Robinson *et al.*, 2010) in R software. Normalization was performed within the library according to reads per kilo base per million (RPKM). Heatmap and hierarchical cluster analysis between individual libraries was done in R software in order to verify the quality of biological replication. For DET calling, only transcripts with expression levels ≥0.9 $\log_{10}$RPKM in at least two libraries were retained. DETs were called at a false discovery rate (FDR) <0.00001.

Variant calling of the RNA sequencing reads

Mapped and quality filtered reads were subjected to variant calling using function "mpileup" to generate genotype likelihoods at each genomic position with maximum 1000 read depth and function "call" for making the actual calls by bcftools-1.9 in SAMtools (Li *et al.*, 2009). To reduce the number of false-positive SNP calls, predicted SNP candidates were filtered with function "view" from bcftools-1.9 based on sequence quality ≥60. Then, variants that exist in at least two biological samples of each genotype were used for further analysis. The variants that were shared by both DH lines were removed.

RNA extraction, cDNA synthesis and gene expression analysis by RT-qPCR

RT-qPCR was used to investigate gene expression in DH lines as well as validating the results obtained from RNA-seq. For this purpose, the total RNA was extracted using TRIzol™ Reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) and residual DNA was removed using my-Budget DNase I (Krefeld, Germany, Bio-Budget Technologies). RNA extraction and DNase treatment were performed according to the manufacturer's instructions. The total RNA concentration and integrity were determined using gel electrophoresis and was quantified spectrophotometrically (ND-1000 Spectrophotometer, NanoDrop Technologies, USA). DNase-treated total RNA was reverse-transcribed using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Vilnius, Lithuania) according to the manufacturer's instructions. The expression levels of the target genes were quantified by RT-qPCR using DyNAmo ColorFlash SYBR Green qPCR Kit (Thermo Fisher Scientific Inc, Massachusetts, USA) and Applied Biosystems 7500 Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. RT-qPCR experiment for each gene in each tissue included as least three biological samples. The dissociation analysis (82°C) was performed at the end of each run (after cycle 45) to confirm the specificity of PCR products. *HvActin* was selected as the reference gene in the RT-qPCR assays. Relative quantification analysis was performed using comparative CT method (Livak and Schmittgen, 2001) according to the Applied Biosystem's instructions. Significant differences were calculated using Student's t-test. The gene-specific primers used in RT-qPCR experiments are listed in Supplementary Table 3.1.

Sequence analysis of promoter region and conserved regions of the candidate gene and phylogenetic analysis

The alignment of sequenced regions was performed by MegAlign Pro (Lasergene 7.1: DNASTAR Inc., Madison, WI). Identification of DNA binding motifs within the promoter was performed by MULAN analysis (Ovcharenko *et al.*, 2005) and the plant cis-acting regulatory DNA elements (PLACE) database (Higo *et al.*, 1999). The primers used for sequencing the promoter region are listed in Supplementary Table 3.1. All the RNA-seq reads for a gene were extracted by SAMtools (Li *et al.*, 2009) and assembled using SeqMan Pro (Lasergene 7.1: DNASTAR Inc., Madison, WI). Conserved domains of the gene were identified based on Ensembl database (http://plants.ensembl.org). To determine the phylogenetic relation, multiple sequence alignments for protein sequence of the gene of interest from barley with protein sequences of 10 model organisms was performed using NCBI BLAST server (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The selected model organisms included *Arabidopsis thaliana, Homo sapiens, Saccharomyces cerevisiae, Mus musculus, Dictyostelium discoideum,*

Schizosaccharomyces pombe, *Plasmodium falciparum*, *Glycine max*, *Drosophila melanogaster*, *Danio rerio*. Then, the phylogenetic tree was built by neighbor-joining method via NCBI BLAST server.

Results

Genotyping HvHeading QTL interval and major flowering time genes

The *HvHeading* QTL interval spanned from 67.13 cM (BOPA1_1670-369, 473.58 Mbs) to 76.84 cM (SCRI_RS_181353, 488.82 Mbs) and contained 160 high confidence genes with predicted function (HC-G). The genotyping this interval with SNPs revealed low level of recombination events. The order of genetic position of SNPs corresponded to their physical position. Major genes involved in epistatic interactions with *HvHeading* including *Ppd-H1*, *Vrn-H1* and *Vrn-H3*, were genotyped (Table 3.1). DH lines 1-4 and 1-20 carry a photoperiod-sensitive *Ppd-H1* as appose to the insensitive version, *ppd-H1* that is carried by DH lines 1-67 and 10-70. All four DH lines have the insensitive *vrn-H3* and sensitive *Vrn-H1*. SNP genotyping showed that SNPs in the *HvHeading* interval for DH lines 1-20 and 1-67 corresponded to Danubia.

Phenotypic effect of HvHeading

Apex development analysis was performed for each pair of comparable DH lines to investigate the flowering effect of *HvHeading* (Figure 3.3A). Evaluation of apex development stages using

Table 3.1. Genotyping of *Ppd-H1*, *Vrn-H1* and *Vrn-H3* genes in parental lines of spring barley MAGIC population

	Ppd-H1										*Vrn-H3*			*Vrn-H1*	
Parents	SNP4	SNP 5	SNP 6	SNP 7	SNP10	SNP 12	SNP14	SNP 15	SNP 16	Allele	SNP 270	SNP 384	Allele	Deletion in intron1	Allele
Ack. Bavaria	C	C	C	C	C	C	G	T	G	*ppd-H1*	T	C	*vrn-H3*	Del	*Vrn-H1*
Ack. Danubia	C	C	C	C	C	C	G	T	G	*ppd-H1*	T	C	*vrn-H3*	Del	*Vrn-H1*
Criewener403	C	C	C	C	C	C	G	T	G	*ppd-H1*	T	C	*vrn-H3*	Del	*Vrn-H1*
Heils Franken	C	C	C	C	C	C	G	T	G	*ppd-H1*	T	C	*vrn-H3*	Del	*Vrn-H1*
Heines Hanna	C	C	C	C	C	C	G	T	G	*ppd-H1*	T	C	*vrn-H3*	Del	*Vrn-H1*
Pflugs Intensiv	C	C	C	C	C	C	G	T	G	*ppd-H1*	T	C	*vrn-H3*	Del	*Vrn-H1*
Barke	C	C	C	C	C	C	G	T	G	*ppd-H1*	T	C	*vrn-H3*	Del	*Vrn-H1*
Ragusa	G	T	T	G	T	A	A	C	A	*Ppd-H1*	A	G	*Vrn-H3*	_	*vrn-H1*
Reference	Turner *et al.*, 2005										Yan *et al.*, 2006			Wang *et al.*, 2010	

paired student's t-test showed difference among each pair of DH lines. The development gap of the photoperiod-sensitive pair, 1-4 and 1-20 was larger (Figure 3.3B). DH line 1-20 showed slower development at early double ridge (W1.5) on 19 days after germination (DAG). On 23

DAG (W2.5), its development accelerated compared to the 1-67 and 10-70 pair that were in double ridge (W2) and triple mound stage (W2.25) respectively. From 23 to 27 DAG the development of the pair 1-4 and 1-20 moderately accelerated and at 27 DAG the pair 1-4 and 1-20 were at stamen primordium (W3.5) and lemma-floret primordium (W3.0) respectively. Their development sped up sharply from 27 DAG to 33 DAG and continued with slower pace from 33 DAG to 37 DAG. However DH line 1-20 was constantly delayed compared to 1-4. The main shoot of 1-4 and 1-20 headed at 44 and 49 DAG, respectively. The slope of regression line from 7 to 19 DAG for 1-4 and 1-20 was 0.15 and 0.09 respectively which revealed faster development rate for 1-4; however, it was almost similar from 23 DAG to heading day; 0.34 for 1-4 and 0.30 for 1-20. The pair 1-67 and 10-70 showed slower developmental rate from 23 DAG. DH line 1-67 was slightly delayed compared to 10-70. This pair did not show the biphasic pattern that was observed in 1-4 and 1-20. The main shoot of 10-70 and 1-67 headed at 72, 74 DAG.

From 7 to 27 DAG, broken line regression analysis showed change in rate of development for each pair (Figure 3.3C). The pair, DH lines 1-4 and 1-20, which showed more distinguished development were considered for further analysis.

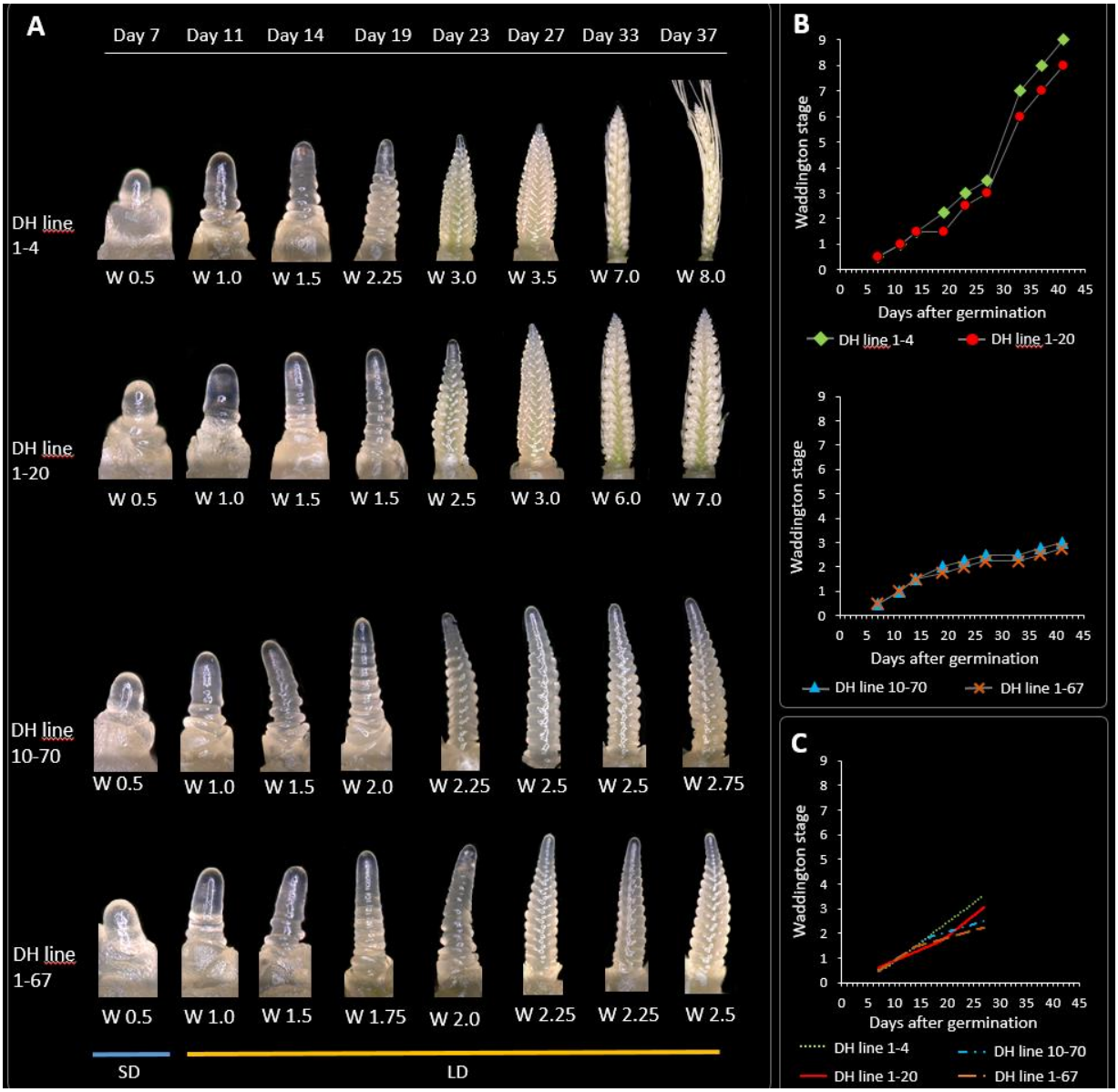

Fig. 3.3. Comparing development of main shoot apical meristems in two comparable pairs of MAGIC DH lines 1-4 vs 1-20 and 10-70 vs 1-67. Phenotypes of apex development under LD from 7 to 37 days after germination (A). Plots describing the apex development of under LD from 7 to 37 days after germination (B). Broken-line regression analysis shown for apex development among DH lines under LD from 7 to 27 days after germination (C).

Characterization of transcriptional changes and variants for HvHeading in apex and leaves

Transcript expression for DH lines 1-4 and 1-20 at three time-points revealed quantitative differences in apex and leaf tissue in *HvHeading* interval (Figure 3.4). Hierarchical cluster analysis distinguished and grouped all biological replicates of each time-point and genotype for apex tissue. However, for leaf tissue one biological replicate from time-point 19 days for each DH line and one biological for DH line 1-4 from time-point 33 did not cluster with other biological replicates and therefore, were not included in downstream analysis. Variant calling showed 31 high confidence genes (HC-G) carrying variants in this region (Supplementary Table

3.2). Results revealed differentially expressed transcripts for studied time-points in apex and leaf tissue for both DH lines in *HvHeading* region. RNA sequencing narrowed down the number of candidate genes based on differentially expressed genes. Differential expression analysis identified the same region using apex and leaf tissue; an interval of 8.00 Mbs in apex containing 105 high confidence genes (HC-G) and 8.40 Mbs in leaf which harbored 107 high confidence genes respectively according to the barley reference genome (Beier *et al.*, 2017; Mascher *et al.*, 2017). The region was located inside the interval detected by Afsharyan *et al.* (2020). Differential gene expression analysis was performed to compare DH lines 1-4 and 1-20 within the region. The results showed 3, 3 and 6 differentially expressed genes for apex tissue (Supplementary Table 3.3) and 4, 8 and 8 differentially expressed genes for leaf tissue (Supplementary Table 3.4) for time-points one (11 DAG), two (19 DAG) and three (33 DAG) respectively. The strongest differentially expressed gene in apex tissue was HORVU1Hr1G067820, annotated as a transcription elongation factor (*Spt6*) which was up-regulated in DH line 1-20 compared to DH line 1-4 (Figure 3.5A). The differential gene expression analysis of *Spt6* by RT-qPCR showed up-regulation in time-point two in apex tissue of DH line 1-20 during early stages of apex development (Figure 3.5B), which suggests that this gene could be considered for further analysis as candidate gene for *HvHeading*. Differential transcript expression analysis showed that gene HORVU1Hr1G067990 annotated as nuclear transcription factor Y subunit C-3 (*NFYC3*) was differentially expressed in leaves (Figure 3.5A)

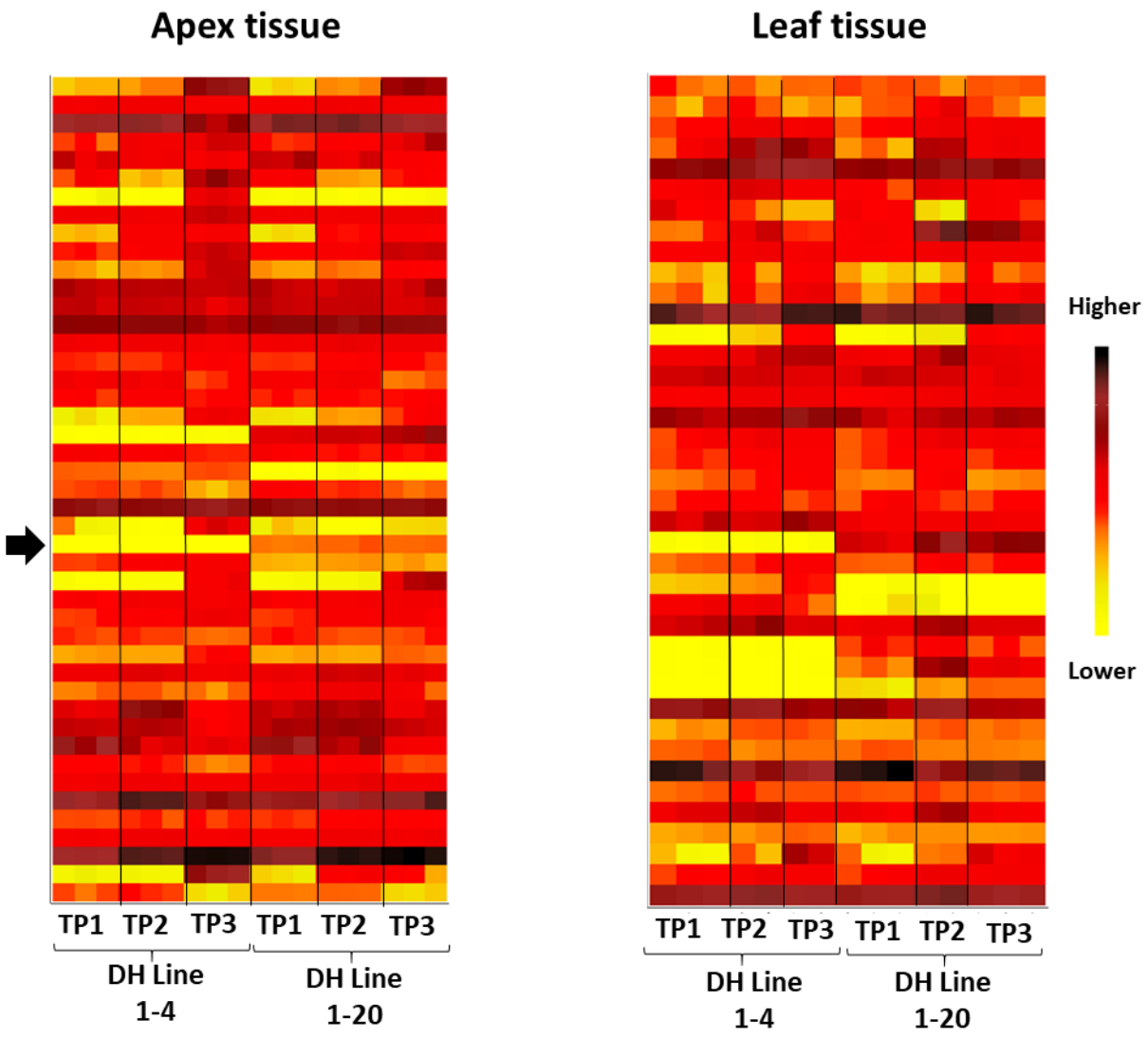

Fig. 3.4. Heatmap of transcript expression for *HvHeading* QTL interval in apex and leaf tissue of DH lines 1-4 and 1-20 in three time-points (TP); 11 (TP1), 19 (TP2) and 33 (TP3) days after germination. The selected candidate gene "HORVU1Hr1G067820" in apex tissue based on differential transcript expression and functional annotation is shown by black arrow.

and revealed a SNP polymorphism in variant calling. The differential gene expression analysis by RT-qPCR did not validate the RNA-seq results for *NFYC3* (Figure 3.5B). To sum it up, the results revealed that RNA-seq approach successfully narrowed down the number of candidate genes for *HvHeading*.

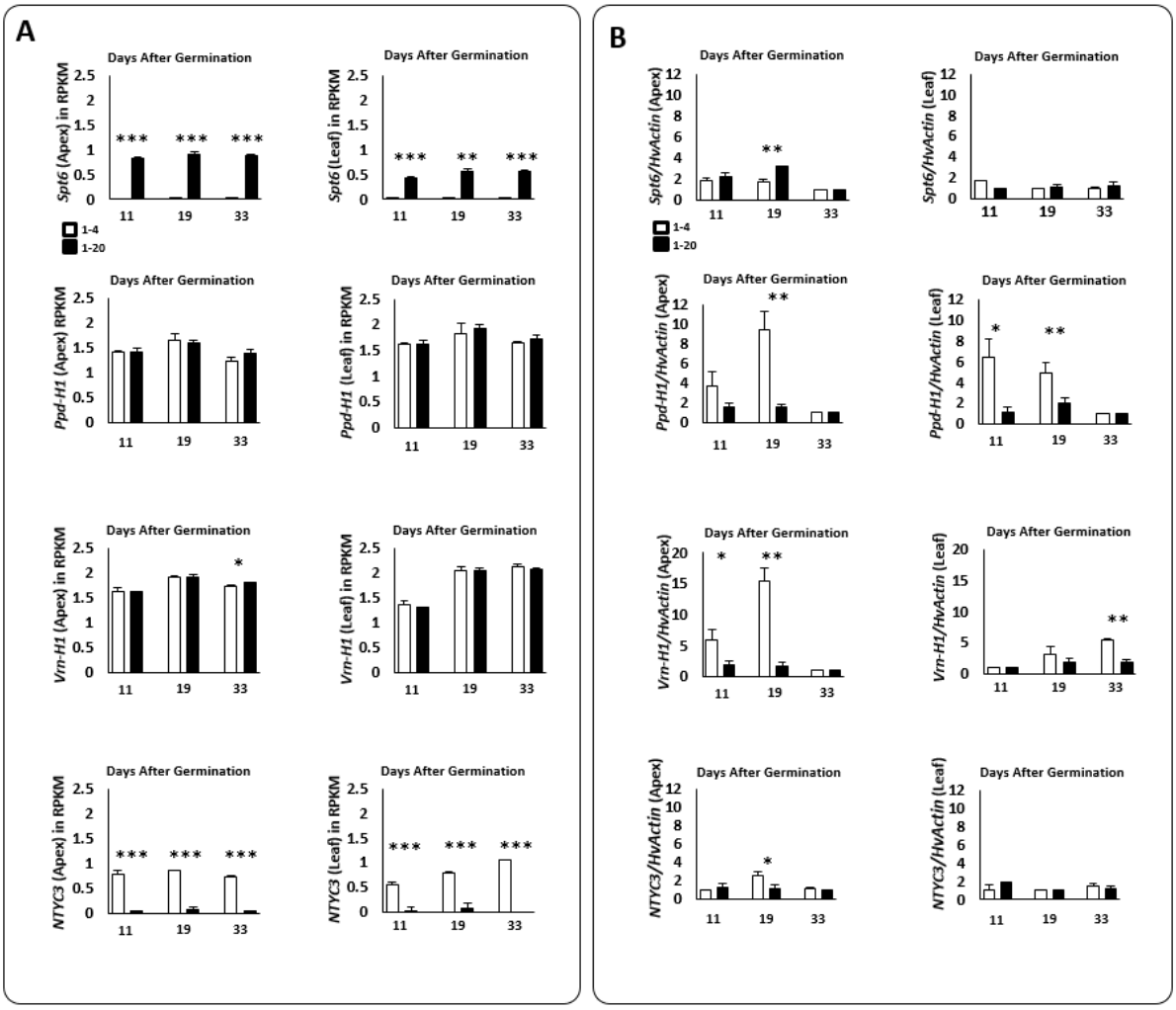

Fig. 3.5. Expression of selected genes in photoperiod-sensitive DH lines 1-4 and 1-20 using RNA-seq and real-time PCR in apex and leaf tissue under LD. A) Differential transcript expression of *Spt6*, *Ppd-H1*, *Vrn-H1* and *NTYC3* in RPKM. B) Differential gene expression of *Spt6*, *Ppd-H1*, *Vrn-H1* and *NTYC3*; the expression of each gene is shown relative to expression of *HvActin*. Significant of differential expression is indicated with asterisks with **P* <0.05, ***P* <0.01 and ****P* <0.001.

Differential expression analysis of major flowering time genes by RT-qPCR

Analysis of RNA-seq results showed that genes *Ppd-H1* and *Vrn-H1* had enough expression level to pass the threshold (expression levels ≥0.9 $\log_{10}$RPKM in at least two libraries) (Figure 3.5A); *Vrn-H3* had lower expression level and was not retained. The Differential gene expression analysis of *Ppd-H1* and *Vrn-H1* in the pair of photoperiod-sensitive DH lines 1-4 and 1-20 was investigated using RT-qPCR in apex and leaf tissue. Their relative expression was studied in time-points 11, 19 and 33 days after germination. The results distinguished DH

line 1-4 and 1-20, and showed that earlier-flowering DH line 1-4 had strong up-regulation in second time-point for *Ppd-H1* and *Vrn-H1* in apex tissue (Figure 3.5B).

Sequence analysis of conserved region and promoter analysis for the putative candidate gene HORVU1Hr1G067820 (Spt6)

Performing protein BLAST using *Spt6* protein sequence from Morex showed link to proteins in other model organisms (Figure 3.6) which indicated conserved regulatory elements within the coding sequences. Comparing *Spt6* gene transcript sequences of 1-4 and 1-20 by aligning them against the reference (Morex) showed a splice variant in DH line 1-20 (Figure 3.7) which was different from Morex. Sequencing of the promoter of HORVU1Hr1G067820 (*Spt6*) was performed to investigate the variations in gene promoter for DH lines 1-4 and 1-20. The sequenced region covered most (87.21%) of 2221 bp upstream of ATG. The sequence comparison revealed mutations across different putative transcription factor binding sites between DH lines 1-4 and 1-20 (Figure 3.8A). Further analysis is needed to determine the role of these mutations in regulation of gene expression by promoter. Sequencing about 1000 bp of the promoter in all parents of spring barley MAGIC population revealed a mutation only in Barke (Figure 3.8B).

Discussion

Effectiveness of targeted background effect elimination approach based on epistasis

Identifying the gene underlying *HvHeading* QTL required removing the background effects that could distort the effect of *HvHeading.* This QTL is prominently involved in epistatic interaction with regions corresponding to major genes *Ppd-H1*, *Vrn-H1* and *Vrn-H3* (Afsharyan *et al.*, 2020). One of the challenges for removing the background effects was finding one pair of genotypes that carry major epistatic interactions involving *HvHeading* with distinguishing phenotypic effect. Developing NILs using parents of MAGIC population did not seem to be a promising approach in this regard, since the only parent which carries different allele for *Ppd-H1*, *Vrn-H1* and *Vrn-H3* is Ragusa and epistatic interaction of each of their loci with both *HvHeading* alleles was evaluated to be in the same direction (Afsharyan *et al.*, 2020).

The comparable (flowering-time-specific-near-isogenic) pair of DH lines, 1-4 and 1-20, were selected according to similarity in flowering time loci and carried the same alleles *PPD-H1*, *VRN-H1* and *vrn-H3*. The line 1-20 showed a delayed apex and inflorescence development compared to DH line 1-4. The epistasis among *VRN-H1* and *HvHeading* shows opposite direction in 1-4 and 1-20 with a delaying effect in 1-20; the same direction with *vrn-H3* and *HvHeading* epistasis albeit with a much smaller effect (Afsharyan *et al.*, 2020). Therefore

strategy to use mapped epistatic flowering time regions for targeted background effect elimination resulted in finding MAGIC DH lines with flowering-time-specific-near-isogenic regions which successfully revealed the expected flowering-delaying effect of *HvHeading* QTL allele.

Detection of trait-specific-near-isogenic lines relies on precise mapping of QTL and epistasis that control the trait of interest as well as presence of reproducible homozygous lines. A high resolution DH mapping population, powerful mapping model and a proper approach in processing the raw data is mandatory for this purpose. Among mapping approaches, MAGIC strategy provides enough diversity as well as the possibility to control diversity (parents) (Cavanagh *et al.*, 2008), which makes MAGIC DH lines an ideal tool for finding trait-specific-near-isogenic regions.

Efficiency in detecting trait-specific-near-isogenic regions also depends on complexity of trait. When the trait is majorly controlled by few number of large effect QTL as is the case for flowering time in barley, the method is more likely to succeed.

Narrowing down epistatic HvHeading QTL interval

The *HvHeading* QTL is located on the long arm of chromosome 1H. The closest candidate gene to this interval is *Ppd-H2* (*HvFT3*) located at 93.l cM (Halliwell *et al.*, 2016) which is not associated with flowering time in spring barley MAGIC population (Afsharyan *et al.*, 2020). The genotyping of *HvHeading* interval using 910 MAGIC DH lines showed low levels of recombination in this region. Gene identification and map-based cloning in suppressed recombination regions of barley genome are challenging and resource intensive since targeting these regions by performing traditional approaches such as marker saturation and fine mapping is less effective (Sánchez-Martín *et al.*, 2016; Hatta *et al.*, 2019). An alternative approach is to evaluate the gene expression and genetic variants in the interval. Therefore, RNA-seq approach using the selected flowering-time-specific-near-isogenic MAGIC DH lines 1-4 and 1-20 was performed to provide an overview of the differential gene expression and genetic variation in the region and then using a candidate gene approach to narrow down the candidate genes. *HvHeading* was apparently having delaying effect on apex development, which was visible from double ridge stage in main shoot apex. This suggests that the expression of the gene underling this QTL is probably expected before or during double ridge stage. Therefore, we focused on transcripts in first and second time-point. A transcription elongation factor (*Spt6*) gene and a nuclear transcription factor Y subunit C-3 (*NTYC3*) gene showed expression patterns that matched the *HvHeading* phenotype in apex and leaves, respectively. *NTYC3* carried a SNP

variant that according to reference genome was located on intron one. Differential gene expression analysis by RT-qPCR validated up-regulation of *Spt6* in double-ridge stage in DH line 1-20. Additionally, the results showed down-regulation for *PPD-H1*, *VRN-H1* in 1-20 compared to 1-4 in 11 and 19 DAG (Figure 3.5B) suggesting that it could be due to the epistatic

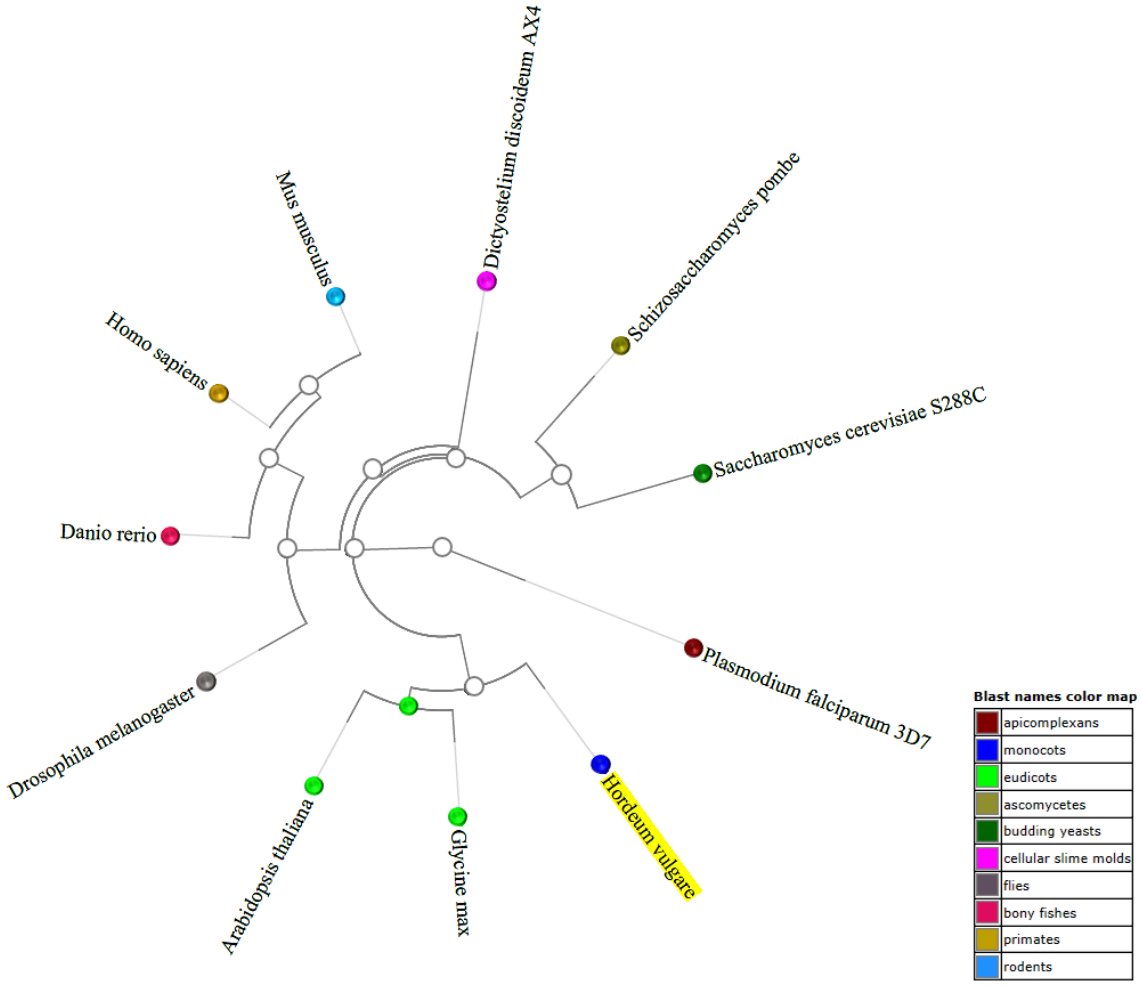

Fig. 3.6. Phylogenetic tree of *Spt6* protein for 10 model organisms including barley from Morex cultivar (highlighted) based on neighbor-joining method

effect of *HvHeading* QTL and was in line with the flowering-delaying effect reported to be linked with *HvHeading* QTL allele (Afsharyan *et al.*, 2020). Effect of different alleles of a gene on regulation of downstream genes has previously been reported. *FT1* (*Vrn-H3*) in the leaf was up-regulated in LD in introgression lines derived from spring barley cultivars Scarlet, Bowmann and Triumph that carried dominant *PPD-H1* allele compared to their respective spring barley allele (Digel *et al.*, 2015*b*). Early maturity 10 (*eam10*) introgression lines Bowman (*Ppd-H1*) and Bowman (*Ppd-H1* + *eam10*) derived from spring cultivar Bowman were compared regarding expression of *HvFT1* (*Vrn-H3*) under long day and short day conditions and *HvFT1* (*Vrn-H3*) was up-regulated in Bowman (*Ppd-H1* + *eam10*) under both conditions (Campoli *et al.*, 2013). Two early maturity 8 (*eam8*) introgression lines derived from

Bowman and Igri showed up regulation of *Ppd-H1* (Igri), *ppd-H1* (Bowman) and *HvFT1* (*Vrn-H3*) in SD (Faure *et al.*, 2012).

The results revealed that this approach successfully showed differential transcript expression in *HvHeading* QTL interval which lead to uncover *Spt6* as candidate gene for *HvHeading* QTL.

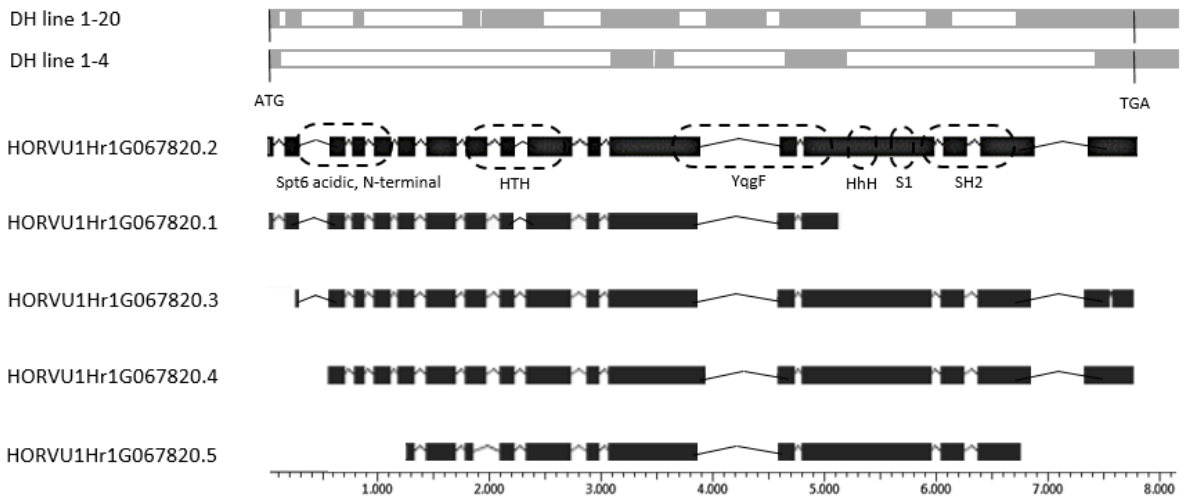

Fig. 3.7. Comparing coverage of RNA-seq reads for *Spt6* gene in DH lines 1-4 and 1-20 (grey) with five reported transcripts (black). Conserved domains of *Spt6* in barley is based on alignment of protein sequence of this gene in barley (HORVU1Hr1G067820.2) and *Saccharomyces cerevisiae*. Regions for conserved domains are shown with broken black lines. Length of the gene (bp) is displayed at the bottom. *In Silico* analysis was performed using Ensembl Plants (http://plants.ensembl.org) (Bolser *et al.*, 2016).

Spt6 as candidate gene underlying HvHeading QTL

Spt6 gene is reported to code a conserved transcription elongation factor (TEF), that controls transcription and chromatin structure and transcript initiation (Doris *et al.*, 2018). Phylogenetic analyses showed that *Spt6* protein from Morex (HORVU1Hr1G067820.2) aligned to similar proteins from 10 other model organisms such as *Arabidopsis*, yeast, human which suggested presence of conserved protein domains among these organisms (Figure 3.6). This was in line with reports that *Spt6* protein is essential for transcription elongation (Swanson *et al.*, 1990; Hartzog *et al.*, 1998; Endoh *et al.*, 2004; Ardehali *et al.*, 2009). Loss of normal *Spt6* function can lead to activation of cryptic promoters within the gene coding region (Kaplan *et al.*, 2003), effect mRNA 3′end formation (Kaplan *et al.*, 2005), splicing, and mRNA export (Yoh *et al.*, 2007). The *Spt6* protein from barley showed closer relation to model plants among other model

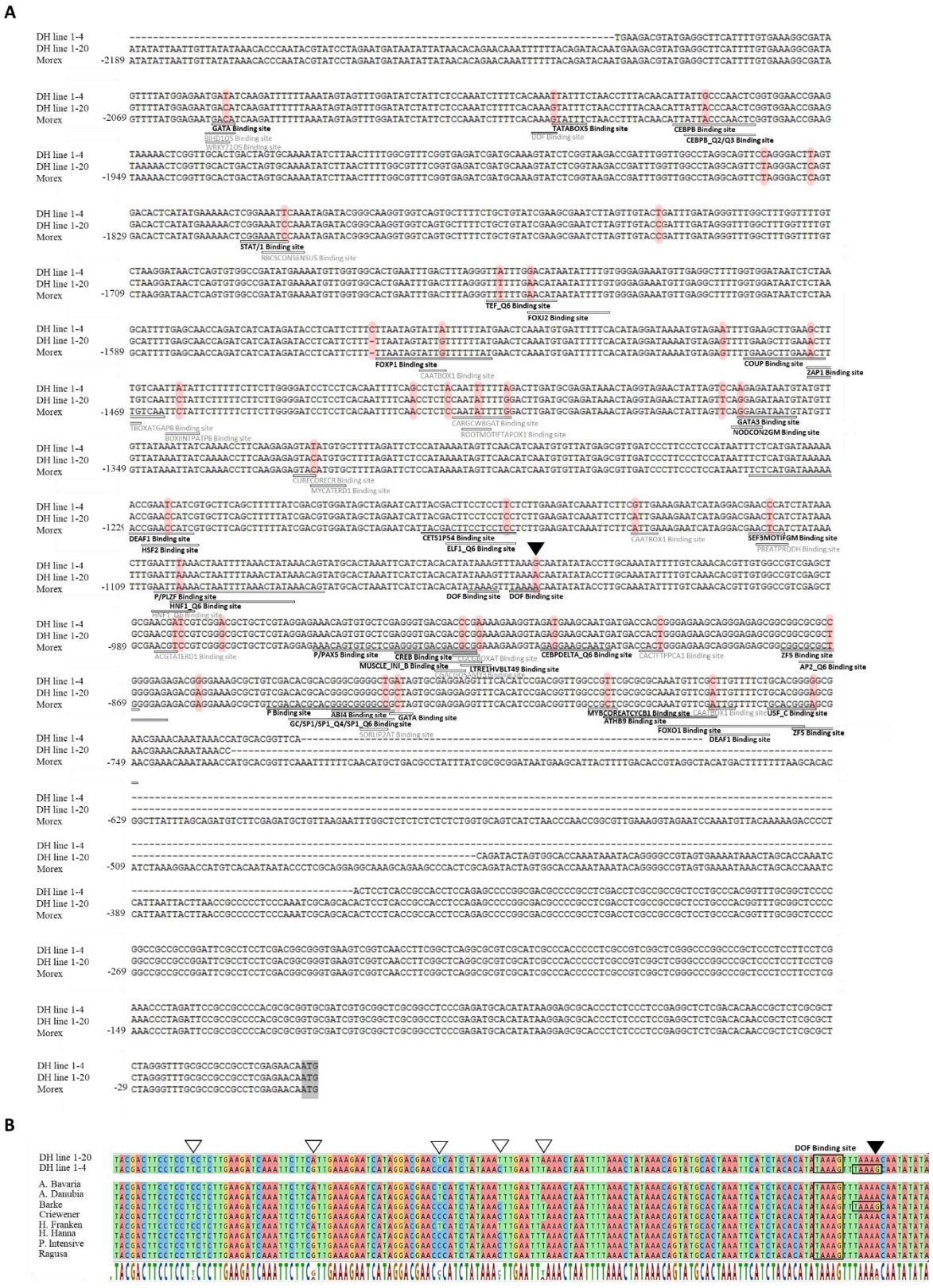

Fig. 3.8. Promoter sequence alignment of *Spt6* gene for DH lines 1-4 and 1-20. A) Promoter sequence alignment of *Spt6* gene for DH lines 1-4, 1-20 and spring barley cultivar Morex. B) Comparison of a portion of promoter sequenced in all parents of spring barley MAGIC population shows a mutation in Barke/line l-4 (shown with black pointer) which creates a double motif DOF binding site. SNP mutations are highlighted with red and pointers. The potential transcription factor binding sites that cover mutations based on DH line 1-4 (black) and 1-20 (grey) are shown with frames underneath the sequence. Broken lines indicate regions that are yet to be sequenced.

organisms as expected. The role of histone and chromatin modifiers has been extensively studied in flowering time pathway of *Arabidopsis* (Berr *et al.*, 2011; Van Lijsebettens and Grasser, 2014). *Spt6* is involved in Histone H3 lysine 36 (*H3K36*) methylation that mediates epigenetic regulation of flowering (Shi *et al.*, 2015) and affects temperature-induced alternative splicing and flowering in plants (Pajoro *et al.*, 2017). The candidate gene of *Spt6* in *Arabidopsis thaliana*, *Spt6l*, is reported to be involved with embryo development and the *spt6l* mutation causes embryonic lethality (Gu *et al.*, 2012). *H3K36* methylation is also reported to have a critical role in timing of flowering in rice (Sui *et al.*, 2013). Considering the involvement of *Spt6* in *H3K36* methylation, which plays a critical role in flowering time in plants and cereals, *Spt6* could be considered as the candidate gene underlying *HvHeading*. Analysis of majority of promoter region of *Spt6* for DH lines 1-4 and 1-20 showed mutations between DH lines 1-4 and 1-20 which influenced binding site of transcription factors (Figure 3.8A). Sequencing of a portion of promoter in parents of MAGIC population showed a mutation in Barke that created a double-motif DOF binding site (Figure 3.8B). DNA-binding protein with one finger (DOF) family is a plant-specific multigene family of transcription factors that were first discovered in maize (Yanagisawa, 1995). Members of this family cover various functions in mostly plant specific biological processes including being involved in negative regulation of biological processes (Boccaccini *et al.*, 2016). Studying the double-motif DOF binding site in promoters in *Arabidopsis* showed that they formed stable complexes with DOF domains compared to single motif binding sites (Sani *et al.*, 2018). Additionally, comparing the coverage of RNA-seq reads for *Spt6* gene in DH lines 1-4 and 1-20 with reported splice variants of *Spt6* (Figure 3.7) suggested that transcript from DH line 1-20 is a different isoform that is not reported before. Due to importance of conserved domains such as acidic N terminal (Swanson *et al.*, 1990) and SH2 (Yoh *et al.*, 2007; Dengl *et al.*, 2009; Close *et al.*, 2011) in *Spt6* protein function, this finding needs to be validated to study its impact further on flowering time in barley. Further studies are needed to elaborate the role of *HvHeading* candidate gene *Spt6* in the flowering time pathway of barley.

Conclusion

We showed that a targeted elimination of background effect approach based on epistasis which used spring barley MAGIC population and RNA-sequencing can be a useful tool to facilitate gene identification process in crops with complex genomes such as barley. Using this approach successfully narrowed down the *HvHeading* QTL interval to *Spt6* gene as a candidate gene in

flowering-time-specific-near-isogenic DH lines. Further studies are needed to functionally characterize the candidate gene and investigate its role in flowering time of barley.

Supplementary Material:

Table 3.1: Primers description for genotyping, RT-qPCR and sequencing to investigate candidate gene underlying *HvHeading* QTL

Table 3.2: Variants in *HvHeading* interval for MAGIC DH lines 1-4 and 1-20 according to RNA-seq data

Table 3.3: Differential expression analysis of *HvHeading* interval in apex for MAGIC DH lines 1-4 and 1-20 according to RNA-seq data

Table 3.4: Differential expression analysis of *HvHeading* interval in leaf for MAGIC DH lines 1-4 and 1-20 according to RNA-seq data

Acknowledgement

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Chapter 4

Genetic dissection of yield-related traits under terminal drought stress in spring barley MAGIC DH lines hints to pleiotropic effect of flowering time regions

Based on:

Afsharyan N. P. *, Sannemann W. *, Ballvora A, Léon J. (in preparation) Genetic dissection of yield-related traits under terminal drought stress in spring barley MAGIC DH lines

*These authors contributed equally to this work

Statistics and QTL mapping, Interpretation and discussion about QTL/candidate genes, Preparing the manuscript: Afsharyan N. P.; Foil tunnel experiment and Phenotyping: Sannemann W.

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Abstract

Barley (*Hordeum vulgare* L.) is the fourth most cultivated cereal worldwide and drought stress is a major cause of its yield loss. Flowering time indicates the transition of plant from vegetative to reproductive development and is involved in adaptation of crops to different environments including abiotic stress as well as determining grain yield. The aim of this study is to improve understanding of yield-related traits and investigate effect of flowering time genetic regions on other yield-related traits under different environments. This study used 534 spring barley MAGIC DH lines constructed of an eight-way cross of barley landraces known as "founders of German barley breeding" and one elite cultivar Barke. Seven yield-related traits were evaluated under well-watered and terminal drought treatments. The analysis of quantitative trait loci (QTL) for each treatment, maker by treatment interaction (M×T) and QTL for drought tolerance was conducted. Results showed that all traits were affected by treatment except for days to heading. Overall 69, 64 and 29 QTL were revealed for well-watered and terminal drought treatments as well as drought tolerance respectively. The M×T analysis detected total 25 loci for four traits. Identified QTL were located at regions that coincided with known genes/QTL as well as newly found regions. The results showed genetic regions for various traits which co-located with flowering time loci under well-watered and terminal drought, suggesting pleiotropic effect of flowering time genes such as *Ppd-H1*, *Vrn-H1*, *Vrn-H3* and *denso/sdw1* on grain yield and plant development. There were QTL with favorable effect on grain number, ear number and single grain weight under terminal drought including QTL corresponding to major flowering time gene *Ppd-H1* for grain weight. The results suggested that flowering time regions are involved in controlling grain weight components in barley through timing of flowering as well as their contribution to developmental mechanisms. The findings showed that spring barley MAGIC population can provide valuable insights into genetic control of yield-related traits under extreme environments including complex condition of drought.

Keywords: Barley, pleiotropic effect, terminal drought, flowering time, yield-related traits, MAGIC population, QTL analysis

Introduction

Barley (*Hordeum vulgare* L.), a major source for food and feed, is the fourth most grown cereal worldwide. Its domestication dates back to 10,000 years ago in arid and semi-arid areas in western Asia known as Fertile Crescent (Harlan and Zohary, 1966; Wang *et al.*, 2015). Flowering time is a key event which switches plants life cycle from vegetative to reproductive development and is affected by environment (Cockram *et al.*, 2007). Barley is known to be more tolerant to abiotic stresses and harsh climate compared to further small grain cereals. This feature, combined with self-pollination and diploidy, makes barley a model for complex genetic traits like yield or drought tolerance (Schulte *et al.*, 2009; Ullrich, 2010). Drought stress is one of the most important causes of barley yield losses (Jamieson *et al.*, 1995; Rollins *et al.*, 2013*b*) and is negatively correlated with grain number and thousand grain weight (Pennisi, 2008). Both yield or drought tolerance are highly complex traits which are influenced by various genetic pathways and environmental factors (Ozturk *et al.*, 2018; Sallam *et al.*, 2019). Timing of flowering is crucial in crop survival under drought stress and can influence yield, particularly by determining developmental phases of a plant's life (Slafer *et al.*, 2015).

A shortage of water in root zone results in drought stress; which can cause yield loss during all phases of plant life cycle (Salekdeh *et al.*, 2009). However, sensitivity to drought stress varies in different stages of crop development. Early-season drought stress can damage yield by reducing seedling survival during the vegetative development (Lelièvre, 1981). On the other hand, late-season drought which occurs during reproductive development, also known as terminal drought, can have devastating effect on the yield components grain number per unit area and thousand grain weight and is more likely to occur in field (Jamieson *et al.*, 1995; Farooq *et al.*, 2014; Shavrukov *et al.*, 2017). Grain number is determined in the pre-anthesis stage by number of spikelets and number of florets within each spikelet during floral meristem differentiation. Drought stress can reduce number of grains by causing loss of spikelets, increasing floret sterility and loss of seed set (Svobodová and Míša, 2004; Dolferus *et al.*, 2011). After anthesis and seedset, the main effect of drought is by reducing the grain fill rate and duration by restricting the final number of endosperm cells or limiting the rate and duration of starch accumulation in the endosperm (Setter and Flannigan, 2001). In barley, starch starts to accumulate in the endosperm cells from approximately 10 days to 45 days after anthesis which is decreased under drought stress due to restricted supply of assimilates into the grain (Wallwork *et al.*, 1998; Radchuk *et al.*, 2009).

During evolution, plants have developed several strategies including avoidance or tolerance to reduce the effect of stresses (Schulze *et al.*, 2005; Verslues *et al.*, 2006; Fitter and Hay, 2012).

Drought tolerance is controlled by various complex mechanisms that induce different proteins in response to abiotic stresses (Beck *et al.*, 2007; Anjum *et al.*, 2011). Abscisic acid (ABA) is a major phytohormone that plays an essential role in environmental stress response such as closure of stomata (Shinozaki and Yamaguchi-Shinozaki, 2007) and chlorophyll breakdown which leads to premature leaf senescence (Lim *et al.*, 2007). Other proteins that play crucial roles in abiotic stress response are involved in various mechanisms including regulation of protein degradation (Qin *et al.*, 2008; Lee *et al.*, 2009), protection of functional proteins (Xu *et al.*, 1996; Wang *et al.*, 2003; Cattivelli *et al.*, 2008; Augustine *et al.*, 2015), protein activation or deactivation (Zhu, 2002) as well as osmotic adjustment (Sayed *et al.*, 2012).

Flowering time is majorly regulated by pathways that interact with environment such as photoperiod and vernalization (Blümel *et al.*, 2015). Genes that control these pathways including photoperiod gene *Ppd-H1* (Turner *et al.*, 2005) and vernalization genes, *Vrn-H1* (Fu *et al.*, 2005) and *Vrn-H3* (Yan *et al.*, 2006), are reported to have pleiotropic effects on plant development and grain yield as well as on timing of developmental phases that contributes to maximize yield formation under harsh environmental conditions (Wang *et al.*, 2010; Wiegmann *et al.*, 2019). Therefore, it is important to better understand effect of flowering time genetic pathway on yield-related traits under different environments such as drought; which can provide more insights into genetic regulation of yield-related traits in barley in order to develop cultivars for different environments that maintain high yield (Cattivelli *et al.*, 2008; Wiegmann *et al.*, 2019). Past QTL mapping efforts in barley under drought stress used various approaches such as advanced-backcross (AB) population (Sayed *et al.*, 2012), interrogation lines (ILs) (Honsdorf *et al.*, 2014, 2017) and association panel (Wehner *et al.*, 2015). However, large genotype by environment (GxE) interactions and reduced trait heritability under drought condition resulted in detecting small effect QTL for traits and their drought tolerance (Abdel-Haleem *et al.*, 2012; Li *et al.*, 2013; Honsdorf *et al.*, 2014; Wehner *et al.*, 2015; Sallam *et al.*, 2019). One study used a multiparental population for QTL mapping of traits such as dry weight, fresh weight, plant height, tiller number under control and drought stressed condition (Pham *et al.*, 2019). However, reports for using multiparental populations as a more advanced mapping approach are rare. Multiparent advanced generation intercross (MAGIC) strategy was designed to increase the power of QTL mapping (Cavanagh *et al.*, 2008; King *et al.*, 2012; Bandillo *et al.*, 2013). In this study, we used a spring barley MAGIC population constructed from eight-way cross of seven landraces known as "founders of German barley" and one elite cultivar Barke (Sannemann *et al.*, 2015) which has been utilized recently in a series of flowering time studies (Sannemann *et al.*, 2015; Maurer *et al.*, 2017; Mathew *et al.*, 2018; Afsharyan *et al.*,

2020). Using this MAGIC population, showed very precise recognition of known flowering time loci as a proof of concept in addition to identifying new loci.

Here we present evaluation and QTL mapping of seven yield-related traits including flowering time as well as investigation of effect of flowering time genetic regions on other yield-related traits using spring barley MAGIC DH lines under well-watered and terminal drought treatments. The aim is to (1) evaluate the effect of well-watered and terminal drought treatments on MAGIC DH lines, (2) explore genetic regions that control the traits under both treatments as well as genetic regions that control marker by treatment interactions and drought tolerance, (3) investigate pleiotropic effect of flowering time regions under both treatments, and (4) identify loci linked with flowering in barley that improve ear number, grain number and grain weight under terminal drought.

Material and methods

Plant material and Experimental set up

A set of 534 spring barley (*Hordeum vulgare* ssp. *vulgare*) MAGIC DH lines were randomly chosen from an eight-way cross population constructed as described by Sannemann *et al.,* (2015). To study yield-related traits under well-watered and terminal drought treatments, MAGIC DH lines and a set of controls consisting of eight parents and spring barley varieties were sown into 19.5 × 25.5 cm plastic pots filled with 5.5 liter of Terrasoil®. For each treatment, four seeds per genotype were sown in each pot on April 4th (mean temperature: 11.99 °C), and April 3rd (mean temperature: 12.51 °C) in 2011 and 2012 respectively. An augmented experimental block design was used under foil tunnel which consisted of 20 varieties as checks with replicates every 20 pots at Campus Poppelsdorf (50°43'34.1"N; 7°05'14.6"E) of University of Bonn, Institute of Crop Science and Resource Conservation, Chair of Plant Breeding. Daily temperature from sowing to the end of growth season was recorded for each year. Daily minimum, mean and maximum temperature for growth season in each year is presented in Supplementary Dataset 4.1. The pots were irrigated three times a day (6:15 am, 0:15 pm, 6:15 pm) using a computer mediated drip irrigation system to keep the volumetric water content (VWC) at 40%. The terminal drought was imposed for five weeks starting from 35 days after sowing (DAS) and was composed of three stages. During the first 21 days the water content in the pots was reduced to the permanent wilting point (15% VWC), then for seven days the water content was stabilized at 15% and finally, at 65 DAS the pots under terminal drought treatment were re-watered slowly to 30% VWC, and finally at 73 DAS re-watered to 40% VWC. For well-watered treatment, VWC was sustained at 40% throughout the experiment.

Phenotypic parameters

The phenotypic values for days to heading (DHE), grain filling period (GFP), plant height (PLH), above ground biomass (AGB), number of ears (NE), number of kernels (NK), and thousand kernel weight (TKW) were measured as described in Table 4.1. For evaluating stress tolerance (ST) for traits that were affected by terminal drought treatment (≤0.001), stress tolerance index (STI) was calculated for each DH line in each year using the following equation:

$$STI = \frac{y_p + y_s}{\bar{y}_p}$$

where y_p: the trait for genotype under well-watered treatment, y_s: the trait for genotype under terminal drought stress, and $\bar{y}_p$: the mean for the trait for genotype under well-watered treatment (Fernandez, 1992).

Statistical analysis

Analysis of phenotypic data

Descriptive statistics were calculated for seven traits across two years by using summary function from core generic functions of R software to determine minimum, maximum, mean. Functions std.error; var and sd were used to calculate standard error, standard deviation (SD) and coefficient of variation (CV) respectively using R software (R core team 2015). Analysis of variance (ANOVA) was performed for each trait using the restricted maximum likelihood (REML) method by PROC MIXED procedure in SAS (9.4 version, SAS Institute Inc., Cary, NC, USA) to fit the following mixed model:

$Yijk = \mu + Li + Tj + Ck + Li \times Tj + \varepsilon ijk$

Where $Yijk$ is response variable; μ is general mean; Li is the random effect of i-th DH-line; Tj is the fixed effect of j-th treatment; Ck is the random effect of the k-th calendar year; $Li \times Tj$ is the random effect of interaction of i-th DH-line with j-th treatment and εijk is the residual.

Variance components were estimated for each treatment by taking genotype (non-replicated MAGIC DH lines), year and genotype × year interaction as random effects using REML method by PROC VARCOMP (all effects as random) in SAS and broad sense heritability (H^2) for each trait was estimated as:

$$H^2_{(well-watered\ or\ drought)} = \frac{V_G}{V_G + \frac{V_{GY}}{y} + \frac{V_E}{y}}$$

where V_G: variance of genotype; V_{GY}: variance of genotype × year; V_E: variance of experimental error; and y: number of years.

Effect of row and column were evaluated separately for well-watered and terminal drought treatment in R software with lm function to fit a fixed model by taking non-replicated MAGIC DH-lines, row, column and controls as fixed effects. LSmeans was calculated for each trait using lsmeans function from "emmeans" package; then LSmeans was used to calculate correlations between traits for each treatment. Correlation coefficients was computed by the Pearson's coefficient (r) using rcorr function from package "Hmisc" in R software.

Marker-trait association analysis

For marker-trait association analysis, this study used a processed set of 5,199 SNPs (MAF ≥1%) from Afsharyan *et al.,* (2020) which described handling of missing data and minor allele frequency (MAF). This data set was produced by genotyping 534 spring barley MAGIC DH lines by Illumina 9k iSelect SNP array (Comadran *et al.*, 2012) processed by Sannemann *et al.*, (2015). Due to un-replicated experiment design, the BLUP (Best Linear Unbiased Prediction) values for each treatment in each year were produced separately using lmer function from package "lme4" in R software (R Core Team 2015). The analysis was performed using REML to fit a mixed model by considering non-replicated MAGIC DH-lines as random effects. Then ranef function from the same package was used to estimate BLUP values for QTL analysis under well-watered and terminal drought treatments. For traits affected by treatment, values for drought tolerance index was used to analyze QTL that control drought stress tolerance (ST) for each respective traits.

The QTL analysis was performed separately for well-watered treatment, terminal drought treatment, marker × treatment interaction and drought stress tolerance (ST) using the PROC MIXED procedure in SAS 9.4 by the following linear model:

$Yij = \mu + Mi + Tj + Mi \times Tj + \varepsilon ij$

Where *Yij*: response variable; μ: general mean; *Mi:* the fixed effect of *i*-th marker; *Tj*: the fixed effect of the *j*-th treatment (only included for marker by treatment interaction analysis); $Mi \times Tj$: the fixed interaction effect of *i*-th marker with *j*-th treatment (only included for marker by treatment interaction analysis) and εij: the residual. The possibility of detecting false positive QTL was addressed by implementing multi-locus analysis (Sillanpää and Corander, 2002; Kilpikari and Sillanpää, 2003; Bauer *et al.*, 2009) and incorporating the control of the QTL false-discovery rate (FDR value ≤0.05) inside the model using PROC MULTTEST procedure. The QTL intervals were determined in the first iteration of multi-locus by clustering SNPs based on their significance. The model defined the significant of the SNPs by a threshold of *P*-value ≤0.001 with 1000 permutations and FDR value ≤0.05 (Doerge and Churchill, 1996). The significance of QTL were validated by calculating the mean p-value of 20 rounds of a

‘‘leave20%out’’ cross-validation procedure. The codes and procedure regarding QTL analysis was described in detail by Afsharyan *et al.* (2020). The confident interval was set as 3.5 cM on both sides of the most significant SNP marker based on LD (linkage disequilibrium) of the population (Sannemann *et al.*, 2015). Genetic variance explained by a single SNP marker (R_M^2) was conducted as:

$R_M^2 = SQ_M/SQ_g$

where SQ_M: sum of squares of *M*; SQ_g:type I sum of squares of the barley MAGIC DH lines in an ANOVA model (Von Korff *et al.*, 2006). Additionally, total explained genetic variance by all QTL was determined.

Based on population LD, a window of 7 cM was determined to compare detected QTL positions that co-locate in the same region as known genes/QTL described in literature that used the same genetic map and markers (Comadran *et al.*, 2012; Alqudah *et al.*, 2014, 2016, 2018; Maurer *et al.*, 2015, 2016; Sannemann *et al.*, 2015; Mathew *et al.*, 2018; Pham *et al.*, 2019; Afsharyan *et al.*, 2020). *In silico* analysis was performed using the IPK barley BLAST server (Colmsee *et al.*, 2015).

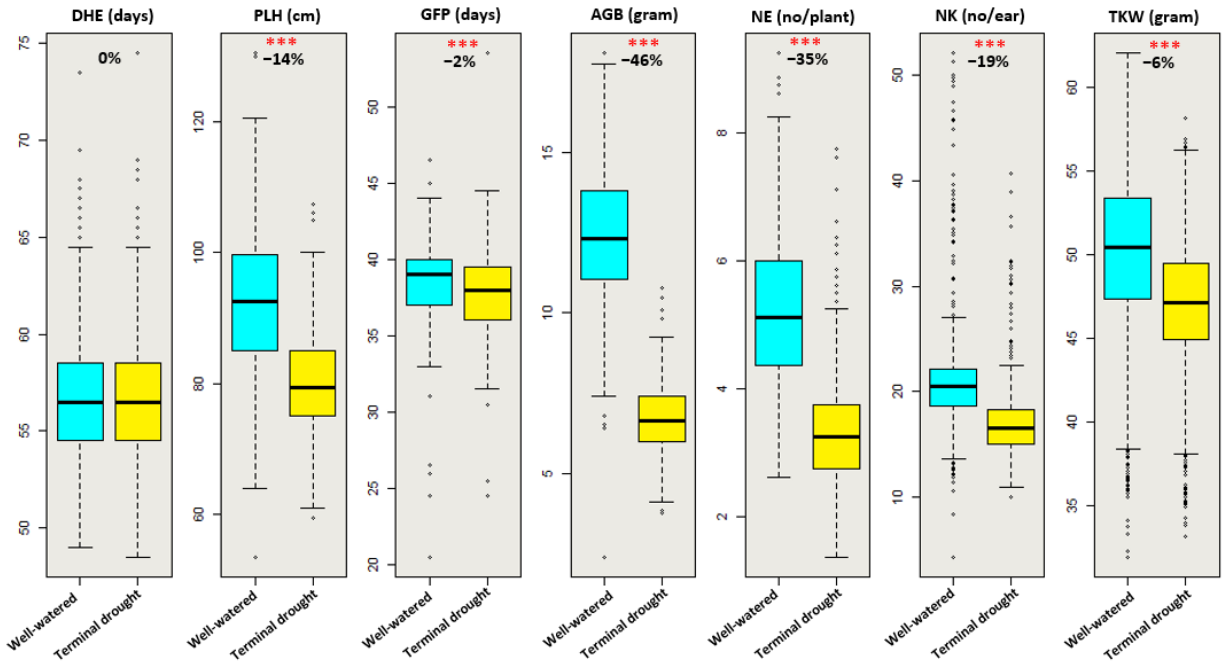


Fig. 4.1. Box plots describe the variation for seven traits in 534 spring barley MAGIC DH lines under well-watered and terminal drought treatments using mean values for 2011 and 2012. Trait names and units are indicated above their respective sub-plot. Trait abbreviations are DHE: days to heading, PLH: plant height, AGB: above ground biomass, GFP: grain filling period, NE: number of ears, NK: number of kernels, TKW: thousand kernel weight. Significant treatment effect is indicated with red asterisks with **P* <0.05, ***P* <0.01 and ****P* <0.001. Change in trait mean (%) under terminal drought treatment compared to well-watered is shown below the asterisks.

Results

Evaluating traits under well-watered and terminal drought treatments in MAGIC DH lines

A set of 534 spring barley MAGIC DH lines were used in a pot experiment in a foil tunnel to study seven yield-related traits under well-watered and terminal drought treatments in two consecutive years. The investigated traits showed lower mean values for terminal drought treatment compared to well-watered except for DHE (Figure 4.1, Table 4.1). Comparing the CV, DH lines had lower variation under terminal drought treatment for PLH, NK, TKW and DHE than under well-watered treatment; while for GFP, NE, AGB the variation under terminal drought was higher. Apart from the column effect for TKW under terminal drought, the row and column effects ($p < 0.001$) did not differ significantly from zero for either trait under both treatments. Heritability (H^2) was estimated to be >50% for DHE, PLH, NK and TKW in both well-watered and terminal drought. ANOVA revealed that treatment effects were significant ($P < 0.001$) for all traits except for DHE (Supplementary Table 4.1) and depending on the trait, showed a reduction ranging from 2% to 46% under terminal drought treatment (Figure 4.1). Correlation coefficients (Supplementary Table 4.2) revealed higher correlation between AGB and PLH (r=0.69) as well as AGB and NE (r=0.66) for well-watered treatment and AGB and

Table 4.1. Descriptive statistics, heritability (H^2) under well-watered and terminal drought treatments for spring barley MAGIC population

					Parents	MAGIC population						
Trait	Description	Methods of measurement	Unit	Treat[a]	Mean[b]	Min[b]	Max[b]	Mean[b]	SE[b]	SD[b]	CV[b]	H^2
DHE	Days to heading	number of days from sowing until emergence of 3 cm of awns	days	Well-watered	54.90	44.00	76.00	56.87	0.15	5.00	8.79	0.73
				Terminal drought	55.10	44.00	76.00	56.80	0.15	4.78	8.40	0.75
GFP	grain filling period	number of days from heading to hard dough ripening	days	Well-watered	38.20	8.00	53.00	38.50	0.10	3.38	8.79	0.23
				Terminal drought	38.00	10.00	65.00	37.76	0.11	3.47	9.21	0.27
PLH	plant height	distance between soil ground level and tip of awns in cm	cm	Well-watered	95.90	44.00	140.00	92.16	0.48	15.75	17.09	0.54
				Terminal drought	85.00	52.00	119.00	79.53	0.31	10.27	12.92	0.66
AGB	above ground biomass	amount of dry above-ground biomass	g/plant	Well-watered	11.60	1.26	19.15	12.00	0.09	3.00	24.05	0.28
				Terminal drought	6.70	2.04	11.24	6.74	0.06	1.88	27.97	0.002
NE	number of ears	number of ripe ears	no/plant	Well-watered	4.20	0.50	11.25	5.31	0.05	1.59	29.82	0.33
				Terminal drought	2.90	1.00	10.00	3.43	0.04	1.30	37.88	0.24
NK	number of kernels	amount of grains per ear	no/ear	Well-watered	25.50	1.65	73.33	21.06	0.20	6.40	30.39	0.70
				Terminal drought	20.80	2.25	46.00	16.96	0.14	4.70	27.73	0.59
TKW	thousand kernel weight	weight of 1000 grains	gram	Well-watered	52.20	28.57	64.46	49.65	0.20	6.38	12.86	0.69
				Terminal drought	50.30	17.03	69.87	46.81	0.15	4.97	10.62	0.70

[a] Well-watered and terminal drought stress treatment

[b] Mean, minimum, maximum, mean, standard error, Standard deviation in % (SD), coefficient of variation (CV) (standard deviation divided by mean)

NE (r=0.63) for terminal drought. The highest average and CV for stress tolerance (ST) belonged to NE-ST and the lowest was for PLH-ST. The heritability ranged from 21% for AGB-

ST to 78% for TKW-ST (Supplementary Table 4.3). Correlations coefficients for stress tolerance were highest for NE-ST and AGB-ST (r=0.65) (Supplementary Table 4.4).

Analysis of QTL under well-watered and terminal drought treatment and marker by treatment interaction

QTL mapping was performed to identify the associated genetic regions under well-watered and terminal drought treatments for each trait (Figure 4.2). Results showed that some genetic regions harbored QTL for various traits or treatments such as region on 2H chromosome (18.90 - 28.70 cM), but others contained trait and/or treatment specific QTL such as region on 1H (71.03 cM) for flowering time and region on 6H (118.4 - 119.0 cM) for well-watered treatment (Figure 4.3). In total 69 and 64 QTL were found for well-watered (Supplementary Table 4.5) and terminal drought (Supplementary Table 4.6) treatments respectively.

The number of total loci associated with each trait under both treatments ranged from ten QTL for AGB to 25 QTL for PLH. Genetic variation explained by detected QTL for each trait under well-watered treatment ranged from 11.29% for AGB to 60.10% for NK and under terminal drought spanned from 8.71% for AGB to 52.88% for TKW. Among investigated traits, DHE was controlled mostly by the same genetic regions under well-watered and terminal drought treatments. The most significant QTL was located in the same loci under both treamtmnet for most of the traits. The prominat region associated QTL was located on chromosome 2H (19.90 cM) for GFP, 2H 76.66 cM for NK and TSW, 3H (109.21 cM) for PLH, 5H (95.14 - 121.25 cM) for NE and 7H (32.79 cM) for DHE. For AGB, the most significant QTL was detected on 4H chromosome (97.17 cM) under well-watered treatment and on 7H (32.79 cM) under terminal drought. The results revealed three, four, five, six, seven and nine treatment specific QTL for terminal drought associated with GFP, NK, AGB, TKW, NE and PLH respectively.

Analysis of marker by treatment interaction was conducted for traits affected by treatment (Figure 4.2). The results revealed overall 25 genetic regions interacting with treatment for four traits from which 19 loci were also detected under well-watered and/or terminal drought treatments (Supplementary Table 4.7). The number of detected loci for each trait ranged from ten for GFP to four for PLH. The prominat region interacting with treatment for GFP and NK was identified on charomosome 2H at 19.90 cM and 76.66 cM respectively. The most significant locus involved in treatment intaraction for PLH and AGB was located on 3H (49.29-51.20 cM).

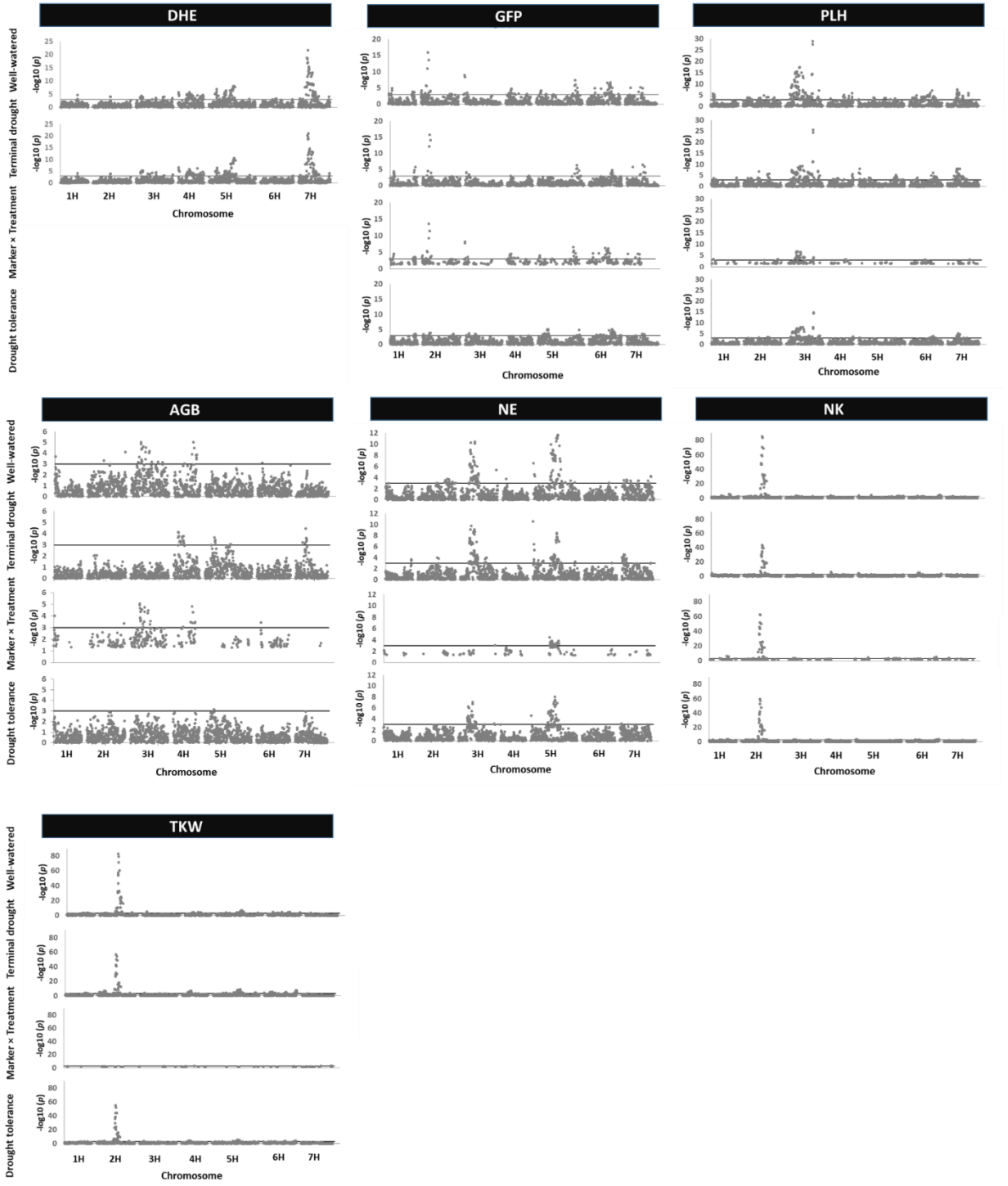


Fig. 4.2. Manhattan plots describes QTL analysis of seven traits for well-watered condition, terminal drought condition, marker × treatment interaction and drought tolerance using the spring barley MAGIC population. Trait abbreviations are DHE: days to heading, PLH: plant height, AGB: above ground biomass, GFP: grain filling period, NE: number of ears, NK: number of kernels, TKW: thousand kernel weight. The y axes denote the significance of SNP markers as -log10 (*P*); the chromosomes are denoted on the x axes. The SNP markers above the cut-off line are significant by a threshold of $P \leq 0.001$ with 1000 permutations plus 20 times cross-validation.

QTL analysis for drought tolerance

QTL regions associated with drought stress tolerance were evaluated for each trait (from here on referred as trait-ST) affected by treatment (Figure 4.2). In total 29 QTL were identified (Supplementary Table 4.8) from which overall 24 genetic regions co-localized with QTL identified for the respective traits under two treatments. For GFP-ST 11 QTL regions were found that explained total genetic variation of 28.59%. four QTL regions on chromosome 1H (122.10 cM), 2H (19.9 cM), 5H (155.56 cM) and 6H (72.18 cM) co-localized with locus found under terminal drought treatment and were also involved with treatment interaction. Another region on 3H chromosome (2.41 cM) coincided with a QTL for well-watered treatment which was also detected for treatment interaction. All estimated seven QTL regions for PLH-ST explained 36.57% of genetic variation. Among them, regions on 3H (109.21 cM) were commonly found for PLH under both treatments as well as treatment interaction. While loci on 7H (41.43 cM) and 3H (62.54 cM) co-localized with regions for both treatments and terminal drought respectively. Three QTL were identified for NE-ST that explained 17.51% of genetic variance. The region on 3H (51.35 cM) was commonly located for NE under well-watered and terminal drought treatments. QTL analysis for NK-ST revealed two regions including a QTL on 2H (79.89 cM) which co-localized with the prominent locus (2H, 76.66 cM) for both well-watered and terminal drought treatments as well as treatment interaction. For TKW-ST, six positions were located from which one on 6H (48.80 cM) co-localized with a terminal drought spesific QTL. No QTL was identified for AGB-ST.

Genetic regions with pleitropic effect

The loci for all identified QTL (well-watered, terminal drought and ST) were compared in a window of 7 cM to determine genetic regions that controled various traits (Figure 4.3; Supplementary Table 4.5, 4.6, 4.8). The findings revealed 15 common regions linked to at least three traits. The most common region was located on 5H (44.24-51.46 cM) and was associated with five traits including, DHE (well-watered, terminal drought), GFP (well-watered, terminal drought, ST), AGB (terminal drought, ST), NE (terminal drought), TKW (well-watered, ST).

Discussion

Effect of different environments (treatment) on spring barley MAGIC population

Spring barley MAGIC DH lines were investigated for seven traits under well-watered and terminal drought treatment in pot experiment under foil tunnel. The traits showed various extent

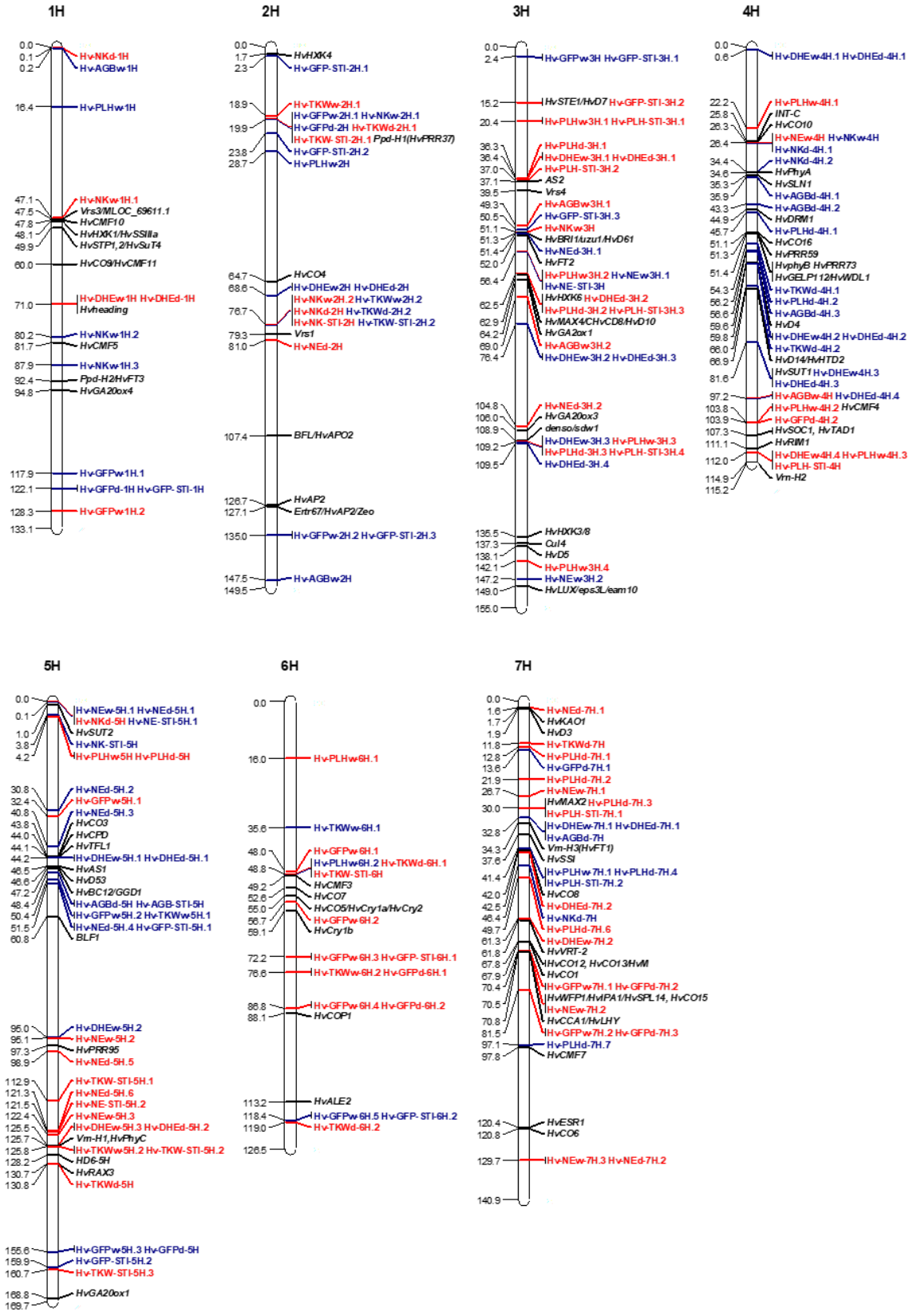


Fig. 4.3. Genetic map of QTL detected for seven traits under well-watered and terminal drought treatments as well as stress tolerance in spring barley MAGIC DH lines. The located QTL are detailed in supplementary tables 4.5, 4.6 and 4.8. Barley chromosomes are exhibited with white bars. The position for the peak SNP marker represents each QTL which is colored according to increasing (red) or decreasing (blue) effect of the minor allele of QTL. The name of known genes as described for the Barke × Morex RILs by (Mascher *et al.*, 2013*a*) are italicized in black indicating their position on chromosomes.

of decrease under terminal drought stress, except for DHE; suggesting that starting terminal drought treatment less than two weeks before start of heading did not affect its timing. Loss in NE and NK was observed under terminal drought. Increase in number of sterile ears per plant (Mogensen, 1992; Sánchez-Díaz *et al.*, 2002; Samarah, 2005) and loss in seed set (Mogensen, 1992; González *et al.*, 1999; Del Moral *et al.*, 2003) is a major reason for reduction in number of ears and grains of barley during drought. Competition of spikes and elongating stems for assimilates during late reproductive phase is reported to be linked with higher possibility of floret abortion under drought stress (Miralles *et al.*, 2000; González *et al.*, 2003). However, number of sterile fully developed florets of MAGIC DH lines was not affected by treatment in this study; implying that it could be due to timing of treatment which began in a more advanced stage of inflorescence development (shortly before start of heading) and reached maximum severity during grain filling period (after seed set). Therefore, other reasons might be responsible for loss in number of grains such as grain abortion. Height reduction under terminal drought in MAGIC DH lines might be result of shorter vegetative development period due to accelerated plant life cycle (González *et al.*, 1999; Samarah *et al.*, 2009), decreased gross photosynthetic rate and osmotic potential (Hopkins and Wilhelmova, 1997; González *et al.*, 1999; Taiz and Zeiger, 2002). AGB showed more reduction under terminal drought compared to other traits in MAGIC DH lines which might explain its very low heritability. Also traits that are positively correlated with AGB such as NE and PLH were negatively affected by terminal drought treatment. The research has shown strong loss in dried above ground biomass under drought condition (Samarah *et al.*, 2009) which is reported to be connected to decreased plant height (González *et al.*, 1999; Sánchez-Díaz *et al.*, 2002). Loss of grain yield components including grain weight, grain number and ear number is an indication of yield reduction under drought in barley. However, since grain number is the most important component of cereal yield (Slafer, 2003; Reynolds *et al.*, 2009; Sreenivasulu and Schnurbusch, 2012), reduction of ear number and grain number is reported to be strongly connected to yield loss under drought stress (Samarah *et al.*, 2009). GFP, TKW, NK and NE reduced in MAGIC DH lines under terminal drought. In barley, remobilization of assimilates from straw to grains is accelerated under drought stress which leads to faster loss of grain moisture, faster maturity and earlier senescence (Mogensen, 1992; González *et al.*, 1999; Sánchez-Díaz *et al.*, 2002; Asseng and Van Herwaarden, 2003; Del Moral *et al.*, 2003; Samarah, 2005; Ehdaie *et al.*, 2008). Therefore, as a result of shorter grain filling period and limited assimilates, rate and duration of starch accumulation in the endosperm of grains is decreased which leads to loss of grain weight (Brooks *et al.*, 1982; González *et al.*, 1999; Sánchez-Díaz *et al.*, 2002; Del Moral *et al.*, 2003).

On the other hand, correlation of TKW with NK and NE was stronger and in opposite direction of its correlation with GFP; suggesting that rise in source–sink ratios by reduction in total number of grains (Serrago *et al.*, 2013) led to increase in weight of each grain in DH lines and partly compensated the negative effect of shorter grain filling period on single grain weight.

QTL analysis to investigate the traits under well-watered and terminal drought treatment

Genetic regions that control studied traits under well-watered and terminal drought treatment were detected on all chromosomes of barley (Figure 4.2). This study identified distinctive QTL for each treatment for six traits affected by treatment as well as recognizing QTL involved in marker by treatment interaction for four of them (Supplementary Table 4.5 - 4.7). The majority of detected QTL coincided with known gene/QTL regions with high precision (Supplementary Table 4.5 – 4.8) including the peak marker BK_12 on chromosome 2H which is gene-specific for *Ppd-H1* gene (Colmsee *et al.*, 2015). DHE was not influenced by terminal drought, nevertheless it was included in QTL analysis for both treatments for comparison to other traits. More QTL were detected for DHE in this study compared to Sannemann *et al.* (2015) which utilized different method for preparing phenotypic and genotypic data including higher MAF threshold as well as QTL analysis. The genetic regions associated with drought tolerance were detected for five traits and many of them co-localized with loci identified for same trait under well-watered and terminal drought treatment and marker by treatment interaction (Supplementary Table 4.8). Compared to marker by treatment interaction, QTL analysis for drought tolerance found more loci for more traits; including QTL that coincided with regions associated with one treatment. This suggested that, this analysis was more efficient in explaining the regions involved in tolerance under drought compared to marker by treatment interaction in this study. Research has shown that QTL mapping for traits under complex condition of drought and their drought tolerance is challenging (Abdel-Haleem *et al.*, 2012; Li *et al.*, 2013; Wehner *et al.*, 2015). Involvement of many genes for drought tolerance would produce many small effect marker-trait associations (Sallam *et al.*, 2019) that could be missed during the attempt for mapping (Honsdorf *et al.*, 2014). However, the approach presented in this study successfully detected regions which majority of them overlapped with QTL identified for respective traits under both or either treatments including regions corresponding known genes. Genetic regions for genes *Ppd-H1*, *Vrn-H1* and *Vrn-H3* which were reported to be associated with flowering time in spring barley MAGIC population (Sannemann *et al.*, 2015; Mathew *et al.*, 2018; Afsharyan *et al.*, 2020) were detected for DHE (well-watered, terminal drought) in this study as well. The locus for *Vrs1 (Six-rowed spike 1),* the yield component gene

involved in determining the number of grains in barley inflorescence (Komatsuda *et al.*, 2007), aligned with QTL for yield component traits; TKW (2H, 76.66 cM; well-watered, terminal drought, ST), NK (2H, 76.66 cM; well-watered, terminal drought, ST) and NE (2H, 80.95 cM; terminal drought) (Figure 4.3). This region showed interaction with treatment for NK. QTL for PLH (3H, 109.21 cM; well-watered, terminal drought, ST) was also involved in treatment interaction. This region corresponds to semi-dwarf locus (*denso/sdw1*) and is reported to be associated with height in barley under drought (Pham *et al.*, 2019) and normal condition (Maurer *et al.*, 2016; Pham *et al.*, 2019). QTL region (3H, 49.29 cM; well-watered) detected for AGB, was reported to be associated for dry weight in barley under control and drought stress treatment (Pham *et al.*, 2019). The previously reported QTL are presented in Supplementary Table 4.5, 4.6 and 4.8. Overall, a number of 26, 20 and 11 novel loci were identified for well-watered, terminal drought and drought tolerance respectively.

Results revealed that spring barley MAGIC population is a valuable genetic resource that can be used to gain insights into genetic control of yield-related traits under different environments including complex condition of drought and their drought tolerance in barley.

Pleiotropic effect of flowering time regions under well-watered and terminal drought treatment

Various flowering time regions were revealed to be associated with other yield-related traits in this study (Figure 4.2 - 4.3; Supplementary Table 4.5 - 4.8), suggesting that they pleiotropically regulate other traits in spring barley MAGIC DH lines. For instance, *PSEUDO-RESPONSE REGULATOR Ppd-H1* (*HvPRR37*) gene region (Alqudah *et al.*, 2014; Maurer *et al.*, 2015; Afsharyan *et al.*, 2020) was detected regarding GFP (well-watered, terminal drought, ST), TKW (well-watered, terminal drought, ST) and PLH (well-watered) (Figure 4.3). *Ppd-H1* region was reported to be linked with grain filling period under normal condition in barley (Maurer *et al.*, 2016). Other *PSEUDO-RESPONSE REGULATORs*, *HvPrr59* and *HvPRR73* (Cockram *et al.*, 2012) as well as phytochrome gene *HvPhyB* (Szűcs *et al.*, 2006) were close to the position detected for PLH (terminal drought), TKW (terminal drought) and AGB (terminal drought). Another phytochrome gene *HvPhyA* (Szűcs *et al.*, 2006) was near to QTL for AGB (terminal drought) and NK (terminal drought). QTL loci for AGB (terminal drought) and PLH (terminal drought, ST) were detected in region corresponding to *Vrn-H3 (HvFT1).* This locus was reported to be associated with dry weight and plant height under well-watered and drought condition (Pham *et al.*, 2019). The *Vrn-H1*/*HvPhyC* region co-located with QTL identified for NE (well-watered, terminal drought, ST) and TKW (well-watered, terminal drought, ST). This region was reported to be mapped for tiller number (Alqudah *et al.*, 2016) and grain weight

(Maurer *et al.*, 2016) under normal condition. Higher tiller number is strongly linked to more ears in barley (Kebrom *et al.*, 2013; Evers and Vos, 2013; Hussien *et al.*, 2014) and increasing the number of tillers that carry fertile ears is one of strategies for improving grain yield (Sreenivasulu and Schnurbusch, 2012; Xie *et al.*, 2016). *Vrn-H1* is a gene involved in plant development that is linked to number of tillers (Arifuzzaman *et al.*, 2016; Voss-Fels *et al.*, 2018). *Vrn-H1* region was found to have increasing effect under terminal drought treatment for QTL associated to NE and reducing effect for TKW, suggesting that more tillers increases the number of spikes and number of grains and therefore results in loss of grain weight in DH lines. *CONSTANS (CO)* genes including *HvCO1, HvCO3, HvCO5, HvCO6, HvCO7, HvCO8, HvCO9, HvCO10, HvCO12, HvCO13, HvCO15, HvCO16,* co-located with various traits (well-watered, terminal drought, ST) which demonstrates the diverse role of circadian clock genes in plant development under different environments (Griffiths *et al.*, 2003). Loci associated with GFP (well-watered, terminal drought) are near *HvCOP1 (CONSTITUTIVELY PHOTOMORPHOGENIC 1)* gene which is known to be a late flowering time gene (Matsumoto *et al.*, 2011). The QTL for GFP (well-watered, ST), AGB (terminal drought, ST), TKW (well-watered) and NE (terminal drought) were found close to genes *HvTFL1 (NARROW LEAF AND DWARF 1/ TERMINAL FLOWER1 (HvND1/TFL1))* (Alqudah *et al.*, 2016) and *HvCO3*.

Flowering time regions overlapped with many QTL associated with traits under well-watered and terminal drought treatments as well as drought tolerance. Considering terminal drought treatment did not affect time to heading in this study, this suggests the contribution of flowering time genes in other developmental mechanisms in barley under both treatments aside from influencing by timing of flowering.

Loci linked with flowering showing favorable effect on grain weight, grain number and ear number under terminal drought

QTL analysis of ear number, grain number and grain weight revealed five, two and four QTL respectively with desirable effect under terminal drought treatment from which four were novel (supplementary table 4.5, 4.6, 4.8). The region corresponding to *Ppd-H1* gene (Turner *et al.*, 2005), which is known for regulating flowering time in response to environmental cues (Turner *et al.*, 2005; Afsharyan *et al.*, 2020), had favorable effect on grain weight under terminal drought and was also associated with drought tolerance. This region was not detected for flowering time in this study; which suggests that *Ppd-H1* might control grain weight under terminal drought and its drought tolerance in this population by ways other than timing of flowering or is linked to genes that control this trait. Loci associated with *Vrs1* gene, which is linked with flowering by being involved in controlling fluorescence structure (Komatsuda *et*

al., 2007), showed favorable effect on ear number and grain number and unfavorable effect on grain weight. This QTL was found to be linked with drought tolerance for grain number and grain weight. Another QTL region that showed desirable effect on grain number under terminal drought was located on 5H chromosome (0.14 cM). This region was not reported as a flowering time region; however was located close to position of sucrose transporter gene *HvSUT2* which was also detected for ear number. This QTL along with majority of identified QTL for ear number was previously reported for tiller number in control (Alqudah *et al.*, 2016) and drought stress condition (Pham *et al.*, 2019) in barley; suggesting that sugar-related genes might be involved in controlling tillering in barley development (Pham *et al.*, 2019). There were three other loci with desirable effect on grain weight from which two located on 6H chromosome (48.80 cM and 118.98 cM) were previously reported for grain weight and flowering time under normal condition (Maurer *et al.*, 2016). The locus at 6H, 48.80 cM was also found for drought tolerance.

The results identified loci with favorable effect associated with grain weight, grain number and ear number under terminal drought as well as for drought tolerance which co-located with known flowering loci. These findings could be further investigated to be used for improving yield in breeding programs.

Conclusion

The findings showed that yield-related traits and their drought tolerance is being regulated by many genetic regions including the loci corresponding to known regulators as well as newly-found regions. The results revealed association of flowering time loci with other yield-related traits suggesting their pleiotropic effect. This included flowering time loci with favorable effect on grain yield components and their drought tolerance which can be examined further in future to explore strategies for yield improvement in barley under extreme environments.

Supplementary Material:

Dataset 4.1. Daily temperature (°C) for growth season in 2011 and 2012

Table 4.1. Analysis of variance for 534 MAGIC DH lines

Table 4.2. Pearson correlation coefficient (r) for traits using LSmeans under well-watered and terminal drought treatments

Table 4.3. Descriptive statistics, heritability (H^2) for stress tolerance (ST) for six traits

Table 4.4. Pearson correlation coefficient (r) for stress tolerance index (ST) for six traits

Table 4.5. Significant QTL for seven traits under well-watered treatment

Table 4.6. Significant QTL for seven traits under terminal drought treatment

Table 4.7. QTL × Treatment interactions via cross-validated multi-locus QTL analysis of six traits

Table 4.8. Significant QTL for Stress tolerance (ST) for six traits

Acknowledgement

We thank the *German Research Foundation* (*DFG*) under the priority program 1530, flowering time control: from natural variation to crop improvement and *Federal Ministry of Education and Research (BMBF)* under program CROP.SENSe.net (Förder-Nr. 0315529) for funding this research.

Chapter 5

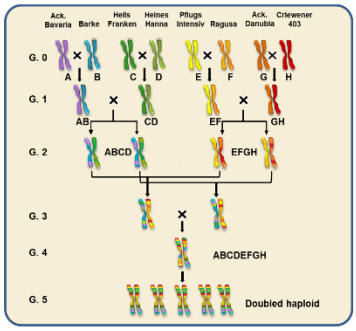
General discussion

General discussion

For annual crops such as barley, timely transition from vegetative to reproductive growth can ensure successful completion of crop's life cycle and has a large impact on yield. Therefore, understanding environment dependent regulation of flowering time pathway is crucial to develop plants with high yield in different environments. Flowering time is known to be partly controlled by various environmental factors, such as temperature, photoperiod, and stress (Mouradov *et al.*, 2002; Johansson *et al.*, 2013). This trait is complex, highly heritable and a number of major genes have been identified including *Ppd-H1* and *Ppd-H2* in photoperiod pathway (Turner *et al.*, 2005; Casao *et al.*, 2011), *Vrn-H1* and *Vrn-H3* in vernalization pathway (Yan *et al.*, 2003, 2006) and *HvCO1* in circadian clock pathway (Campoli *et al.*, 2012*a*). However the mechanism of environment-dependent regulation of flowering time in barley is still not well understood compared to the knowledge that is available for other model species such as *Arabidopsis thaliana* (Blümel *et al.*, 2015). Spring barley MAGIC population, constructed from inter-crossing eight parents (Sannemann *et al.*, 2015), was used to investigate this mechanism in barley in the present thesis and this chapter focuses on the general discussion of the main findings as summarized in Figure 5.1.

5.1 Power and precision of QTL mapping for flowering time in spring barley MAGIC population

Populations based on MAGIC strategy are expected to increase the power and precision in QTL mapping as a result of their linkage-based design, multiple generations of recombination and having higher allelic diversity compared to bi-parental populations (Cavanagh *et al.*, 2008; King *et al.*, 2012). The binary (single SNP) statistical approach commonly used for QTL analysis of bi-parental populations faced limitations for explaining the trait of interest in MAGIC populations and determining the origin of a given allele when more than two parents were involved. Therefore, QTL analysis based on haplotype-phasing data was used as complementary approach (Sannemann *et al.*, 2015; N'Diaye *et al.*, 2017; Ogawa *et al.*, 2018). Sannemann *et al.* (2015) used single SNP



Spring Barley MAGIC Population

1

To better understand the interconnected dynamics of epistasis and environment and look for novel regulators

- ✓ Investigating epistasis, environment and their interactions, in addition to single QTLs, is an effective approach
- ✓ Identification of novel epistatic QTL *HvHeading* which is engaged in environment interactions
- ✓ Barley can adapt flowering time to the environment via alternative routes within the pathway

2

To identify candidate gene(s) underlying barley flowering time QTL "HvHeading" by a targeted background effect elimination approach based on epistasis

- ✓ Narrowing down *HvHeading* QTL interval
- ✓ Proposing *Spt6* gene as a candidate gene for *HvHeading*
- ✓ This approach for targeted background effect elimination based on epistasis can be useful to facilitate gene identification

3

To investigate effect of flowering time genetic regions on yield-related traits under different environments

- ✓ Flowering time regions revealed pleiotropic roles under different environments including favorable effect on grain yield componants under terminal drought
- ✓ Involvement of flowering time regions in controlling yield-related traits aside from the timing of flowering
- ✓ Spring barley MAGIC population is useful to study yield-related traits under different environments in barley

Main Findings

Fig 5.1. Schematic overview of main findings of this thesis regarding the environment-dependent regulation of flowering time in barley according to chapters.

approach (SA) and haplotype approach (HA) to study flowering time in spring barley MAGIC population in pot experiment under foil tunnel (semi-controlled condition) and successfully detected major loci including *Vrn-H1* (Yan *et al.*, 2003) and *Vrn-H3* (Yan *et al.*, 2006) regions as a proof of concept.

Afsharyan *et al.* (2020) improved the QTL mapping approach in this population by allowing more SNP markers into the analysis, refining the haplotype-phase data by hand-correction as well as using an improved SAS macro for QTL/epistasis mapping (Chapter 2; Afsharyan *et al.*, 2020). To identify genetic regions that control flowering time under field condition, QTL analysis was performed for days to heading data collected from field experiment for two consecutive years using SA and HA (Chapter 2; Afsharyan *et al.*, 2020). The results revealed 11 QTLs by SA which explained 48.43% of total genetic variance and seven QTLs by HA which explained in total 52.92% of the genetic variance. All seven chromosomal regions found by HA were also detected by SA which included loci harboring *Ppd-H1* (*HvPRR37*) (Alqudah *et al.*, 2014; Maurer *et al.*, 2015), *denso/sdw1* (Wang *et al.*, 2010; Maurer *et al.*, 2015, 2016; Sannemann *et al.*, 2015; Alqudah *et al.*, 2016), *Vrn-H1* (Wang *et al.*, 2010; Alqudah *et al.*, 2014; Maurer *et al.*, 2015; Sannemann *et al.*, 2015), and *Vrn-H3* (*HvFT1*) (Wang *et al.*, 2010; Alqudah *et al.*, 2014; Maurer *et al.*, 2015; Sannemann *et al.*, 2015). Infinium iSelect 9K SNP array includes gene-specific SNP markers BOPA2_12_30893, BOPA2_12_30894, BOPA2_12_30895, BK_05 that are located on *Vrn-H3* gene (Comadran *et al.*, 2012). SA successfully located *Vrn-H3* position by detecting BOPA2_12_30895 as the peak marker for the QTL that had strongest association with flowering time and explained 9.96% of genetic variance which showed the high precision of locating QTL by this method. HA identified the most significant QTL in *Ppd-H1* region (SCRI_RS_140819) which explained 14.96% of genetic variance. Novel loci were identified by SA and HA; particularly a QTL on chromosome 1H, which we named "*HvHeading*". Days to heading data from Sannemann *et al.* (2015) was used to perform QTL analysis as described in Chapter 4 (Afsharyan *et al.* under preparation) and identified 14 QTL that explained 51.75% of genetic variation including QTL for major flowering time genes *denso/sdw1* (Laurie *et al.*, 1995), *Vrn-H1* (Yan *et al.*, 2003), *Vrn-H3* (Yan *et al.*, 2006) *and HvCO1* (Griffiths *et al.*, 2003). The position corresponding to *Ppd-H1* gene was detected only under field condition. Comparing the results to Sannemann *et al.* (2015) showed that the method used in the present thesis detected more QTL linked to major genes and therefore increased the precision and power of QTL analysis in this population.

HA estimated the effect of alleles originating from each parent and overall calculated higher explained genetic variance by QTL compared to SA. This could be explained by detection of larger QTL intervals as a result of potential contribution of all SNP information in one haplotype block (Sannemann *et al.*, 2015; N'Diaye *et al.*, 2017; Ogawa *et al.*, 2018). Also, the lower resolution of haplotype-phase data could result in larger size of identified QTL intervals by HA compared to SA; even though, hand correction of haplotype-phase data improved the data resolution. The resolution of haplotype-phase data can be influenced by generations of recombination and similarity among founders of population. The MAGIC DH lines were produced from fourth generation hybrids, and therefore underwent three rounds of crossing-over which possibly limited the amount of shuffling. A higher number of selfing rounds could be useful in creating more fragmentations in the genomes of the offspring and shortening the parental phase. Nevertheless, the current state of fragmentation in MAGIC DH lines seems still sufficient for QTL mapping, since the size of the genomic intervals or haplotyping blocks did not interfere with locating the QTL of the major flowering time genes in HA, as confirmed by results from SA and Sannemann *et al.* (2015). Regardless, lower resolution of haplotype data is a result of high amount of unassigned regions which effects the QTL size (Wang *et al.*, 2002). Higher number of assigned genetic regions could not be assured by more cross-over rounds even by including more selfing steps (Stadlmeier *et al.*, 2018) due to presence of similarity among population founders. However, still the efficiency of converting the existing data to haplotype phase data can be maximized by improving the algorithms for phase-haplotyping which would eliminate the requirement of labor-some and time-consuming manual corrections. Therefore to produce higher resolution of phase-haplotyping data, strategies such as including more crossing-overs in population construction process and developing an efficient phase-haplotype algorithm should be performed in the future.

5.2 Detection of the effects of epistasis and environment and their contribution to flowering time

There is increasing interest to better understand mechanism of optimal timing of flowering since it directly affects grain yield (Hill and Li, 2016). Mapping flowering time QTL in barley has been extensively reported (Bezant *et al.*, 1996; Wang *et al.*, 2010; Alqudah *et al.*, 2014; Maurer *et al.*, 2015, 2016; Sannemann *et al.*, 2015). However, the number of studies that attempted to decipher epistatic interactions that control this pathway (Maurer *et al.*, 2015; Mathew *et al.*, 2018) are scarce; probably due to difficulty of accurate estimation of epistasis. To our knowledge, there are no reports on mapping epistasis × environment interaction in barley for flowering time.

Chapter 2 described performing genome-wide analysis of epistatic interaction, QTL × environment interaction and epistasis × environment interaction for flowering time using spring barley MAGIC DH lines under field and semi-controlled conditions by SA and HA (Chapter 2; Afsharyan *et al.*, 2020). Epistasis interaction analysis mapped 1139 (SA) and 1199 (HA) epistatic interactions ($P \leq$0.1E-15) in semi-controlled and 55 (SA) and 27 (HA) interactions ($P \leq$0.1E-15) in field conditions. The *Vrn-H3* region showed the strongest epistatic interaction in both environments. The results were in line with previous reports regarding epistasis mapping for flowering time pathway in barley (Maurer *et al.*, 2015; Mathew *et al.*, 2018) which showed that this population can be used to conduct QTL and epistasis mapping for complex traits. Interestingly, distinctive epistatic interactions were identified under semi-controlled condition and field condition; suggesting that it could be due to adaptation of the DH lines to environment. Analysis of QTL/epistasis × environment interactions revealed interactions with environment both at QTL and epistasis level including prominently mapped regions corresponding to *Ppd-H1* (QTL × environment: semi-controlled, four years; epistasis × environment: four years) and *Vrn-H3* (QTL × environment: four years; epistasis × environment: semi-controlled, field) and *Vrn-H1* region (epistasis × environment: semi-controlled). The effect of environment in field and semi-controlled condition was due to temperature difference and these findings revealed regions corresponding to genes known to be regulated by temperature including *Ppd-H1* (Turner *et al.*, 2005; Ejaz and von Korff, 2017), *Vrn-H3* (Yan *et al.*, 2006) and *Vrn-H1* (Karsai *et al.*, 1997; Yan *et al.*, 2003; Von Zitzewitz *et al.*, 2005; Distelfeld *et al.*, 2009; Ejaz and von Korff, 2017; Gol *et al.*, 2017).

There are well-known examples that show the role of epistasis and environmental factors in flowering time pathway such as, the interaction among vernalization genes *Vrn-H1* and *Vrn-H3* and temperature (Hemming *et al.*, 2008; Cockram *et al.*, 2015; Gol *et al.*, 2017); as well as interaction of photoperiod pathway with day-length which leads to *Ppd-H1* gene promoting down-stream genes *Vrn-H1*, *Vrn-H3* and *CONSTANS* (*CO*) (Turner *et al.*, 2005; Yan *et al.*, 2006). Therefore, investigating only QTL is not enough to comprehend the mechanism of a complex trait such as flowering time. Analyzing epistasis showed a larger effect of gene-gene interactions compared to single loci and therefore explained larger genetic variance. Combined analysis of QTL, epistasis, and environment interaction proved to be more capable in explaining flowering time in spring barley MAGIC population.

5.3 *Identifying Spt6 as candidate gene underlying novel epistatic QTL HvHeading*

Chapter 2 (Afsharyan *et al.*, 2020) described detection of a novel flowering-delaying QTL allele on 1H chromosome using spring barley MAGIC population which we named "*HvHeading"*. The findings revealed that this region was involved in epistasis and epistasis × environment interactions with regions harboring *Ppd-H1*, *Vrn-H3*, and *Vrn-H1* and *sdw1/denso* suggesting that it might have an important role in environment-dependent regulation of flowering time in barley. Therefore, the study presented in chapter 3 (Afsharyan *et al.*, under preparation) aimed to investigate epistatic *HvHeading* QTL interval to identify the underlying candidate gene.

According to chapter 2 (Afsharyan *et al.*, 2020) the interval contained relatively high number of 160 high confidence genes and *HvHeading* was involved in epistatic interactions that could influence the phenotypic effect of this QTL. We assumed that, these interactions are probably responsible for majority of background effect; particularly the ones including *Ppd-H1*, *Vrn-H3*, and *Vrn-H1* genes (Afsharyan *et al.*, 2020). The genetic background has major impact on determining the phenotypic effect of a gene. It is reported that the same mutations that cause disease, failed to produce symptoms in a different genetic background in human (Chen *et al.*, 2016). In barley, the dominant *Ppd-H1* allele, originated from wild/winter type, shows stronger effect for early flowering in a spring background (Turner *et al.*, 2005). To investigate the phenotypic effect of gene alleles in barley, various methods have been used to maintaining the same genetic background; including developing near isogenic lines (NILs) (Liller *et al.*, 2017), mutants (Hänsel and Zakovsky, 1956) and introgression lines (Schmalenbach *et al.*, 2008; Faure *et al.*, 2012). Epistatic interactions of both *HvHeading* QTL alleles with *Ppd-H1*, *Vrn-H3*, and *Vrn-H1* regions showed the same direction in parental line Ragusa according to chapter 2 (Afsharyan *et al.*, 2020). This led to assumption that using this parental line to develop NILs is not favorable for distinguishing the effect of *HvHeading*. On the other hand, finding low level of recombinations in the *HvHeading* interval suggested that executing traditional strategies for gene identification which are based on marker saturation and fine-mapping might not work efficiently (Sánchez-Martín *et al.*, 2016; Hatta *et al.*, 2019). Therefore, to correspond to these challenges, we evaluated the gene expression and genetic variants in the interval as an alternative approach. We executed this strategy by using RNA-sequencing and focusing on finding comparable spring barley MAGIC DH lines, since these DH lines could provide different combinations of QTL alleles. Assuming that the detected flowering time regions and epistatic interactions (chapter 2) compose the majority of background effect, MAGIC DH lines can be used for finding flowering-time-specific-near-isogenic regions to eliminate the background effect of other flowering time regions.

After rigorous screening, two DH lines 1-4 and 1-20 were selected that majorly have the same background of known flowering time genes including the same alleles for *PPD-H1*, *VRN-H1* and *vrn-H3*. DH line 1-20 has the Danubia haplotype harboring the flowering-delaying *HvHeading* QTL allele. This phenotypic effect was validated when the inflorescence development of two selected MAGIC DH lines was compared and the phenotypic effect was observed after vegetative-to-reproductive transition of apex. Furthermore, RNA-sequencing was performed using apex and leaf tissues which showed a distinctive expression pattern in the region which helped refine the region to a smaller interval. Differential transcript expression analysis showed strong up-regulation of *Spt6* in DH line 1-20. *Spt6* gene codes for a conserved transcription elongation factor (TEF) involved in controlling transcription and chromatin structure as well as transcript initiation (Doris *et al.*, 2018). It is reported to be involved in Histone H3 lysine 36 (*H3K36*) methylation that contributes to control epigenetic regulation of flowering time (Shi *et al.*, 2015) and is associated with temperature-induced alternative splicing and flowering in plants including rice (Gu *et al.*, 2012; Sui *et al.*, 2013; Pajoro *et al.*, 2017). This suggests that *Spt6* gene could be studied further as a candidate gene for *HvHeading*. In the next step, differential single gene expression by RT-qPCR validated up-regulation of *Spt6* in double-ridge stage in DH line 1-20 and revealed down-regulation of *Ppd-H1* and *Vrn-H1* genes. Down-regulation of these genes is in line with the reported flowering-delaying effect of *HvHeading* QTL allele from parental line Danubia (Afsharyan *et al.*, 2020).

Sequencing majority of the promoter region from DH lines 1-4 and 1-20 found many single nucleotide variations which influenced the binding sites of transcription factors and might have a role in regulation of *Spt6* expression in DH lines 1-20 and 1-4. Also, analysis of gene structure based on transcript sequences from DH line 1-20 suggested that this line carries a *Spt6* splice variant different from isoforms published so far according to Ensembl Plants (www.plants.ensembl.org). Complete sequencing and comparison of coding frame of *spt6* in both DH lines needs to be performed to validate the novelty of *Spt6* isoform in DH line 1-20. Further investigation are required to provide more insights into the role of *HvHeading* candidate gene *Spt6* in the flowering time pathway of barley.

5.4 Studying pleiotropic effect of flowering time regions under different environments

Aside from day length and temperature, there are other environmental factors such as stress that can influence genetic components of flowering time pathway in barley (Blümel *et al.*, 2015;

Ibrahim *et al.*, 2018). Among abiotic stresses, drought is one of major causes of yield loss in barley (Jamieson *et al.*, 1995; Rollins *et al.*, 2013*b*) and is more likely to occur at the end of the growth season; also known as terminal drought (Jamieson *et al.*, 1995; Farooq *et al.*, 2014; Shavrukov *et al.*, 2017). To investigate the flowering time pathway and its pleiotropic effect under different environments, days to heading and six other yield-related traits were evaluated under well-watered and terminal drought treatments in spring barley MAGIC DH lines. QTL analysis was performed under both treatments as well as for drought tolerance (Chapter 4; Afsharyan *et al.* under preparation). QTL × treatment interaction analysis was also conducted to identify QTL affected by treatment. In this study terminal drought treatment started close to heading time and therefore did not affect timing of flowering. Other traits had some extent of decrease under terminal drought. There were total 17 QTL identified for flowering time and QTL for both treatments were generally mapped to same regions. Genetic regions for major flowering time genes *Vrn-H1* and *Vrn-H3* (Sannemann *et al.*, 2015; Mathew *et al.*, 2018; Afsharyan *et al.*, 2020) as well as the novel flowering time QTL *HvHeading* (chapter 2; Afsharyan *et al.*, 2020) were detected for flowering time under both treatments. For other traits, detected QTL co-located with flowering time regions under both treatments, which suggested pleiotropic effects of flowering time genes on yield-related traits. For instance, *Vrn-H1/HvPhyC* region (Wang *et al.*, 2010; Alqudah *et al.*, 2014; Maurer *et al.*, 2015; Sannemann *et al.*, 2015; Afsharyan *et al.*, 2020), was associated with number of ears and thousand grain weight under both treatments and for stress tolerance. The opposite direction of QTL effects in this region for number of ears and thousand grain weight under terminal drought suggested that increase in ear number has negative effect on grain weight. On the other hand, the region for another major flowering time gene *Ppd-H1* (Alqudah *et al.*, 2014; Maurer *et al.*, 2015; Afsharyan *et al.*, 2020) was found for grain filling period and thousand grain weight under well-watered and terminal drought treatments as well as for stress tolerance and showed favorable effect on grain weight under terminal drought. The peak marker BK_12 is gene-specific SNP marker located on *Ppd-H1* gene (Comadran *et al.*, 2012). The *Ppd-H1* locus was not detected for flowering time in Chapter 4; which suggests that this gene might control grain weight under terminal drought and its drought tolerance by ways other than timing of flowering. Flowering time genes such as *Ppd-H1*, *Vrn-H1* and *Vrn-H3* are reported to have pleiotropic effects on plant development and grain yield components (Drosse *et al.*, 2014) and favorable effect on yield under harsh environment (Wang *et al.*, 2010; Wiegmann *et al.*, 2019).

These finding suggested that flowering time regions showed pleiotropic roles regarding yield-related traits under different environments in spring barley MAGIC DH lines including favorable effect on grain yield components under terminal drought. Stress treatment in this study did not affect the timing of flowering which reveals that these regions have direct or indirect role in controlling yield and developmental mechanisms aside from their involvement in determining the time of flowering.

5.5 Conclusions

This thesis used a multi-disciplinary approach and advanced our understanding of environment-dependent regulation of flowering time in barley, sheding light on novel regulators. This refers specifically to exploring QTL, epistasis and interactions with environment (e.g. temperature, terminal drought) that control flowering time, investigating epistasis and environment influence in gene and transcriptome level as well as identifying candidate gene underlying newly-found epistatic QTL "*HvHeading*" using a systemic approach. Important outcomes of this thesis are:

1. It is important to improve our understanding of complicated mechanisms that determine the time of flowering in barley. The critical role of epistasis and environment interactions in flowering time pathway is established; however, not enough studies have been dedicated to explore them. Therefore, it is important that more studies focus on flowering time beyond classical QTL studies and improve their approaches by exploring epistasis and environment interaction as well.

2. A suitable mapping population is needed to provide the possibility of precise mapping of QTL, epistasis and environment interaction. Spring barley MAGIC population is a powerful tool which provided the possibility to map known flowering time genes and their well-known interactions with other genes and environment. For instance, detecting distinguishable epistatic interactions in different conditions showed that barley adapts flowering time to the environment by using alternative routes within the flowering time pathway.

3. Studying epistasis and environment interaction that control flowering time can uncover novel regulators. Flowering-delaying epistasis QTL allele *HvHeading* was detected which apparently is also interacting with environment

4. A reliable evaluation of epistasis interactions can be used as a powerful tool to develop new strategies for eliminating background effect to facilitate gene identification process in post-genomic era. A systemic approach for targeted elimination of background effect based on epistasis revealed *Spt6* as a candidate gene for *HvHeading*. This approach used flowering-time-specific-near-isogenic MAGIC DH lines.

5. Flowering time regions were found to be associated with yield-related traits under different environments (well-watered and terminal drought treatments) suggesting their pleiotropic effects and showed favorable effects on grain yield components. Flowering time regions might be involved in controlling yield-related traits by engaging in developmental mechanisms which are not necessarily related to changing the timing of flowering.

5.6 Outlook

The studies described in this thesis provided insights into novel regulators of environment-dependent flowering time in barley. The findings uncovered novel epistatic QTL *HvHeading* on chromosome 1H and identifed *Spt6* gene as a candidate gene underlying this QTL. Therefore, in future sequencing of this putative *HvHeading* gene should be performed and its function in flowering time pathway of barley should be characterized and validated.

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List of publications

- **Peer-reviewed journal publications:**

  Afsharyan N. P., Sannemann W., Léon J., Ballvora A. (2020) Effect of epistasis and environment on flowering time in barley reveals a novel flowering-delaying QTL allele. Journal of Experimental Botany. https://doi.org/10.1093/jxb/erz477

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- **Manuscripts in preparation:**

  Afsharyan N. P., Léon J., Ballvora A. (in preparation) A systemic approach for targeted background effect elimination based on epistasis identifies transcription elongation factor Spt6 as candidate gene underlying HvHeading QTL in barley (Internal review).

  Afsharyan N. P. *, Sannemann W. *, Ballvora A, Léon J. (in preparation) Genetic dissection of yield-related traits under terminal drought stress in spring barley MAGIC DH lines (Internal review).

Conferences or workshops:

- **Afsharyan N. P.**, Benaouda S., Sannemann W., Léon J., Ballvora A. (2018) Genetic and molecular analysis of epistatic interactions in flowering time pathways identified in a spring barley MAGIC population and wheat populations. III. International PP1530 Symposium: Genetic Variation of Flowering Time Genes and Applications for Crop Improvement: Kiel, Germany, 14th - 16th March 2018 (Poster).

- **Afsharyan N. P.**, Sannemann W., Léon J., Ballvora A. (2017) Flowering regulation in barley using a MAGIC population. Breeding the future, young investigator workshop: Bonn, Germany, 01th September 2017 (Talk).

- **Afsharyan N. P.**, Sannemann W., Léon J., Ballvora A. (2017) Genetic and molecular analysis of epistatic interactions in flowering time pathways identified in a spring barley MAGIC population. PP1530 6th Annual Status Meeting: Gatersleben, Germany, 14th - 16th March 2018 (Talk).

- **Afsharyan N. P.**, Sannemann W., Léon J., Ballvora A. (2016) Genetic and molecular analysis of epistatic interactions in flowering time pathways identified in a spring barley MAGIC population. GPZ Conference 2016: Bonn, Germany, 08th – 10th March 2016 (Poster).

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