Developmental and genetic analysis of pre-anthesis phases in barley (*Hordeum vulgare* L.)

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"The sun of hope doesn't know sunset"

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List of Abbreviations

AE:	Anther Extrusion
AM:	Americas
AP:	Awn Primordium
BY:	Biological Yield
CV:	Coefficients of Variation
EA:	East Asia
EU:	Europe
FDR:	False Discovery Rate
GDD or °C*D:	Growing Degree Days or Thermal Time
GH:	Greenhouse
GNP:	Grain Number per Plant
GNS:	Grain Number per Main Spike
GWAS:	Genome-Wide Association Scan
GWP:	Grain Weight per Plant
GWS:	Grain Weight per Main Spike
H^2**:**	Broad-sense Heritability
HD:	Heading
HI:	Harvest Index
Hrv:	Harvest
LA:	Leaf Area
LAGR:	Leaf Area Growth Rate
LD:	Long-Day
LDMC:	Leaf Dry Matter Content
LDW:	Leaf Dry Weight
LDWGR:	Leaf Dry Weight Growth Rate
LFWGR:	Leaf Fresh Weight Growth Rate
LMA:	Leaf Mass per Area
LWGR:	Leaf Weight Growth Rate
MSDW:	Main Spike Dry Weight
MSHI:	Main Spike harvest index
QTL:	Quantitative Trait Locus
REP:	Repeatability
SD:	Short-Day
SE:	Stem Elongation
SFI:	Spike Fertility Index
SNP:	Single Nucleotide Polymorphism
SNS:	Spikelet Number per Main Spike
TGW:	Thousand Grain Weight
TIP:	Tipping
WANA:	West Asia and North Africa

1.0 CHAPTER ONE: General Introduction

Barley (*Hordeum vulgare* L.) belongs to the monocotyledonous angiosperms. The genus *Hordeum* contains 32 species and 45 taxa from the Triticeae tribe in the grass family Poaceae (Bothmer et al., 2003). Species of *Hordeum* are temperate annuals or perennials with a basic chromosome number $x = 7$ at different ploidy levels including diploid ($2n = 2x = 14$) for cultivated barley (*Hordeum vulgare* L. ssp. *vulgare*) and its wild ancestor (*H. vulgare* L. ssp. *spontaneum)* (C. Koch.) Thell, whereas other wild species are tetraploid ($2n = 4x = 28$) or hexaploid ($2n = 6x = 42$). The annuals are mainly inbreeding species whereas the perennials are highly variable in their breeding systems (von Bothmer and Jacobsen, 1986;Komatsuda et al., 1999). Compared to other cereals, barley is able to grow in wide-ranging environmental conditions and shows spring and winter growth habits, including extreme conditions as in northern Scandinavia, the Himalayan mountains and the Arabic desert, where other cereal crops such as wheat fail to grow (Wahbi and Gregory, 1989;Nevo et al., 1992). The barley genome is exceeding 5 Gbp (International Barley Genome Sequencing et al., 2012) and thereby, it's larger than other cereal crop genomes such as rice, but only ~30% of the wheat genome size. These characteristics in addition to rich natural and induced genetic diversity and high collinearity with other cereal crops contributed to the role of barley as a prominent model system for cereal genetic research.

1.1 The origin and domestication of barley

Wild barley (*H. spontaneum* (C. Koch) Thell, or *H. vulgare* ssp. *spontaneum*) is the progenitor of cultivated barley (*H. vulgare* L. ssp. *vulgare*), which is considered as one of the oldest domesticated cereal crops in the world (since 10,000 years) (Badr et al., 2000;Kilian et al., 2009). The earliest domesticated barley belongs to the two-rowed class while domestication of six-rowed barley occurred ~1000 years later (Zohary and M, 2000). The area of Fertile Crescent is one of the important regions of barley domestication as being the center

of origin and diversity of wild cereals, such as wild barley (Zohary et al., 2012). The region of West Asia and North Africa (WANA) is defined as the geographic region of wild barley and genetic evidence has confirmed its relation to barley domestication (Nevo, 2006;Zohary et al., 2012). Molina-Cano et al. (2005) showed that wild barley discovered in areas outside of the Fertile Crescent range from Ethiopia, Morocco to Tibet and subsequently, provide strong evidence that barley domestication occurred more than once. According to Kandemir et al. (2004) the genetic analysis of the most important domestication trait in barley (non-brittle spike) showed that different loci control the trait in worldwide landraces, confirming multi independent origins of barley domestication (Takahashi, 1955). Large wild barley collections with high genetic diversity were found in Tibet region which display close genetic relationship to cultivated barley (Tingwen, 1982), additionally providing genetic evidence for Tibet as one of barley domestication regions (Dai et al., 2012). Morrell and Clegg (2007) found two domestication regions of barley, one within the Fertile Crescent which is important for diversity in European and American cultivars, whereas the second region is located in 1,500 – 3,000 km separation east of the Fertile Crescent, which might be responsible for the diversity in Central Asia to the Far East. In general, these findings indicate that barley was domesticated more than once (i.e. polyphyletic or multiple origins).

1.2 World production and uses

Since long time, barley ranks as one of the most important cereal crops in the world. Globally barley is ranked as fourth important cereal crop in terms of total production after maize (*Zea mays* L), rice (*Oryza sativa* L.), and wheat (*Triticum spp*) (**Figure 1-1a**). In terms of seed production it is ranked as the third important cereal crop after wheat and rice (**Figure 1-1b**) in 2012 (FAOSTAT, 2014). According to FAOSTAT (2014) barley production decreased at 3% (4 million tons) in last 10 years and 25% (33 million tons) compared to 1992. More than 65% of global barley production in 2012 came from Europe (**Table 1-1**), with Russia on the head followed by Turkey, Ukraine and Germany as top barley producer. Similar to total

production, barley is the fourth important cereal crop globally based on harvested area after wheat, rice and maize (**Figure 1-1c**). The distribution of harvested area across the continents showed that Europe covered more than 50% of barley harvested area over last 50 years. In Europe, the harvested area was decreased by 12 million hectares [(91 million hectares in 2002 to 79 million hectares in 2012 (FAOSTAT, 2014)]. World barley production has fallen in 2012 by 3.6% (132 million tons in 2012 compared to 137 million tons in 2002) (FAOSTAT, 2014). Moreover, FAOSTAT (2014) explained a significant decline in the area planted due to diversion of land to more profitable crops in addition to dry weather at early growing time. This report provides proof about the importance of improving high yielding cultivars as a one of the major goals of breeding programs.

In Germany, barley production decreased 14.5% in the last two decades (i.e. 1992-2012) as a result of reduction in harvested area and seed production (30% and 24% respectively, FAOSTAT (2014)). The maximum of barley production in Germany was at the early eighties of the last century (FAOSTAT, 2014). The erratic nature of climate change and planting new crops, such as maize in large scale, might be the major reasons for such reduction in barley production in Germany. To overcome the reduction in barley production, obvious breeding strategies are needed for improved productivity.

Because barley has a high ability of adaptation to a wide range of environments and high nutritive values, barley is widely used as fodder for livestock, for human food or beverage making. Baik and Ullrich (2008) reported that more than two-thirds of the barley production has been used for animal feeding and one-third for malting and little amount for food. In the ancient times barley grains were used for human food production and are still used in some parts of Asia and Africa for food preparation. Because of the high nutritional value, some reports highlighted the importance of barley as human food source (Baik and Ullrich, 2008). The barley grain consists of about 65-68% starch, 10-17% protein, 4-9% β-glucan, 2-3% free lipids and 1.5-2.5% minerals (Quinde et al., 2004). The importance of β-glucan on human

health is well known as it lowers blood cholesterol levels and glycemic index (Pins and Kaur, 2006). β-glucans also control glucose levels which in turn impact the cardiovascular health and diabetes (Baik and Ullrich, 2008). Barley flour can easily be added to wheat products such as bread, cakes, cookies, noodles and snacks (Newman and Newman, 1991). However, compared to other cereal grains, barley consumption in human food is still insignificant (approximately 2%) (Baik and Ullrich, 2008) and more efforts are needed to develop new processes for using barley grain as human food.

Beverage production (normal and alcoholic) is one of the major applications for barley grains. Barley is the primary cereal used for production of malt. The features of grains are important in malting production, e.g. hulled barley grains are preferred for malting and brewing due to the impact on beer flavor (Baik and Ullrich, 2008). Hulled barley production has been emerged rapidly as the best economical benefit from barley grains. Barley is used broadly for animal feeding because it has high carbohydrate and protein contents and is comparatively cheaper in production than the other cereals such as wheat. More than 60% of world barley production is used for livestock feed (Baik and Ullrich, 2008). Whole above ground parts of barley can be used for feeding (grain and straw) in different forms of forages, such as silage (Heuzé, 2013). Barley silage is fed to beef cattle and dairy cows because of its high digestibility and nutritive value for meat and milk production (Walsh et al., 2008;Wallsten and Martinsson, 2009). Barley grain is also used for feeding beef cattle in the United States (Heuzé, 2013). In most of the European countries, barley grain is the most important cereal commonly used in poultry feeds (Inborr et al., 1993).

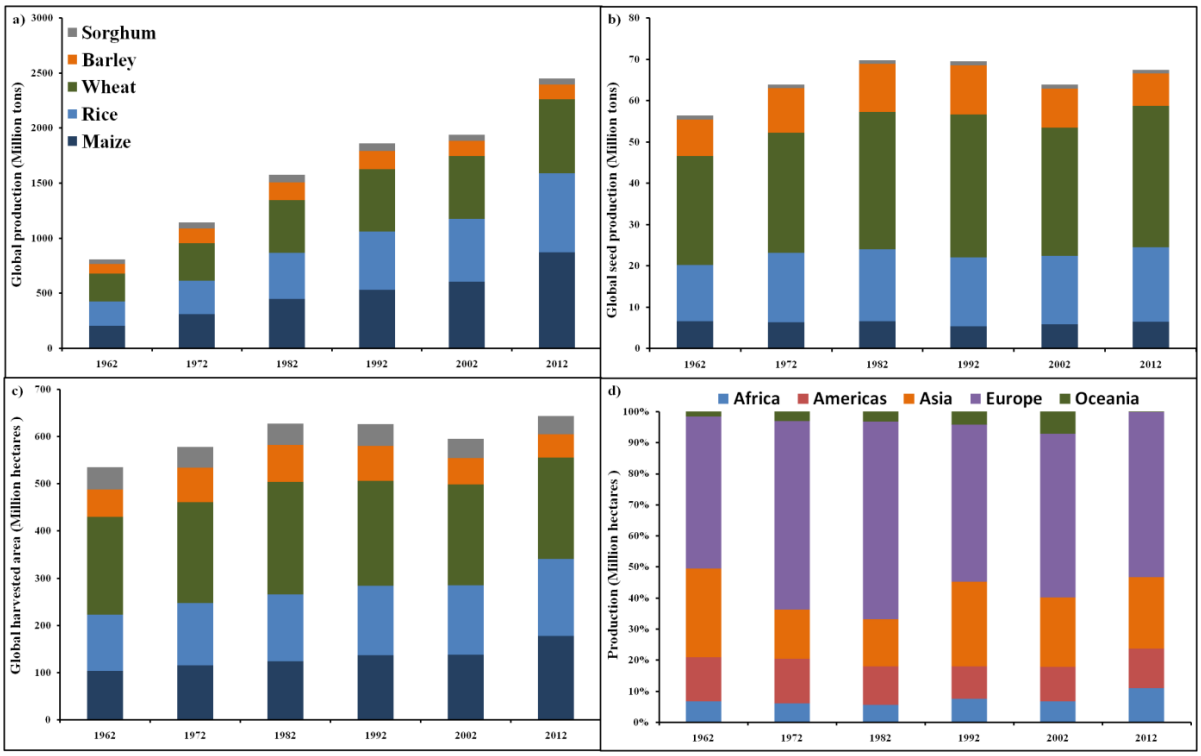

Figure 1-1: Global production of major cereal crops over the last 50 years (a), global seed production (b), global harvested area (c), and continents harvested area for barley over the last 50 years (d).
FAOSTAT | © FAO Statistics Division 2014 | 07 March 2014 (FAOSTAT, 2014)

Table 1-1: Top barley producers over the last 50 years.

	Barley seed production (tonnes.year^{-1})					
Country	**1962**	**1972**	**1982**	**1992**	**2002**	**2012**
Russian Federation				3685000	2879000	2600000
Turkey	570000	510000	580000	697000	680000	599960
Ukraine				595000	724000	440000
Germany	237653	339776	481423	367000	319000	277000
Canada	223360	424600	407200	394400	441700	257000
France	396000	417000	360000	290000	234000	248000
Kazakhstan				1100000	200000	240000
Poland	263000	179800	225500	240000	227000	232000
Belarus				396000	244000	220000
Australia	62000	134000	155000	154000	171000	174000
Morocco	157333	163304	172064	172064	181320	151450
United Kingdom	311000	374000	330000	181000	162000	135000
United States of America	457000	392000	374500	285000	188900	130365
Argentina	94000	83000	32100	26700	40500	128385
Iran	75600	94320	144473	151295	108729	120960

FAOSTAT | © FAO Statistics Division 2014 | 07 March 2014 (FAOSTAT, 2014)

1.3 Barley germplasm classification

Knowledge about the genetic relationships among different genotypes is important for the utilization of the germplasm resources in plant breeding and also for genetic studies. Barley germplasm can be divided into different groups based on distinctive features, such as it can be classified into two- and six-rowed forms based on spike morphology, growth habits (winter, spring and facultative) or germplasm can be classified according to geographical dispersion (Malysheva-Otto et al., 2006).

The major classes in barley growth habit are based on sensitivity to environmental cues, i.e. temperature (vernalization), light (photoperiod) and endogenous factors, which are associated with barley growth and development. Vernalization sensitivity depends on the exposure to low temperature for enhancing heading (flowering). Winter barley varieties are vernalization-sensitive whereas facultative and spring types are not. In terms of photoperiod, sensitivity means the plant will not flower until the day length reaches a critical threshold. A winter barley is planted in late fall and harvested in the following summer whereas a spring barley is planted in spring and harvested during summer. A winter barley sown in spring will not flower or flower too late and a spring barley planted in fall will die due to cold-injury of shoot apex. The facultative barley varieties can be planted in spring or fall, as they are cold-tolerant. Despite differences between barley growth habit classes in response to environmental cues, the phase duration and transition in barley are controlled by environmental factors in addition to genetic factors (Appleyard et al., 1982;Kernich et al., 1995a;1997). Phase duration is considered as a key trait for adaptation, yield and yield-related traits (see chapter 2). In terms of genetic, allelic diversity at the major photoperiod gene *PHOTOPERIOD RESPONSE LOCUS 1 (Ppd-H1*) can explain variation in pre-anthesis phase durations between diverse origins of accessions (chapter 4).

Barley germplasm was classified into three classes of gene pools, which are important for genetic diversity studies for crossing of related species (Harlan and de Wet, 1971). According

to Harlan and de Wet (1971), the primary gene pool comprises cultivated barley and wild species *H. vulgare* ssp. *spontaneum*, the secondary gene pool consists of *H. bulbosum* L., and the tertiary gene pool includes the remaining 30 *Hordeum* species. Generation of fertile hybrids by intercrossing the members of the primary gene pool is easy, because they are closely related. However, generation of fertile hybrids by crossing members of primary and secondary gene pools is difficult. The tertiary gene pool is more distantly related to other gene pools and some techniques such as chromosome doubling can be used for gene transfer (Harlan and de Wet, 1971).

1.4 Morphological classification of barley spikes

The barley spike is considered to be one of the major features for morphological and genetic variation among barley cultivars. Morphologically, the indeterminate spike possesses many rachis nodes and each node has three spikelets. If all three spikelets are fertile, it is called six-rowed barley (six spikelet in both sides) and when only the central spikelet is fertile and the laterals are sterile it is called two-rowed barley (Bonnett, 1966). Morphology, growth and development of spikelets as well as differences in yield and yield components between the two major row-type classes will be covered deeply in chapter 2. Barley row-type is regulated by five known loci which include spontaneous (induced by nature) or/and induced (by chemical mutagenesis) mutants (Lundqvist and Lundqvist, 1988).

Genetically, Six-rowed spike 1 (*Vrs1*) is the major gene controlling spike row-type phenotype (two- and six-rowed). The row-type is also controlled by other genes like, *vrs2, vrs3, vrs4* and *vrs5* (*intermedium–c, int–c*), which show different degrees of lateral spikelet fertility. Komatsuda et al. (2007) isolated *vrs1* (2HL) and found that wild type allele form of *Vrs1* (*HvHox1*), suppressed the lateral spikelet fertility in two-rowed barley. As mentioned in section 1.1, the domesticated barley was the wild barley (*H. vulgare* ssp. *spontaneum*) carrying two-rowed phenotype (*Vrs1*) which confirmed that two-rowed class is the ancestral state of barley. With regards to other *vrs* genes, *vrs2* and *vrs3* are being cloned (Schnurbusch

and Waugh labs, unpublished) whereas *vrs4* was recently cloned at 3H by Koppolu et al. (2013). *Vrs4* (*HvRA2*) is the ortholog of maize *RAMOSA2* and it has a central role in controlling spike architecture by modulating *Vrs1* activity (Koppolu et al., 2013). Ramsay et al. (2011) showed that *int–c* (*vrs5*, 4HS) is an ortholog of the maize domestication gene *TEOSINTE BRANCHED 1* (*TB1*) and *int-c* controls the modification of lateral spikelets. These findings confirmed that there is a high diversity in barley spike architecture which is controlled by several genes, and further work to improve yield potential by these features would be a promising task for the future.

1.5 Growth and developmental stages

1.5.1 Scales for growth and development

Since long time, several attempts have been made to describe crop growth and developmental life cycle. Some scales describe the stages based on external (visual) attributes (Large, 1954;Zadoks et al., 1974) without looking at the apical meristem (spike) development. The time for apical meristem development (prior to heading) is important for spike and/or spikelet development and subsequently for yield potential. The dissection of apical meristems at early stages is required to find out the sequence of spike/spikelet developmental stages and the impact of these stages on yield potential. The oldest documented barley apical development scale was published by Lermer and Holzner (1888), since then, many studies described morphological changes in apical meristem development (Bonnett, 1935;Waddington et al., 1983;Kirby and Appleyard, 1987). There was a comprehensive study by Landes and Porter (1989) to compare twenty-three developmental scales in cereals, and they recommended to use the scale of Kirby and Appleyard (1987), because it is the most extensive and accurate scale covering the external and internal (apical meristem) developmental changes. In our study we used the scale of Kirby and Appleyard (1987), because it is the most accurate scale to detect spike/spikelet changes prior to heading stage. For external signs/changes of spike

emergence and development, Zadoks et al. (1974) scale (**Figure 1-2**), which is divided into 10 major phases, was used (Landes and Porter, 1989).

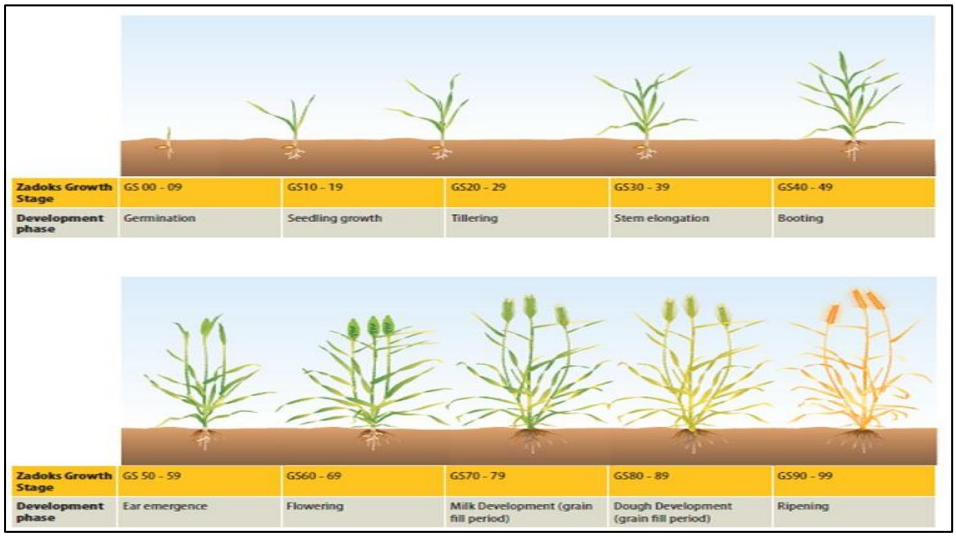

Figure 1-2: Zadoks *et al*. (1974) scale for cereals.
Modified after Zadoks *et al*. (1974).

Barley development passes through three major phases: vegetative, reproductive and grain-filling phases to reach maturity (Slafer et al., 2002). The first two phases (vegetative and reproductive phases) can be assigned to pre-anthesis phase. The vegetative phase (leaf initiation) starts with the seed germination process until collar initiation whereas the reproductive phase starts afterwards (Kirby and Appleyard, 1987;Sreenivasulu and Schnurbusch, 2012). The reproductive phase is divided into two phases: early-reproductive phase (spikelet or floret initiation) starting from collar initiation to AP stage and the late-reproductive phase (spike growth and development) starting from AP to anthesis stage (Kirby and Appleyard 1987; Sreenivasulu and Schnurbusch 2012). The last phase (grain-filling phase) starts with the onset of grains dry-matter accumulation and ends at the maturity stage. The present dissertation focussed on the late-reproductive phase (spike growth and development) as part of the pre-anthesis phases (vegetative and reproductive phases).

1.5.2 Vegetative phase

In the mature grain, the embryo has primordia of the first three to four leaves (Kirby and Appleyard, 1987). Appearance of the leaf primordia visible as dome formation is the major sign for the beginning of vegetative phase (**Figure 1-3a**), which continues until collar initiation stage (Kitchen and Rasmusson, 1983;Kirby and Appleyard, 1987;Sreenivasulu and Schnurbusch, 2012). The final number of leaves per culm can be detected at the end of this phase and varies between 3-8 leaves depending on variety, sowing date and climatic conditions (Kitchen and Rasmusson, 1983;Kirby and Appleyard, 1987). Variation in the duration of leaf initiation phase was around 10 days and the heritability value was around 0.60 in three bi-parental populations studied by Kitchen and Rasmusson (1983). The duration of leaf initiation phase was significantly longer in wild barley than cultivated barley (Kernich et al., 1995b) and two-rowed barley has longer leaf initiation phase than six-rowed (Kirby and Riggs, 1978). Formation of tiller buds starts at early vegetative stages (ridge of meristematic tissue in the axial of basal leaf), sequentially with leaf development (Kirby and Appleyard 1987). The first tiller developed from the axial of leaf number two which is about 3 mm long and enclosing the shoot apex (**Figure 1-3b**; Kirby and Appleyard 1987). Extended duration of this phase leads to increase the number of developed leaves, tillers and spikelet primordia, but has negative impact on survival of spikelet primordia (Kitchen and Rasmusson, 1983).

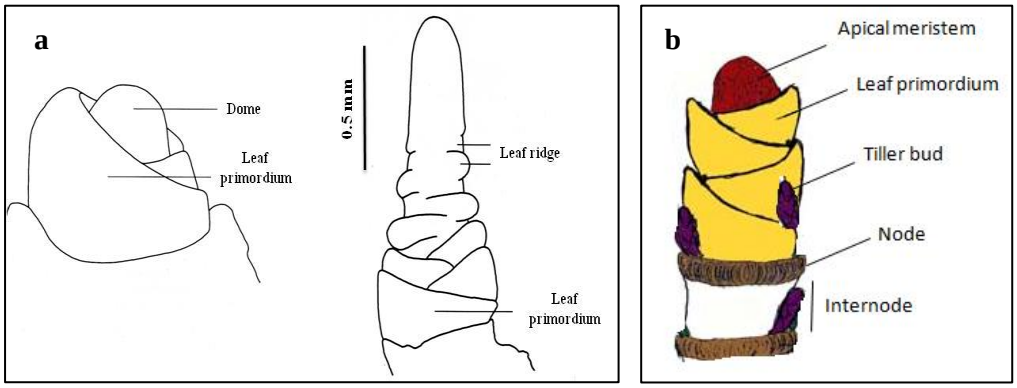

Figure 1-3: Leaf and tiller initiation phase, vegetative phase (Kirby and Appleyard, 1987).

1.5.3 Reproductive phase

Collar initiation is considered as the transition point from vegetative to reproductive phase (Kirby and Appleyard, 1987;Sreenivasulu and Schnurbusch, 2012). Reproductive phase is the longest phase in barley life cycle and it is divided into two sub phases: the first (early-reproductive or spikelet initiation phase) belongs to spikelet outgrowth (i.e. the stage at which spikelet forms) whereas the second one (late-reproductive or spike growth and development phase) belongs to early spike formation from developed spikelet (Kirby and Appleyard, 1987;Sreenivasulu and Schnurbusch, 2012).

1.5.3.1 Spikelet initiation phase or early-reproductive phase

The early-reproductive phase consists of distinct stages: double ridge, triple mound, glume primordium, lemma primordium, stamen primordium and finally awn primordium (**Figure 1-4**, Kirby and Appleyard (1987). The name double ridge was coined because leaf (lower) and spikelet (upper) primordial ridges form one unit (**Figure 1-4a**) and at this stage several undeveloped spikelets can be distinguished. The upper spikelet ridge develops into three mounds, one central (middle spikelet) and two lateral (lateral spikelets, **Figure 1-4b**), which is known as triple mound stage (Kirby and Appleyard, 1987). Glume, lemma and stamen primordium appear consecutively (**Figure 1-4c, d, e**) in short time. Glume primordium (**Figure 1-3c**) is small and very difficult to see before lemma primordia distinguished as a crescent shape under central and between lateral spikelets (**Figure 1-4d**). During the stamen primordium stage, three stamen primordia will be developed, one in the anterior median and two posterior laterals, **Figure 1-4e**. Other hidden parts such as carpel, lodicule and palea are also formed during this stage (Kirby and Appleyard, 1987). The last stage in early-reproductive phase is AP (when AP visible, **Figure 1-4f**) characterized by the maximum number of spikelet primordia (maximum yield potential). This stage is considered as the transition point from early- to late-reproductive phase and more information about this stage will be given in chapter 2. Kitchen and Rasmusson (1983) found 10 days of variation between

10 barley cultivars in early-reproductive phase while extension of this phase had high positive impact on spikelet number and survival.

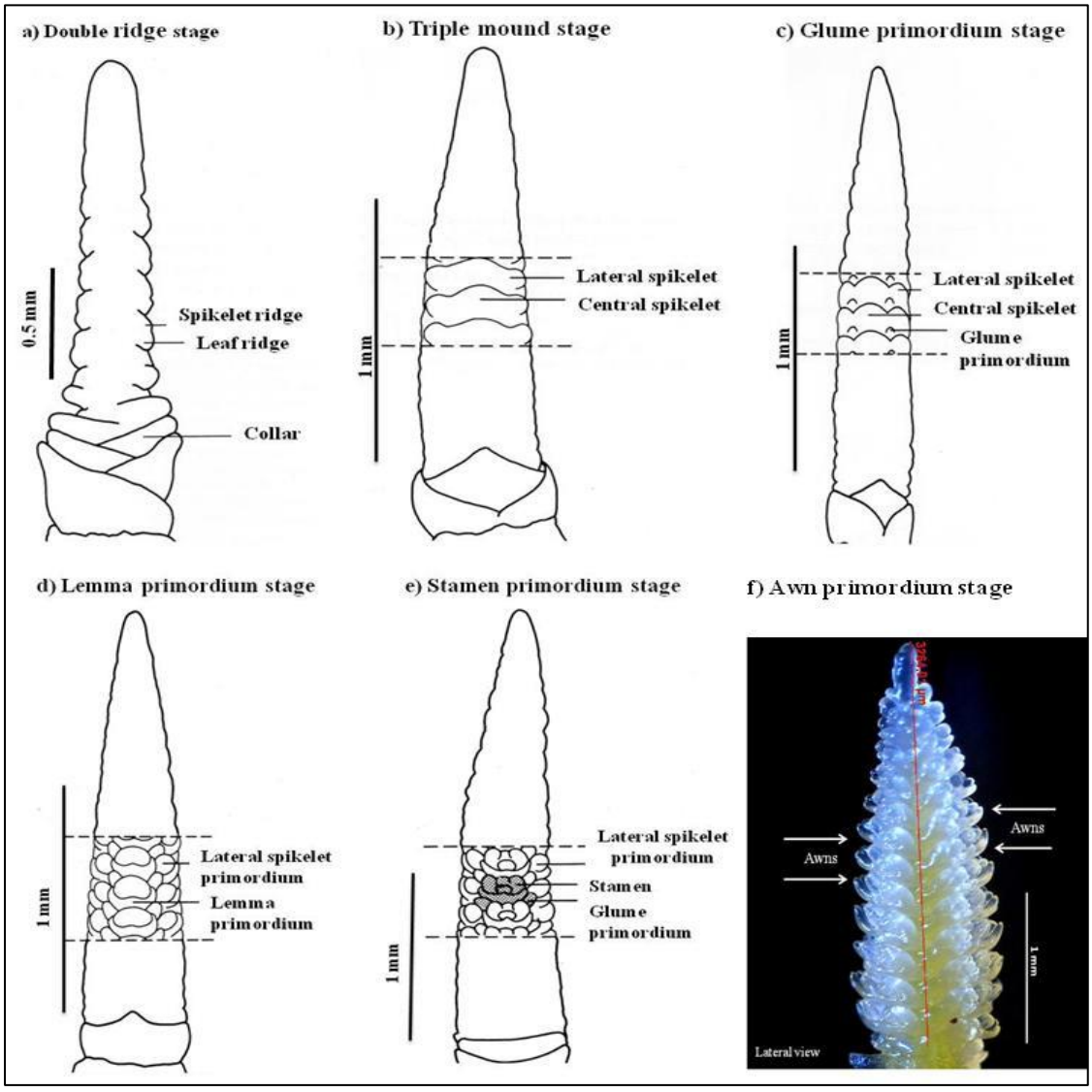

Figure 1-4: Spikelet initiation phase.
a) double ridge, b) triple mound, c) glume, d) lemma, e) stamen and f) awn primordium stages according to Kirby and Appleyard (1987).

1.5.3.2 Spike growth and development phase or late-reproductive phase

The late-reproductive phase which starts at AP stage is followed by awn tipping (TIP), heading (HD) and anther extrusion (AE) stages. The TIP, HD, and AE stages are elaborated in chapter 2, 3 and 4. Here, we provide the details of stages that are formed AP to TIP phases in which the spike is enclosed in the sheath of flag leaf (Kirby and Appleyard, 1987). Barley anther development includes three developmental stages (white-, green- and yellow-anther)

that occur between AP and TIP stages (Kirby and Appleyard, 1987). The first stage, white-anther is characterized by white (translucent) anthers which are located beyond the carpel and in front of the awn (**Figure 1-5a**). Each anther consists of four segments and during this stage the anthers starts to elongate to produce a filament. Meanwhile, the top of the carpel, which has two lumps, starts to develop style and stigma. Thereafter, the anther gets green, known as green anther stage and new structures, such as the style appear at this stage. Meiosis of anther and carpel is an important event during this stage, meiotic abnormalities during this stage lead to sterility in carpel (female part) while those in anther (male part) result in pollen sterility and lack of grain set (Kirby and Appleyard, 1987). Yellow-anther is the last stage of AP-TIP phase (**Figure 1-5b**), when the pollen is formed and stigma becomes ready to receive pollen (Kirby and Appleyard, 1987). This phase i.e. AP-TIP phase was found as the most important phase for spikelet survival, yield and yield components (chapter 2), leaf growth and development (chapter 3) and for heading time (chapter 4). More studies (developmentally and genetically) based on these findings are required to complete our knowledge about this phase.

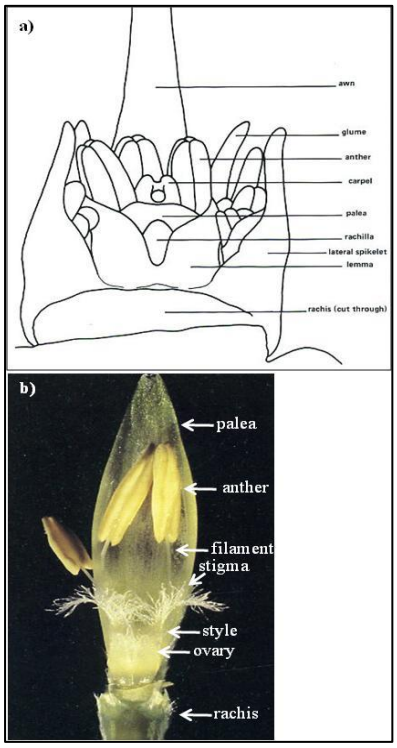

Figure 1-5: Spikelet structure at a) white anther stage and b) at yellow anther stage according to Kirby and Appleyard (1987).

1.5.4 Grain-filling phase

Anthesis or fertilization happens between TIP to HD stage, and grain-filling phase starts around 10 days subsequent to fertilization (i.e. between HD to AE) until physiological maturity (PM, Z92; Zadoks et al. (1974)). This is the last phase in barley development which is important for yield, because the developed fertile florets/spikelets grow into a caryopsis. Caryopsis development passes several stages such as milk, dough and ripen (Zadoks et al., 1974). It is characterized by dry matter accumulation, increase in size and decrease in moisture content (Kirby and Appleyard, 1987). The caryopsis development is affected by environmental conditions, for instance Alqudah et al. (2011) reported that drought stress (water deficit) leads to decrease in barley yield and yield components as a result of shortened grain filling period. In this respect, increasing the yield by lengthening grain-filling duration is the goal of several barley breeding programs.

Hence, each developmental phase has a particular role in barley growth and development. Vegetative phase is responsible for producing leaves and tillers while early-reproductive phase has a role in spikelet development. Spikelets/florets are developed during late-reproductive phase, whereas the grain-filling phase is important for accumulation of dry-matter in caryopses. Manipulation of the duration of these phases is important in improving specific traits.

1.6 Recent advances in heading time

Transition of barley plants from vegetative to reproductive phase is the fundamental step which is regulated by a complex network of genetic pathways. For instance, the genetic dissection of heading time in barley lead to the identification of a large number of genes controlling this crucial stage including *VERNALIZATION (VRN-H), CONSTANS (HvCO), FLOWERING LOCUS T (HvFT), PSEUDO-RESPONSE REGULATOR* (*HvPRR*) or *Ppd-H*, *PHYTOCHROME* (*HvPhy*) and *CIRCADIAN CLOCK* genes, see the review by Higgins et al. (2010);Andres and Coupland (2012);Shrestha et al. (2014). Despite this progress, several

questions remain, for example how these genes are working or/and interacting through phase transition in response to diverse environmental conditions. Explaining the interaction processes between these genes in response to environmental signals is of great importance in understanding heading time in barley. The current knowledge of the molecular genetics controlling heading time in barley comes based on the results of other crops (Fjellheim et al., 2014;Shrestha et al., 2014). For example, *Arabidopsis* (long-day plant -LD) and rice (short-day plant -SD) were used to understand photoperiod pathway in barley see the review by (Higgins et al., 2010;Shrestha et al., 2014). These model plants have greatly increased our knowledge to understand the molecular mechanisms that regulate heading time in barley. Even though there are a lot of heading time researches had been done in barley during the last decades, still there is a lack of information about the role of some gene families e.g. *HvCO*-like genes. Likely, because most studies were focused on major heading time family-genes e.g. *Vrn-H, HvFT* and *Ppd-H* (Turner et al., 2005;Faure et al., 2007;Szucs et al., 2007). Up to date, the genetic network model regulating barley heading time, consists of these families' genes (see the review by Shrestha et al. (2014)) and considering the role of other gene families is important. Recently, several genes were newly introduced into barley molecular heading time networks, e.g. *HvPhyC* (Nishida et al., 2013); however, the role of these genes is not well understood. In chapter 4, we propose the most recent advances genetic network model of heading time in barley. The model is based on photoperiod responses to long-day condition, which includes several newly identified genes, such as several *HvCMF*- and *HvCO-like* genes, belonging to different heading time pathways in barley. Comprehensive studies including gene function and interactions with other genes under various conditions are required for better understanding barley heading time mechanism. In addition, more attention to natural genetic variation and/or allelic diversity using different kinds of population is important to detect desired alleles.

1.7 QTL mapping

Revealing the genetic variation and causal genetic factors underlying the phenotypic variation of complex traits (controlled by many genes, quantitative trait loci (QTL)) and their interaction with environmental factors is a fundamental goal of molecular genetics. To this end, there are two common approaches to identify QTL underlying complex traits in crops; such as bi-parental mapping (linkage mapping) and linkage disequilibrium mapping (association mapping).

1.7.1 Bi-parental mapping

The most common and classical approach for mapping of phenotypic traits is bi-parental linkage and QTL mapping which allows detecting causal QTL in complex traits through crosses of parents and their pedigrees (Mitchell-Olds, 2010). Detection of QTL using bi-parental populations started a long time ago (Würschum, 2012) and hundreds of reports were published in barley using this approach in the last two decades (Patrick and Alfonso, 2013). Larger family sizes in bi-parental populations (>500 individuals) are required for small effect QTL detection (Würschum, 2012). Identification of QTL for a trait of interest using bi-parental populations depends on the analysis of the co-segregating trait with genetic markers. There are several studies based on agronomical traits such as heading date in barley using this approach e.g. (Schmalenbach et al., 2009;Xue et al., 2010). The major impediments of this approach are the long time needed to develop mapping populations with low resolution of mapped QTL as an outcome of low number of recombinations caused by the few number of genotypes resulting in a narrow genetic base (Mitchell-Olds, 2010). There are many positive features in using this approach such as it requires only few markers for mapping to minimize the risk of false positive QTLs. Advanced molecular technologies allowed for rapid and cost-effective genotyping which make QTL mapping robust and useful for identifying the target region of complex traits (Ingvarsson and Street, 2011).

1.7.2 Association mapping

Non-random association of alleles at different loci is the key for the association mapping or linkage disequilibrium mapping approach, which detects QTL based on the strength of correlation between causative SNPs and traits (Flint-Garcia et al., 2003). Linkage disequilibrium mapping shows historical recombinations that accumulated over generations. This approach has been studied extensively in humans and also started in plants since the beginning of this century (Flint-Garcia et al., 2003). Early reports using this approach in plants came from a diverse maize population (Tenaillon et al., 2001) and *Arabidopsis* (Nordborg et al., 2002), thereafter the approach was used in other crops and the number of published reports increased, see the review by Rafalski (2010). In barley, this approach was started very recently (Caldwell et al., 2006;Stracke et al., 2007). Linkage disequilibrium is an indicator to detect the distance between loci, which is important to find the number of required markers to cover the genome, i.e. high linkage disequilibrium value means low number of markers are needed to cover the genome (Myles et al., 2009). Map resolution (number of required markers) relies on linkage disequilibrium decay, in other words how quickly the linkage disequilibrium decays over distance, linkage disequilibrium decay differs among species, which mainly depends on the breeding system of the species of interest (Flint-Garcia et al., 2003). Semagn et al. (2010) mentioned that 1.1 million SNP markers were required to cover the barley genome in case of linkage disequilibrium decay at 5 kbp, whereas the required SNPs will be only 57,000, if the decay was at 100 kbp. In self-pollinated species like barley, the decay of linkage disequilibrium was larger than in cross-pollinated species such as maize. The linkage disequilibrium in worldwide spring barley collection disappeared within 25-50 Mbp (Pasam et al., 2012), while it was broken within 500 kbp in maize (Jung et al., 2004). Moreover, Sharma (2013) reported that the extent of linkage disequilibrium varies among different barley gene pools (<1 cM in wild and 14 cM in cultivated winter barley) and they also found that LD decay varies within the genome, i.e.

linkage disequilibrium was higher around centromeres while it was low in telomeric regions. Long-range of linkage disequilibrium increases the chance of false association and therefore, calculation of linkage disequilibrium at the beginning of the association analysis is essential (Balding, 2006).

1.7.2.1 Population structure

Population structure is a statistical approach to calculate relatedness correlation (phenotypic and genotypic) among individuals within the population. The natural population can be divided into subpopulations based on the distribution of individual relationships (population structure), which is important to study the natural variation. Association between phenotypes and marker loci to detect the causative allele of variation can be spurious as a result of population structure (Myles et al., 2009). This problem may appear particularly in mapping adapted traits, like flowering time (Aranzana et al., 2005), because the phenotypic variation is highly correlated with allele frequency. Barley as a inbreeding crop has complex population history leading to generate a complex population structure (Rostoks et al., 2006). The different relatedness among individuals of natural barley populations resulted in the classification of subpopulations based upon row-type (two- and six-rowed) and/or geographical origins (Pasam et al., 2012), while growth habits separated the population into spring and winter subpopulations (Cockram et al., 2010;Comadran et al., 2012). Moreover, in the present dissertation, the spring barley panel was structured based upon the heading time gene (alleles) as described in chapter 4.

Therefore, controlling the population structure using statistical methods is essential for having robust associations. There are several proposed methods to prevent spurious association in general linear model, such as genomic control (Devlin and Roeder, 1999). Pritchard et al. (2000) have developed a highly accurate clustering approach (Q-matrix) using STRUCTURE software which decreased the proportion of spurious associations in quantitative traits by up to 80% (Thornsberry et al., 2001). Yu et al. (2006) developed a mixed-model approach to

control spurious associations through account multiple levels of relatedness. However, this approach is problematic in controlling population structure for large datasets, so additional correction methods are needed as a pre-requisite for association analysis. Genetic markers' relatedness using kinship or principal components analysis (PCA) significantly decreases false association (Price et al., 2006;Zhang et al., 2010). Nowadays, combining mixed model (kinship or PCA) with Q are intensively used in crops association analysis for controlling spurious associations e.g. in barley (Cockram et al., 2010;Comadran et al., 2012). In general, this approach appears to be the most powerful and appropriate way in association analysis.

1.7.2.2 Genome-wide association study

Recently, population analysis using genome-wide association studies (GWAS) to identify QTL in diverse collection based on relations between single nucleotide polymorphisms (SNPs) and phenotypes started in crops as a new approach. Followed by success in human, animal and in model crop species such as Arabidopsis (Zhao et al., 2007), GWAS analysis became more popular in cereals. In comparison to bi-parental mapping, GWAS has higher resolution as a result of more recombination events, and more genotypes can be used in this approach, which generates a broader genetic base (Mitchell-Olds, 2010). For covering the whole genome and detecting QTL, high number of SNPs (thousand) is required with dense positions. Generally, three major elements control the power of associations; the number of accessions, genetic marker density and linkage disequilibrium (Mackay and Powell, 2007).

In the current decade, usage of GWAS analysis in crop species resulted in several publications indicating the importance of this approach for detecting new QTL through genetic dissection of complex traits. In barley, identification of causal alleles using GWAS has started to emerge, which includes dissecting the complex trait by analyzing germplasm to detect significant associations and/or study the phenotypic/genotypic variation (Waugh et al., 2009). Cockram et al. (2010) published a GWAS analysis for 15 traits in 500 cultivars that have been genotyped by 1,536 SNP markers (Illumina's GoldenGate technology, Close et al. (2009))

and confirmed GWAS results by re-sequencing the detected putative candidate gene from anthocyanin metabolism. Using the 'Illumina GoldenGate SNPs array', GWAS has been conducted to analyze agronomical traits such as heading date in worldwide spring barley collection to detect markers trait associations (Pasam et al., 2012) or other agronomical traits in 615 cultivars from the United Kingdom (Wang et al., 2012). In 2012 (Comadran et al.) developed 9K iSelect Illumina™ SNP platform from which they provided a high map resolution with unprecedented genetic marker density. QTL identification by GWAS using a high throughput SNP platform with sufficient marker density to cover the genome can be achieved after controlling population structure and elimination of false association. This new promising approach represents a tremendous step forward in genetic analysis and will undoubtedly prove to be a valuable tool in identification of genes and genetic networks in the future.

1.8 Organization and objectives of the dissertation

The dissertation is written in the form of a cumulative type, which includes a general introduction (chapter 1), three scientific articles (chapter 2, 3 and 4) and a general discussion based on all results (chapter 5). In chapter 1 (general introduction), the general information about barley, its growth scales/stages and QTL mapping approaches are provided. Chapter 2, 3 and 4 are either published or in the reviewing process. In general, chapters 2, 3 and 4 are self-contained i.e. contain own introduction, materials and methods, results and discussion parts. In chapter 5, the major results from chapters 2, 3 and 4 are discussed in order to explain how presented findings are related to improving yield and yield components.

A note on organization: the dissertation is written based on scientific articles wherein I am the first author. However, because these works include one or more co-authors, for this reason I used plural pronoun "we" in this dissertation. Also, this work has been done with the help of many people, most of whom are listed in the acknowledgements.

The overall aim of this study was to developmentally and genetically study pre-anthesis phases in a diverse spring barley collection. To this end, phenotypic analyses of spring barley accessions (i.e. pre-anthesis growth, development and length of stages/sub-phases) and detecting QTL underlying these traits by using GWAS has been performed. Because the dissertation consists of three scientific articles, the specific objectives to address the goals have been covered in depth in chapter 2, 3, and 4.

Chapter 2:

The goal of this chapter was to provide details about the differences between two major barley spike row-type classes, i.e. two- and six-rowed, related to the pre-anthesis phases under different growing conditions (greenhouse (GH) and field). In this part, 32 diverse accessions (14 two- and 18 six-rowed) were dissected at different developmental stages (AP, TIP, HD and AE) to count the number of spikelet per main spike for calculation of spikelet survival in sub-phases. Yield, yield components and broad-sense heritability estimation of these traits were also targeted. Finally, the duration of stem elongation (SE) and the late reproductive sub-phases were correlated with yield and yield components traits to extract the critical duration (in particular spikelet survival). Dissection work of this phase allowed us to describe the spikelet survival process between the sub-phases, which is highly important in improving yield potential.

Chapter 3:

This chapter describes differences between two- and six-rowed barley during pre-anthesis phases but from another point of view. Evaluation of leaf trait performance under different growing conditions (greenhouse (GH) and field) was the major goal. For this purpose 14 two- and 18 six-rowed genotypes were analyzed. The second goal was to estimate the genetic basis of leaf traits and explain how these traits could be implicated in breeding programs by correlating them with single plant grain yield. By performing dissection work, leaf trait

performance during the late-reproductive phase and its importance in improving yield in two- and six-rowed barley was examined.

Chapter 4:

Studying the natural variation in pre-anthesis stages/sub-phases in two photoperiod response groups (*Ppd-H1*/*ppd-H1*; 95/123 accessions, respectively) was one of the aims described in the chapter. Detecting QTLs underlying the variation between photoperiod groups using the 9K SNP platform by GWAS was also one of the main targets. To this end, dissecting pre-anthesis phases at four developmental stages (AP, TIP, HD and AE) in a worldwide spring barley collection (218 accessions) was conducted manually under controlled GH condition. Upon using GWAS analysis with phenotyping (dissection) approach, we were able to propose a new model of heading-time regulation in both photoperiod groups.

2.0 CHAPTER TWO: Awn Primordium to Tipping is the Most Decisive Developmental Phase for Spikelet Survival in Barley

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2.1 Abstract

In small-grain cereals, grain yield is closely associated with grain number. Improved spikelet survival is an important trait for increasing grain yield. We investigated spikelet number, spikelet survival and yield-related traits under greenhouse conditions, and pot- and soil-grown field conditions. Thirty-two spring barley (*Hordeum vulgare* L.) accessions (14 two- and 18 six-rowed accessions) were manually dissected to determine spikelet/floret number on the main culm spike (SNS) at awn primordium (AP), tipping (TIP), heading and anther extrusion. We observed a significant difference between two- and six-rowed barley for SNS and spikelet survival at all stages and growing conditions. Both traits were highly genetically controlled, with repeatability and broad-sense heritability values of 0.74–0.93. The rate of spikelet survival from AP to harvest was higher in two- (~70%) than in six-rowed (~58%) barley. Spikelet abortion, starting immediately after AP, was negatively affected by increased SNS and the thermal time required to reach the AP stage. The largest proportion of spikelet reduction happened during the AP–TIP phase, which was the most critical period for spikelet survival. The duration between AP and the end of stem elongation correlated better with spikelet survival and yield-related characters than the estimated duration of stem elongation using leaf height measurements. Our observations indicate that the main spike plays an important role in single-plant grain yield. Extending the length of the critical AP–TIP phase is

promising for improving yield through increased spikelet development and survival. The results also demonstrate that greenhouse conditions are appropriate for studying traits such as phase duration and spikelet survival in barley.

Keywords: *Hordeum vulgare* L., six-rowed, spikelet survival, stem elongation, two-rowed.

2.2 Introduction

Barley (*Hordeum vulgare* L.) is considered to be the fourth most important cereal food crop in the world (FAOSTAT (2014); http://faostat.fao.org), largely due to its exceptional adaptations toward growing in a variety of different environmental conditions. The barley spike possesses three single-flowered spikelets (one central and two lateral spikelets) at each rachis internode (Forster et al., 2007a). Based on the fertility of the lateral spikelets, barley has been classified into two different row types, namely the two- and six-rowed barleys. All three spikelets are fertile in six-rowed barley, but the two lateral spikelets are sterile in two-rowed barley (Bonnett, 1966). The difference in lateral spikelet fertility between the two germplasm pools is one of the major factors determining barley yield potential.

Improved grain yield is a major objective of crop breeding, and a promising avenue for maximizing yield is through improved spikelet survival. A number of researchers have considered the pre-anthesis development phase as a target for improving yield potential (Appleyard et al., 1982;Kitchen and Rasmusson, 1983;Borras et al., 2009). However, little is known about spikelet survival and its role in improving grain yield. Crop breeding programs have focused intensively on final grain yield directly rather than improving other yield components such as spikelet survival. In barley, the number of spikelets per spike at the awn primordium (AP) stage represents the maximum yield potential per spike (Riggs and Kirby, 1978;Waddington et al., 1983;Kirby and Appleyard, 1987;Kernich et al., 1997). Moreover, the maximum number of spikelet/floret primordia in wheat is genetically controlled (Kirby et al., 1989;Gonzalez et al., 2003). Six-rowed barley has more spikelet primordia per spike at the AP stage than does two-rowed barley (Whingwiri and Stern, 1982;Kirby and Appleyard,

1987;Kernich et al., 1997;Miralles et al., 2000;del Moral et al., 2002;Arisnabarreta and Miralles, 2006) and variation in the number of spikelets per spike at AP is higher in six-rowed barley (Kitchen and Rasmusson, 1983;Kernich et al., 1997).

Several studies have postulated that the differences in spikelet mortality between two- and six-rowed barley arise as a result of competition for assimilates (Kirby, 1988;Arisnabarreta and Miralles, 2004), competition between spikelets per spike (Appleyard et al., 1982) or the position of spikelets within the spike (Arisnabarreta and Miralles, 2006). Spikelet abortion is generally higher in six-rowed barleys (Frank et al., 1992;Kernich et al., 1997;Arisnabarreta and Miralles, 2004;Arisnabarreta and Miralles, 2006) because they possess more fertile spikelet primordia per spike (Whingwiri and Stern, 1982;Kirby and Appleyard, 1987;Kernich et al., 1997;Miralles et al., 2000;del Moral et al., 2002;Arisnabarreta and Miralles, 2006). However, no focused research has been performed to identify the causes of spikelet/floret survival from AP to harvest in both row-type classes of barley.

In barley, the pre-anthesis developmental phases include the vegetative phase (leaf initiation), the early reproductive phase (spikelet/floret initiation; from double ridge until awn primordium) and the late reproductive phase (spike growth and development; from awn primordium until anthesis) (Appleyard et al., 1982;Kirby and Appleyard, 1987;Slafer and Rawson, 1994;Sreenivasulu and Schnurbusch, 2012). Variation in the duration of the pre-anthesis developmental phases and morphological changes, particularly during the late reproductive phase, have been reported (Appleyard et al., 1982;Kitchen and Rasmusson, 1983;Kernich et al., 1995a;1997). The duration of these phases is affected by environmental conditions such as temperature, photoperiod and vernalization, but also by genotypic differences among barley varieties (Appleyard et al., 1982;Kernich et al., 1995b;Kernich et al., 1997). Increasing the duration between the triple mound stage (when the spikelet ridge part of the double ridge has differentiated into three distinct bumps or mounds, Kirby and Appleyard (1987) to heading time (HD) may increase barley grain yield through higher

spikelet/floret fertility (Miralles et al., 2000). In barley and wheat, stem elongation (SE) appeared to be the critical period for determining floret/spikelet survival (Cottrell et al., 1985;Gonzalez et al., 2003) and extending its duration has been proposed as an appropriate way for increasing grain yield (Miralles et al., 2000). Therefore a more complete understanding of the genetic constitution of these pre-anthesis phases could help breeders in improving grain yield (del Moral et al., 2002).

In this study we investigated spikelet survival from the standpoint of developing a more detailed description of the later reproductive phases in barley and their specific influences on fertility. We sought to identify a sub-phase in which the majority of spikelets were aborted and to estimate the broad-sense heritability of this trait. Finally, we examined differences in spikelet survival between two and six-rowed barleys as well as in plants growing in different environments (greenhouse vs. field). In our study of spikelet fertility in the context of later reproductive development, we used 32 diverse spring barley accessions to address the following specific objectives: I) Quantify spikelet number per main culm spike (SNS) at several stages during the late reproductive phase (from awn primordium to anther extrusion) and determine spikelet survival in individual sub-phases, (II) Measure yield, yield components, and the contribution of the main spike to single-plant yield, (III) Estimate the genetic basis for these traits by calculating their repeatability/broad-sense heritability and (IV) Correlate the onset of stem elongation (SE) and the late reproductive sub-phases with spikelet survival in barley.

Analyses of these traits in 32 accessions under greenhouse and field conditions showed that spikelet survival in barley is highly genetically controlled. We narrowed down the critical growing period in which most spikelet abortion occurred and found that most of the reductions consistently happened during the first sub-phase of the late reproductive phase, from AP to tipping (TIP), regardless of different growing conditions. These findings may help

breeders to maximize barley grain yield by focusing on this critical period of grain development.

2.3 Materials and methods

2.3.1 Plant materials and experimental conditions

The study was conducted at the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany. Three growing conditions were used during this study: (i) Greenhouse (GH), or planting into pots in controlled conditions, (ii) Field planting into pots and (iii) Field planting into soil. Temperature, rainfall and humidity data for the field and GH conditions are presented in **Table 2-1**. Seeds for each of the three growing conditions were planted on the same date (1st of April 2012) and each condition was represented as a set of 32 spring barley accessions comprising a worldwide collection of 14 two-rowed and 18 six-rowed accessions (see **Table 2-2**). Thirty plants per accession were planted in each growing condition, resulting in a total of 960 barley plants. Agricultural practices were performed as recommended, including pest, disease and weed control. For the GH experiment (long days, 16h/8h day/night and ~20/ ~16 ^{o}C day/night), seeds were germinated in trays and seedlings were grown for ten days until they reached the 2-3 leaf stage. Seedlings were then exposed to vernalization at ~4 ^{o}C for a period of 28 days. Seedlings were subsequently hardened for a period of 7 days to gradually acclimatize the plants under 12/12 h and ~14/~12 ^{o}C, day/night and temperature respectively. Plants were transplanted into 0.5 liter pots (one plant per pot; 9x 9cm pot diameter and height) and grown in potting substrate (peatmoss fertilized with 14:16:18 N:P:K)under long day conditions (16 h/8 h light/dark and ~20/~16 ^{o}C). Plants were irrigated daily and each 0.5 L pot was fertilized with 1.5 gram (17:11:10 / Nitrogen (N): Phosphorous (P): Potassium (K)) fertilizer to avoid nutritional deficiencies.

Table 2-1: Monthly average temperature (^{o}C), precipitation (mm) and relative humidity (%) in the field and greenhouse during the 2012 growing season at IPK.

Month	Field			Greenhouse‡	
	Temperature ^{o}C	Rainfall (mm)	Relative humidity %	Temperature ^{o}C	Relative humidity %
April	8.8	17.4	75.8	9.3	72.9
May	15.0	48.7	73.0	14.3	71.7
Jun	15.6	72.4	80.3	18.8	77.2
July	18.1	93.4	78.7	19.7	75.9
August	18.7	38.1	75.3	20.0	72.6

Planting date for all growing conditions was April 1st 2012.
‡Greenhouse-grown plants were maintained for10 days at 20 ^{o}C for germination, for 28 days at 4 ^{o}C for vernalization, for 7 days at 14 ^{o}C for hardening and under normal greenhouse growing conditions at 20 ±1 ^{o}C until harvest.

Table 2-2: Spring barley accessions used in this study.

No.	Name	Germplasm status	Origin	Name	Germplasm status	Origin
	Six-rowed			Two-rowed		
1	BCC1453	Cultivar	Finland	BCC1497	Landrace	Kyrgyzstan
2	HOR2835	Landrace	Iran	BCC1541	Cultivar	Yugoslavia
3	BCC1494	Landrace	Kazakhstan	BCC869	Cultivar	Mexico
4	BCC579	Cultivar	India	HOR8006	Landrace	Turkey
5	BCC219	Landrace	Tajikistan	Barke	Cultivar	Germany
6	BCC447	Cultivar	China	BCC1566	Landrace	Greece
7	BCC719	Cultivar	Korea	BCC1589	Landrace	Italy
8	Morex	Cultivar	USA	Triumph	Cultivar	Germany
9	BCC814	Breeder line	USA	BCC801	Cultivar	Canada
10	BCC818	Cultivar	USA	Proctor	Cultivar	UK
11	BCC718	Cultivar	Korea	BCC1370	Cultivar	France
12	BCC551	Cultivar	India	BCC1371	Cultivar	France
13	BCC577	Cultivar	India	BCC903	Landrace	Afghanistan
14	BCC888	Cultivar	Canada	Weeah	Cultivar	Australia
15	BCC942	Cultivar	USA			
16	BCC875	Cultivar	USA			
17	BCC921	Cultivar	Colombia			
18	BCC868	Breeder line	Mexico			

Supplemental light (~300mEm^{-2} s^{-1} PAR) was used to extend the natural light with low intensity incandescent lamps (Philips son-t agro 400 w). Randomization of pots was conducted three times per week to minimize border and temperature-gradient effects on growth and development. For pot-grown plants in the field, seeds were directly planted into identical 0.5 L pots. Plants were grown in potting substrate and fertilized as above. Plants were manually irrigated when required. For soil-grown plants in the field, seeds were planted directly into silty loam soil (10 plants per row; 50 cm-long rows with 20cm spacing between

rows). Three rows for each accession were randomly distributed and 15 grams of fertilizer per row (17:11:10 N: P: K) was applied. Plants were manually irrigated when required.

2.3.2 Phenotyping and data recording

Data were collected at five developmental stages: awn primordium (AP, the stage is reached when the tip of the lemma starts to grow and curves over the stamen primordia (maximum yield potential)), (Kirby and Appleyard, 1987); tipping (TIP, Z49, first awns visible from flag leaf sheath); heading (HD,Z55, the inflorescence is half emerged from the flag leaf sheath); anther extrusion (AE, Z65, the spikes have anthers extruded) and physiological maturity (PM, Z92, yellow spike, physiological maturity) (Zadoks et al., 1974). The time for each stage was recorded when at least 50% of the main culm spike in each accession had reached this stage. Growing degree days (GDD) or thermal time was used to identify the required temperature for each stage and the base temperature was set to 0 ^{o}C.

Initially, three plants per accession were randomly selected and tagged when the youngest leaf (i.e. top leaf) on the main culm had initiated (Karsai et al., 2011). Estimations of the onset, length and end of stem elongation (SE) were calculated by measuring the height of the youngest, fully developed leaf (the distance between the soil surface and youngest leaf) eight times (**Supplementary Figure 2-1**). These measurements started from Z20 (main shoot only) and continued to Z69 (anthesis completed) (Zadoks et al., 1974) and are expressed as thermal time as well as the number of days. SE started to occur when the distance between the soil surface and the youngest leaf increased, while SE reached an end when this distance stopped changing. To identify the critical duration for spikelet survival, SE was calculated in two different ways: 1) estimated onset of SE (based on leaf height) to the end of SE, and 2) from AP to the end of SE. Correlations with grain yield, yield components and spikelet survival using both methods were calculated.

During the first four developmental stages (AP, TIP, HD, AE) three plants per accession were randomly selected to determine each stage and the number of spikelet primordia or spikelets

on each main culm. To accurately determine the AP stage, immature barley inflorescences were prepared for microscopic dissection and image capture (Stereo Microscope Stemi 2000-C with KL 1500 LCD; Axio Vision, 4.8.2, ZEISS Germany) to count the number of spikelet primordia from each main culm. To identify the exact timing for the awn primordium stage (Kirby and Appleyard, 1987), regular dissection work of the main culm apex had to be performed in each accession three times per week. Floral primordia were counted along the spike to score the maximum number of spikelet per main culm spike.

Number of spikelets was counted at each stage to deduce the changes in SNS. For each sample, main spike dry weight (MSDW), and tiller numbers were also recorded. At each stage, SNS and its relation to GDD was calculated to identify the importance of GDD in spikelet survival between different stages. Spikelet survival was calculated based on the total number of spikelets (both sterile and fertile) at AP stage while it was for developed spikelet at other stages (i.e. TIP, HD and AE), and spikelet fertility was calculated based on the yield of fertile grain at harvest. The equations for spikelet survival and fertility are expressed as follows:

Equation 2-1: Spikelet Survival (%)

$$\boldsymbol{Spikelet\ Survival}\ (\%) = \frac{(Developed\ Spikelets\ x)}{(Developed\ Spikelets\ \ x-1, x-2\ldots . x-n)}\, x\ 100\%$$

Where *x* is a specific stage, and *x*-1, -2…-*n* are stages prior to *x*. For example, if x is Harvest, then X-1 is AP, X-2 is TIP.

Equation 2-2: Spikelet Fertility (%)

$$\boldsymbol{Spikelet\ Fertility}\ (\%) = \frac{(\ Grain\ \ at\ Harvest\ stage\ )}{(Developed\ Spikelets\ at\ Heading\ stage)}\, x\ 100\%$$

2.3.3 Yield and yield components

Six plants per accession were randomly harvested by hand to determine biological yield (BY), which was expressed as the total weight of air-dried aboveground tissue. Single-plant grain yield and yield components were measured by counting the number of grains per main spike

(GNS) and per plant, total grain weight per main spike (GWS) and per plant, following hand threshing. Harvest index (HI) per plant was measured as the ratio of grain weight per plant to BY per plant multiplied by a factor of 100. Main spike harvest index (MSHI) was measured as the ratio of GWS to BY per plant. The MSHI ratio to total HI of each plant was measured to identify the contribution of the main spike toward grain yield. The spike fertility index (SFI) was calculated as the ratio between the number of grains per gram of the main culm spike to the dry weight of the main culm spike chaff (non-grain biomass of the spike) at Hrv (grains. g^{-1}). The grain weight of 1000 grains (1000-grain weight or TGW) was measured at harvest.

2.3.4 Statistical analyses

Each growing condition had a completely randomized design with three replications for each stage and six replications for single-plant yield and yield components. The collected data were analyzed using SAS software version 9.3 at probability level $P \leq 0.05$. Student's *t*-test was used to compare between row-types (i.e. two- and six-rowed) from the same growing condition, while Fisher's Least Significant Difference (LSD) was used to compare row-types across growing conditions. Phenotypic correlation analyses (Pearson) between row-types and growing conditions were calculated using PROC CORR (SAS, 2013). The repeatability (REP) of individual traits was calculated for each row-type within growing condition and broad-sense heritability (H^2) across growing conditions as the ratio between genetic and the phenotypic variance components (PROC VARCOMP (SAS, 2013)). Multi-variate analysis has been performed by Principal Component Analysis (PCA-biplot) to interpret and summarize the major pattern of variation between growing conditions by accessions by heading date, yield and yield components (main culm spike and single plant). PCA is an indicator ordination tool for obtaining multivariate data, which can be explored visually in a two dimensional PCA correlation biplot. PCA was calculated based on accession means for

each trait, under each growing condition to study the interrelationship between the components using GenStat 16 (GenStat, 2014).

2.4 Results:

2.4.1 Comparisons between growing conditions

To verify whether GH conditions can be used to study spikelet survival and related traits of interest we performed a correlation analysis of growing conditions for spike-related traits with data obtained from both GH-grown and field-grown plants. Correlations between growing conditions for thermal time, heading date, spikelet number and spikelet survival per main spike showed significant to extremely robust correlations ranging from 0.62 to 0.98 (**Figure 2-1**). The highest correlation among different growing conditions was observed for thermal time from sowing to PM ($r \geq 0.95$), whereby the strongest correlation was observed between soil-grown and pot-grown field plants ($r = 0.98$). Correlations between growing conditions for SNS (from AP to Hrv) were $r \geq 0.89$ and for heading date, $r \geq 0.83$, respectively. Moreover, soil-grown and pot-grown field plants had the strongest correlations among growing conditions ($r = 0.87$ and $r = 0.94$, for SNS and heading date, respectively). However, spikelet survival had the lowest correlation between different growing conditions and ranged from 0.62 (soil vs. GH) to 0.72 (soil vs. pot field). For the four traits analyzed, a general trend was apparent, showing slightly higher correlations between soil-grown and pot-grown field plants and slightly lower correlations between soil-grown field plants and GH-grown plants. To validate results from the correlation analyses we further performed multi-variate analysis of the 32 barley accessions using principle component analysis (PCA; see **Figure 2-2**). The PCA analysis was used to examine whether growing conditions had effects on plant development, yield and yield components. Based upon PC1 and PC2, two groups could be clearly identified due to their differences in row-type (two- and six-rowed barleys). In each row-type group, accessions from different growing conditions were clearly overlapping with no further

clustering, suggesting that row-type differences explained most of the observed variation in these experiments.

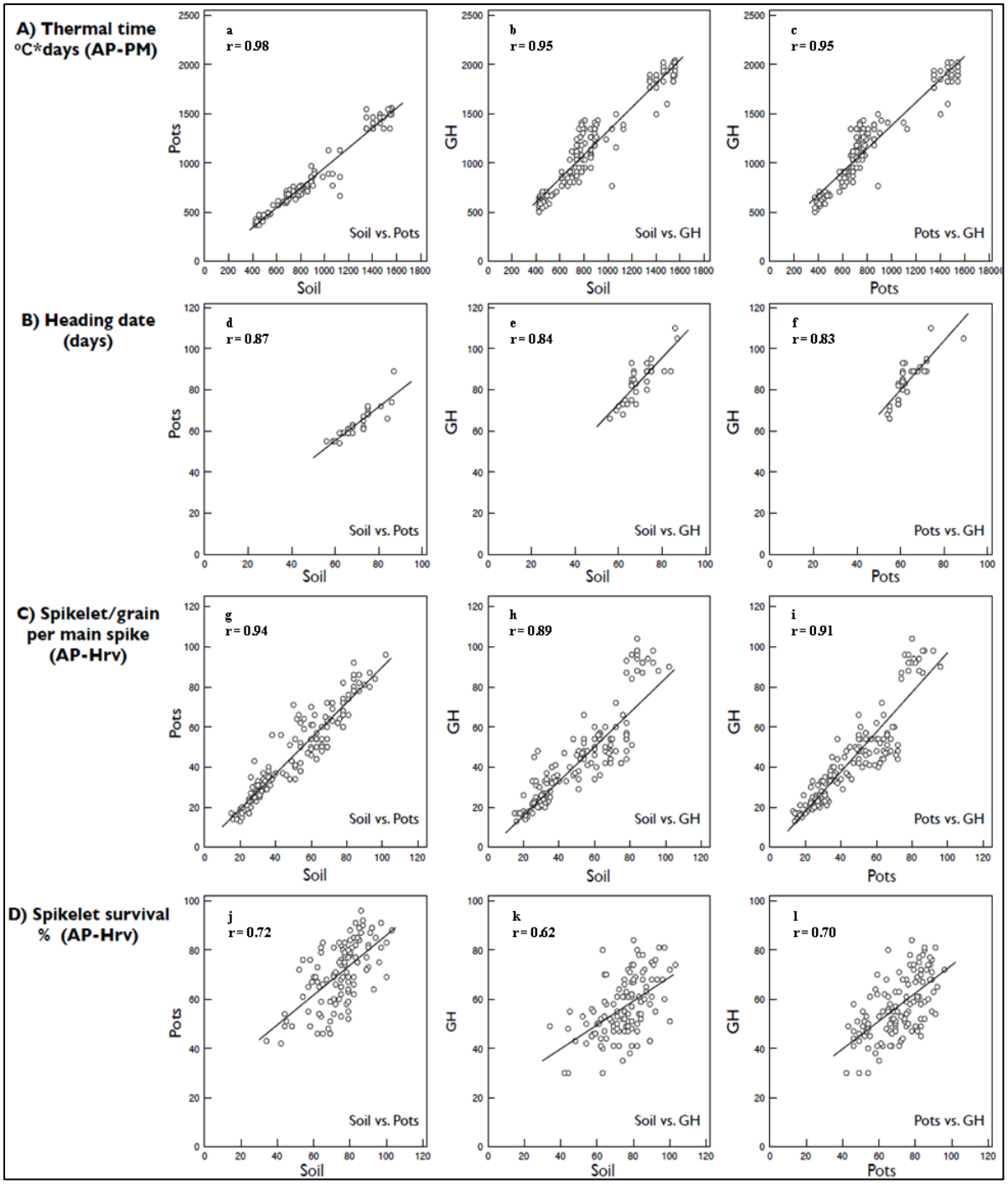

Figure 2-1: Correlation analysis for all barley accessions across stages and different growing conditions

(a, d, g, j) soil versus pots; (b, e, h, k) soil versus greenhouse; (c, f, i, l) pots versus greenhouse in (a, b, c) thermal time (^{0}C.days) during the period between the awn primordium (AP) stage to physiological maturity (PM) (n = 160); (d, e, f) heading date (HD) (n = 32); (g, h, i) spikelet/grain number per main spike during the period between AP and harvest (Hrv) (n = 160) and (j, k, l) spikelet survival (% per main spike) at AP, tipping, HD, anther extrusion and Hrv (n = 160).

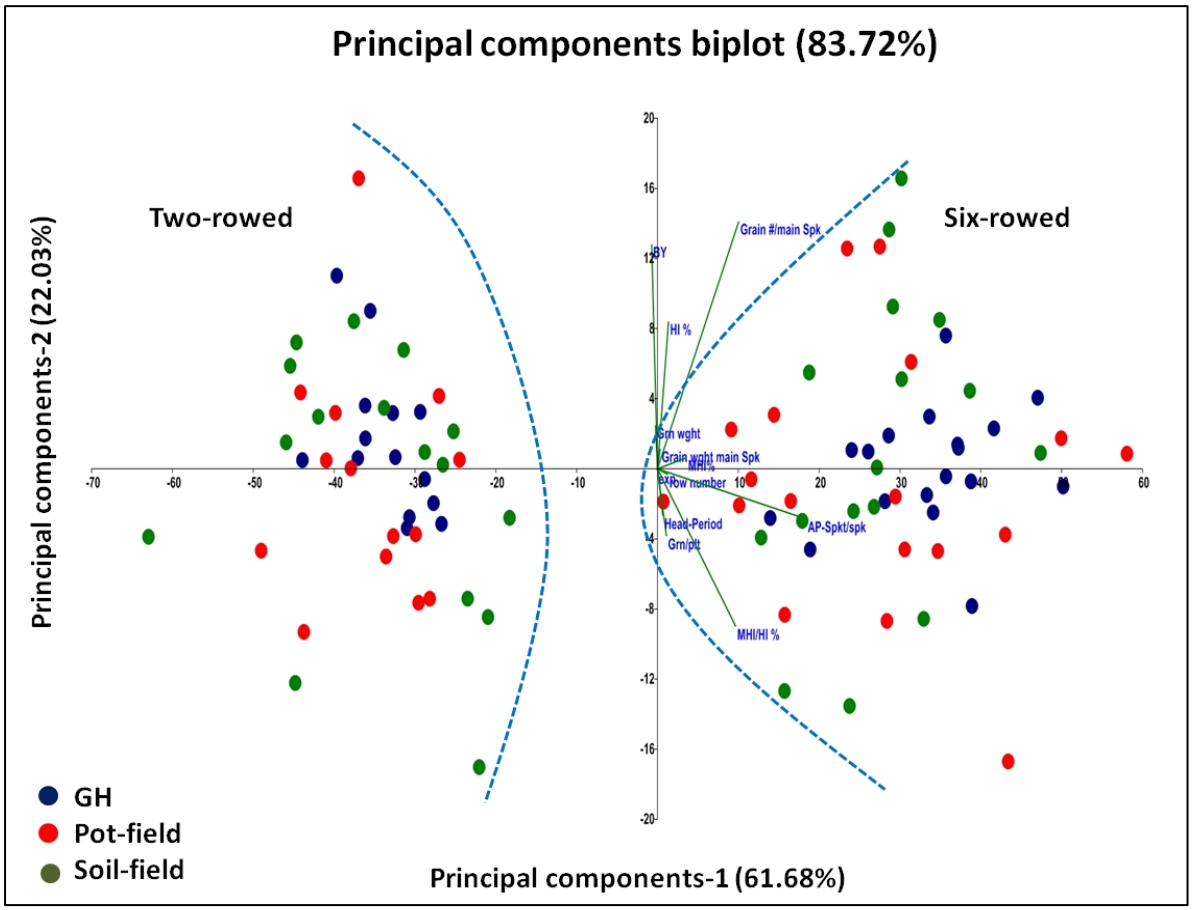

Figure 2-2: Principal component analysis biplot (PCA) based on phenotypic data for different growing conditions by accessions by yield and yield components (single plant and main culm spike) and heading date.
n = 54 (18 x 3 reps) in six-rowed and n = 42 (14 x 3 reps) in two-rowed barley.

2.4.2 Thermal time to reach developmental stages, sub-phases and stem elongation

Comparisons of thermal time to reach different developmental stages in two- and six-rowed barley under the same growing condition yielded no significant differences between the row-type classes ($P \leq 0.05$; **Figure 2-3A**). The duration from sowing to reach each developmental stage was significantly longer in both row-type classes under GH conditions compared with pot-grown and soil-grown field plants (**Figure 2-3A**). Regardless of row-type, the pot-grown field plants showed the most rapid development in all stages prior to anther extrusion. In two-rowed barley, the duration of developmental stages was significantly different between growing conditions at AP, TIP, and HD (**Figure 2-3A**). Very similar trends were found for the six-rowed barley results at all stages at $P \leq 0.05$ (**Figure 2-3A**). The duration between AP-TIP was the longest reproductive sub-phase in comparison to GDD between TIP-HD and HD-

AE under all growing conditions and row-types (**Figure 2-3B**). The shortest reproductive sub-phase for both row-type classes was in pot-grown field plants from AP-TIP and TIP-HD stages. However, soil-grown field plants had the shortest duration between HD-AE (**Figure 2-3B**). Generally, there was no significant difference between two- and six-rowed barley in terms of the duration of the sub-phases in plants growing under the same condition.

Differences between both row-types for the onset of SE, AP, end of SE and the duration of SE in the same growing condition were not significant (**Figure 2-3C**). Spikes from GH-grown plants required more thermal time to reach each stage than plants under pot- and soil-grown field conditions. There were no significant differences between the onset of SE and AP in both row-type classes under field conditions but differences of 124 and 161 GDD in two- and six-rowed barleys were observed under GH conditions (**Figure 2-3C**). SE halted approximately at the HD stage under all growing conditions. The durations from onset to end of SE and AP to end of SE were significantly longer under GH conditions compared to pot- and soil-grown field conditions.

The correlations of durations between the estimated onset of SE (based on leaf height measurements) to the end of SE and AP to the end of SE (GDD) with spikelet survival and yield components traits were calculated for all growing conditions. The correlations for GNS at Hrv, spikelet survival from AP-Hrv, MSDW at HD, GWS, GNP and GWP ranged from 0.11 to 0.82 for the estimated onset of SE to end of SE (**Table 2-3**). A very similar range of correlations was obtained for the duration from AP to the end of SE (0.16 to 0.88) for both row-types under all growing conditions (**Table 2-3**). However, the correlation was higher for spikelet survival in AP to the end of SE (r= 0.68 for two- and six-rowed barley under GH and field grown pot conditions) than the duration from the estimated onset (leaf height measurement) to the end of SE (r= 0.58 for six-rowed barley under field grown pot conditions). For MSDW at HD, the highest correlation was from the two-rowed barley under GH conditions (r= 0.75 for AP to end of SE and r= 0.73 for onset to end of SE). The highest

correlation value for GWS was obtained from two-rowed barleys under pot-growing conditions (r= 0.87 for AP to end of SE compared to r= 0.82 for estimated onset to the end of SE). For GNP, the correlation between the duration of AP to the end of SE was higher than the correlation between the duration of the estimated onset to the end of SE (r= 0.71 and r= 0.65, respectively). For GWP, the highest correlation (r= 0.68) was observed for the duration of AP to the end of SE compared with the onset to the end of SE (r = 0.57). There was a trend of higher correlations with spikelet survival and yield traits with the duration starting from AP compared to the estimated onset of SE (based upon leaf height).

Table 2-3: Correlation analysis of durations (GDD) between the estimated onset of stem elongation (based on leaf height measurements (Karsai et al., 2011) and awn primordium (AP) to the end of stem elongation (SE) with some yield and yield components traits.

Yield components	Onset-End (SE)						AP-End SE					
	GH		Pots		Soil		GH		Pots		Soil	
	2-row	6-row	2-row	6-row	2-row	6-row	2-row	6-row	2-row	6-row	2-row	6-row
Grain number. main spike^{-1} (Hrv)	0.62	0.24	0.70	0.57	0.80	0.34	0.80	0.52	0.78	0.40	0.88	0.58
Spikelet Survival (%) AP-Hrv	0.48	0.20	0.41	0.58	0.18	0.31	0.68	0.40	0.58	0.68	0.51	0.39
MSDW (HD)	0.73	0.55	0.61	0.58	0.61	0.62	0.75	0.62	0.46	0.60	0.70	0.71
Grain weight. main spike^{-1} (g)	0.67	0.43	0.82	0.38	0.75	0.11	0.76	0.38	0.87	0.39	0.79	0.16
Grain number. plant^{-1}	0.64	0.52	0.45	0.55	0.65	0.26	0.71	0.47	0.50	0.53	0.70	0.37
Grain weight. plant^{-1} (g)	0.44	0.35	0.25	0.35	0.57	0.27	0.62	0.49	0.36	0.38	0.68	0.40

Thirty-two barley accessions (two-rowed n=14; six-rowed n=18) were grown in a greenhouse as well as under field conditions (pots and soil).

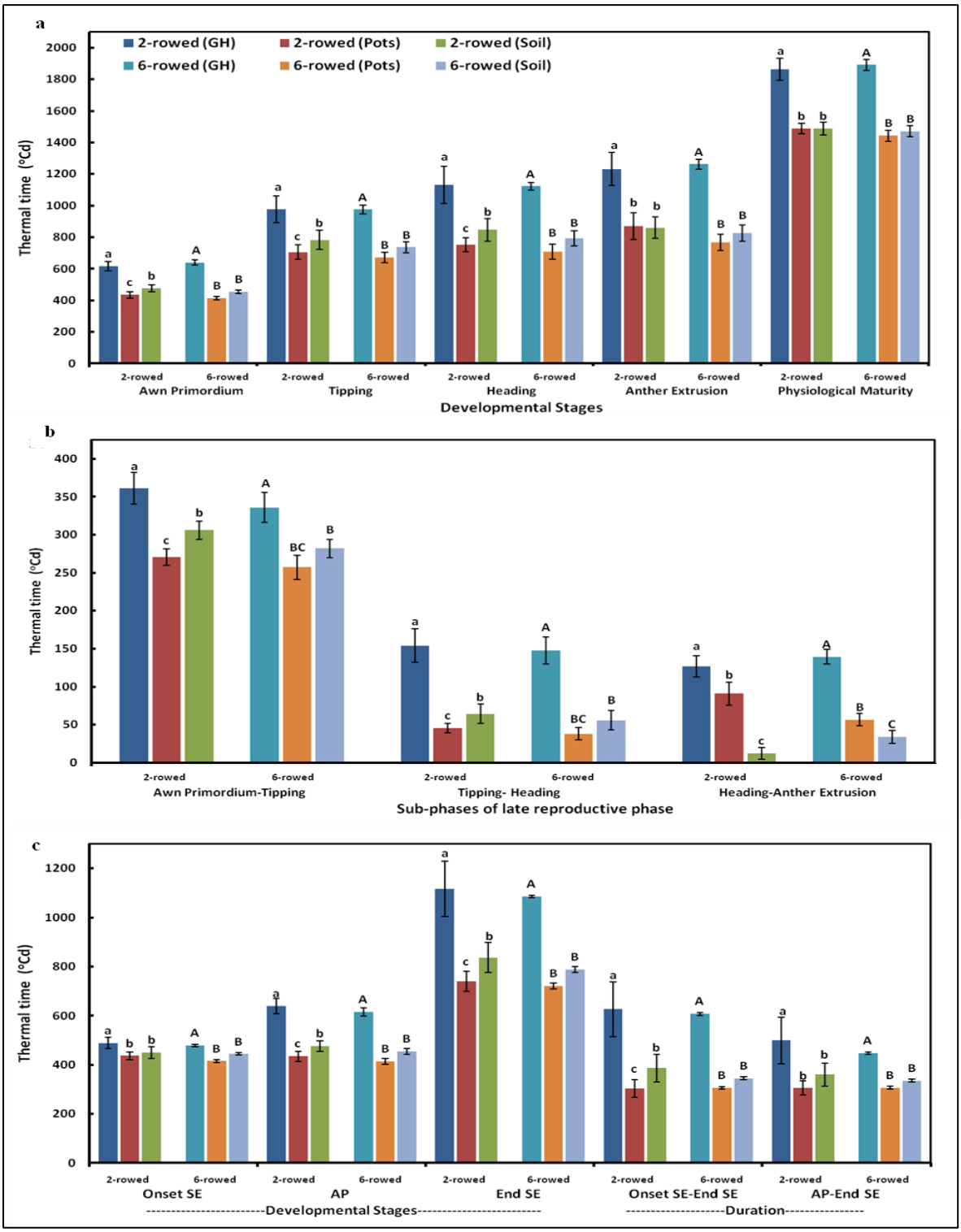

Figure 2-3: Thermal time for different developmental stages and phases.

A) Thermal time from sowing to the beginning of AP (awn primordium), tipping, heading, anther extrusion and physiological maturity (PM) stages. B) Thermal time of the duration between sub-phases. C) Thermal time of the onset of stem elongation (SE), AP, end of SE, and the duration between onset and AP to the end of SE. Small and capital letters differentiate between two-rowed and six-rowed barley, separately at each stage. Among growing conditions, at each stage the same letters for each row-type are not significantly different at $P \leq 0.05$ according to Least Significant Difference. Error bars indicate standard deviation (n = 14 and 18 for two- and six-rowed barleys, respectively).

2.4.3 Spikelet number per main spike, spikelet survival and fertility

Under all growing conditions, there were highly significant differences ($P \leq 0.001$) between both row-type classes for SNS and spikelet survival. Across the three growing conditions, six-rowed barley generally had significantly higher SNS at all stages compared to two-rowed barley (**Table 2-4**). In all growing conditions, two-rowed barley had similar numbers of spikelet primordia at the AP stage, but showed a strong decline in SNS from AP-TIP. After this developmental period, only a gradual reduction of SNS was observed. Significant differences for the highest SNS between growing conditions were found after AP in soil-grown field plants, followed by pot-grown field plants and GH-grown plants (**Table 2-4**). In six-rowed barley, GH-grown plants had the highest yield potential measured as SNS at AP. The reduction in SNS between AP-TIP was evident under GH conditions, but at TIP the spikes from GH-grown plants and pot-grown field plants displayed the same number of spikelets (**Table 2-4**). Consequently, spikelet reduction mostly occurred from AP-TIP in both row-type classes under all growing conditions, with the highest relative reduction occurring in GH-grown plants (**Table 2-4**). Two-rowed barley had significantly higher spikelet survival than six-rowed barley at all stages and in all growing conditions (**Table 2-4**). Spikelet survival from AP-TIP in two-rowed barley was significantly higher in soil-grown and pot-grown field plants than in GH-grown plants. A higher spikelet survival rate was observed in six-rowed barley from AP-TIP in soil-grown field plants, followed by pot-grown field plants and GH-grown plants (**Table 2-4**). In comparisons across all stages, spikelet survival in two- and six-rowed barley from soil-grown and pot-grown field plants was significantly higher than in GH-grown plants (**Table 2-4**). There was no significant difference between two- and six-rowed barley in spikelet fertility in plants growing under the same condition. Spikelet fertility from HD-Hrv was significantly lower in soil-grown field plants than in pot-grown field and GH-grown plants (**Table 2-4**). Notably, the repeatability/broad-sense heritability values for SNS

and spikelet survival ranged from 0.70 to 0.99, indicating that these traits are highly heritable under all growing conditions (**Table 2-4**).

Table 2-4: Spikelet and grain number per main spike (SNS and GNS) at different developmental stages, and spikelet survival for two- and six-rowed barley under three growing conditions.

		Spikelet number (SNS)				Grain number (GNS)	Spikelet Survival %		Spikelet Fertility %
		AP	TIP	HD	AE	Hrv	AP-TIP	AP-Hrv	HD-Hrv
Greenhouse	2-rowed	34a‡	23c	23c	22c	21c	68b	62b	91.4a
	REP†	**0.91**	**0.84**	**0.92**	**0.85**	**0.84**	**0.97**	**0.80**	**0.86**
	6-rowed	91A	55B	48C	46C	44B	60C	48B	91.6B
	REP	**0.99**	**0.99**	**0.95**	**0.90**	**0.80**	**0.99**	**0.70**	**0.74**
	P-value¶	***	***	***	***	***	***	***	ns
Field-Pots	2-rowed	31a	25b	25b	24b	23b	81a	74a	92.0a
	REP	**0.97**	**0.92**	**0.93**	**0.89**	**0.93**	**0.99**	**0.79**	**0.86**
	6-rowed	82C	55B	55B	54B	52A	67B	63A	94.5A
	REP	**0.94**	**0.98**	**0.98**	**0.93**	**0.95**	**0.92**	**0.73**	**0.88**
	P-value	***	***	***	***	***	***	***	ns
Field-Soil	2-rowed	33a	28a	28a	26a	25a	85a	76a	89.2b
	REP	**0.95**	**0.97**	**0.94**	**0.90**	**0.86**	**0.88**	**0.71**	**0.76**
	6-rowed	87B	62A	62A	61A	56A	71A	64A	90.3B
	REP	**0.87**	**0.92**	**0.95**	**0.75**	**0.77**	**0.89**	**0.74**	**0.74**
	P-value	***	***	***	***	***	***	***	ns
2-rowed (H^2)		**0.93**	**0.84**	**0.75**	**0.75**	**0.78**	**0.79**	**0.78**	**0.68**
6-rowed (H^2)		**0.84**	**0.84**	**0.74**	**0.76**	**0.78**	**0.81**	**0.80**	**0.66**

†REP/H^2: repeatability/broad-sense heritability for each row-type in each growing condition and overall growing conditions respectively.

¶*P*-value: Represents a comparison between two- (n=14) and six-rowed (n=18) barley in the same growing condition by Student's t-test. *** denotes a highly significant difference at $P<0.001$ and ns denotes not statistically significant at $P\leq0.05$.

‡Small and capital letters are to compare among growing conditions within two- and six-rowed barleys, respectively. Identical letters in each column indicate no significant difference at $P\leq0.05$ according to the Least Significant Difference (LSD) test.

All plants were sown on 1st April 2012 in the greenhouse as well as in the field.

Spikelet survival was calculated based on the total number of spikelets (sterile and fertile) and spikelet fertility was calculated based on the number of fertile grain at harvest.

2.4.4 Grain yield and major yield components per plant

Analysis of yield and yield components obtained from main culm spikes and single plants showed that there are significant differences between row-type classes and growing conditions. GWS and grain weight per plant (GWP) were significantly different for row-types in the different growing conditions (**Table 2-5**). Six-rowed spikes had significantly higher GWS than two-rowed spikes under all growing conditions. In contrast, two-rowed barley had significantly higher GWP in pot- and soil-grown field plants. GWS and GWP of GH-grown

plants were significantly lower than that of pot- and soil-grown field plants. In general, GNS and GNP were significantly lower under GH conditions (**Tables 2-4 and 2-5**). There were significant differences in tiller number per plant among row-types and growing conditions (**Table 2-5**). Two-rowed barley produced significantly more tillers per plant (13.7±5.2) than six-rowed barley (8.0±3.5). Moreover, GH-grown plants had significantly fewer tillers per plant (4.2±1.5) compared to pot- (14.0±4.1) and soil-grown field plants (14.4±3.9). There were significant differences between row-type and growing conditions for BY (**Table 2-5**). Average BY was higher in two-rowed barley under all growing conditions compared to six-rowed barley. Pot- and soil-grown field plants generated a higher BY regardless of row-type. The lower BY in GH-grown plants was mostly attributed to fewer tillers, reduced grain number, and reduced grain weight (**Table 2-**5). There were significant differences between row-types and between growing conditions for HI. Generally, HI was higher in GH-grown plants than in pot- and soil-grown field plants, and two-rowed barley had a higher HI than six-rowed barley (**Table 2-5**). By using GNS and GWS with BY data, we found that the MSHI and the ratio of MSHI to HI were higher in six-rowed barley under all growing conditions, suggesting that the six-rowed spike contributed more to single-plant yield than MSHI for two-rowed barley. Furthermore, both traits were higher in GH-grown plants than in pot- and soil-grown plants of both row-types. Notably, GWS was the most important contributor to increased HI, particularly under the GH condition. As a consequence of higher GNS in six-rowed barley, SFI at Hrv was significantly higher in six-rowed barley under all growing conditions. Pot- and soil-grown plants generally showed a higher SFI than GH-grown plants (**Table 2-5**). The 1000-grain weight (TGW) was also significantly different between row-types (**Table 2-5**). Two-rowed barley produced significantly higher TGW than six-rowed barley under all growing conditions. The analysis of TGW among growing conditions showed that, in two-rowed barley, soil-grown plants had significantly higher TGW compared to the other growing conditions. In contrast, pot-grown plants showed significantly lower TGW in

six-rowed barley (**Table 2-5**). Repeatability values for grain yield and yield components were above 0.6 for each growing condition, but broad-sense heritability values were smaller across growing conditions.

Table 2-5: Grain yield and yield components per plant for two- and six-rowed barley under three growing conditions.

		Grain yield and yield components									
		GWS g	**GNP** number	**GWP** g	**Tiller** number	**BY** g	**HI** %	**MSHI** %	**MSHI/HI** %	**SFI** grain.g^{-1}	**TGW** g
Greenhouse	**2-rowed**	0.91b‡	70c	3.0b	5.3b	9.7b	30.8b	10.7a	34.3a	61b	42.8b
	REP †	**0.91**	**0.84**	**0.88**	**0.92**	**0.79**	**0.82**	**0.79**	**0.80**	**0.78**	**0.84**
	6-rowed	1.64B	86B	3.5B	3.1B	9.2C	38.1A	18.3A	54.8A	89BC	40.7A
	REP	**0.92**	**0.85**	**0.76**	**0.87**	**0.78**	**0.71**	**0.67**	**0.79**	**0.75**	**0.81**
	***P*-value¶**	***	**	*	*	*	ns	***	***	***	*
Field Pots	**2-rowed**	1.18a	247a	10.2a	17.5a	27.3a	37.3a	4.6b	11.7b	83a	41.2b
	REP	**0.92**	**0.73**	**0.60**	**0.91**	**0.67**	**0.86**	**0.81**	**0.84**	**0.81**	**0.85**
	6-rowed	1.56B	234A	5.8A	10.4A	20.1B	28.7B	9.5B	26.7C	132A	24.7B
	REP	**0.71**	**0.69**	**0.76**	**0.84**	**0.61**	**0.72**	**0.63**	**0.69**	**0.72**	**0.84**
	***P*-value**	***	ns	***	***	***	**	***	***	***	**
Field Soil	**2-rowed**	1.31a	217b	10.2a	18.3a	33.0a	30.8b	5.0b	17b	91a	47.0a
	REP	**0.78**	**0.60**	**0.60**	**0.88**	**0.62**	**0.71**	**0.75**	**0.60**	**0.75**	**0.83**
	6-rowed	2.01A	191A	7.2A	10.5A	28.0A	25.6B	10.0B	42B	108B	37.6A
	REP	**0.88**	**0.71**	**0.64**	**0.90**	**0.70**	**0.62**	**0.64**	**0.61**	**0.74**	**0.82**
	***P*-value**	***	*	**	***	*	ns	***	***	**	**
2-rowed (H^2)		**0.60**	**0.67**	**0.68**	**0.76**	**0.66**	**0.69**	**0.66**	**0.57**	**0.62**	**0.74**
6-rowed (H^2)		**0.70**	**0.59**	**0.61**	**0.65**	**0.65**	**0.59**	**0.65**	**0.75**	**0.69**	**0.76**

†REP /H^2: repeatability/broad-sense heritability for each row-type in each growing condition and overall growing conditions respectively.

¶*P*-value: Represents a comparison between two- (n=14) and six-rowed (n=18) barley in the same growing condition by Student's t-test. *, **, *** denotes significant difference at $P<0.05$, 0.01 and <0.001 respectively and ns denotes not statistically significant at $P\leq0.05$.

‡ Small and capital letters are to compare among growing conditions within two- and six-rowed barleys, respectively. Identical letters in each column indicate no significant difference at $P\leq0.05$ according to the Least Significant Difference (LSD) test.

All plants were sown on 1st April 2012 in the greenhouse as well as in the field.

2.5 Discussion

This study supports the assertion that the maximum yield potential in barley occurs at the AP stage (Kirby and Appleyard, 1987). Spikelet/floret number declined after AP, resulting in significant differences for spikelet/floret survival and final GNS between the two row-type classes (**Figure 2-4**). Moreover, in the present study we identified AP to TIP as the most critical sub-phase related to spikelet reduction and grain yield per main spike (**Figure 2-4**). The main culm spike also had a significant role in improving yield potential. In addition, the

duration between AP to the end of SE showed better correlations with yield and yield components than did the estimated onset of SE (leaf height measurement).

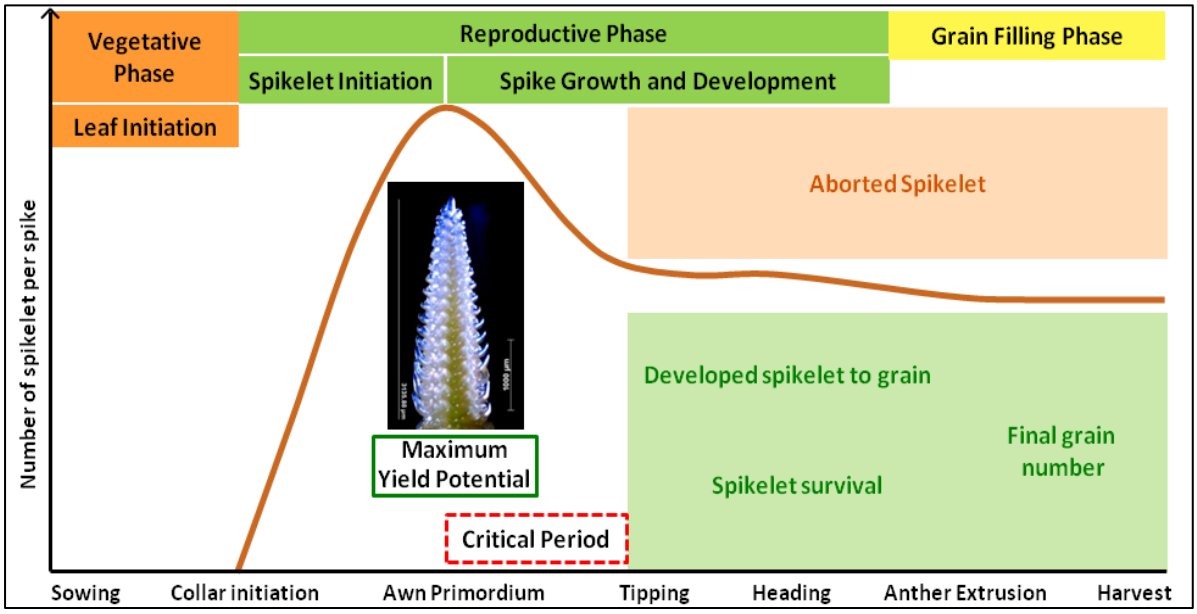

Figure 2-4: General trend of spikelet numbers per spike with its relation to GDD (stages).

2.5.1 Maximum yield potential and spikelet survival in the two row-type classes

In this study we examined the degree of variation in spikelet survival during spike development in different barley row-types and determined its repeatability/broad-sense heritability under different growing conditions. In both row-type classes, mortality of the spikelet primordia started with the onset of fast stem and spike growth under all conditions and lasted until HD and this finding is consistent with the findings of other groups (Kirby, 1988;Miralles et al., 2000;Arisnabarreta and Miralles, 2006). Regardless of the specific growing condition, we found that from all initiated spikelets, approximately 70% and 58% of spikelets survived in two-rowed and six-rowed barley, respectively. Therefore, six-rowed barley had higher spikelet mortality than two-rowed barley. Arisnabarreta and Miralles (2006) reported slightly lower spikelet survival in two- and six-rowed barley near isogenic lines (63 % and 44 %) in comparison to the present study. Several groups have suggested reasons for the differences in spikelet survival observed between two- and six-rowed barley. For example, six-rowed barley possesses a greater number of potentially fertile spikelet primordia

compared to two-rowed barley at the AP stage. Because of the greater sink size and competition among spikelets within a spike, the majority of spikelet primordia are aborted in six-rowed barley (Appleyard et al., 1982). Moreover, Arisnabarreta and Miralles (2006) explained the differences between barley row-types with respect to spikelet survival based on spikelet structure and position. These authors noted that the smaller carpels in six-rowed barley may be a cause for reduced spikelet survival. Moreover, they found that the reduced synchrony between central, basal and apical spikelet/floret primordia explained higher spikelet survival in two-rowed barley. In wheat, most of the floret abortion was observed in more distal spikelets (Whingwiri and Stern, 1982) possibly due to separated vascular bundles between rachis and distal spikelets (Hanif and Langer, 1972). We propose that similar mechanisms for higher spikelet survival in two-rowed barley are at play in our study.

We also showed that the abortion of spikelets was more pronounced under GH conditions than in pot- and soil-grown field plants. This observation could be explained by the increased interval of time required to reach AP under GH conditions. This possibly resulted in a longer spike differentiation phase and hence, in the production of more spikelet primordia. An increase in the number of spikelet primordia, or SNS, under GH conditions may have led to increased within-spike competition, which in turn resulted in a higher proportion of spikelet abortion.

We demonstrated that spikelet survival was similar under all growing conditions (environments) and that it is highly genetically controlled. The high broad-sense heritability suggests a promising, unexplored opportunity to better understand the genetic basis of spikelet survival in barley, thereby opening up a new area of research for increasing yield potential.

2.5.2 Effect of sub-phases on spike growth, development and spikelet survival

The importance of strictly defining pre-anthesis phases for improving yield potential of barley has been previously suggested (Ellis and Kirby, 1980;Kitchen and Rasmusson, 1983;del Moral et al., 2002). However, few studies have explored the specific contribution of pre-

anthesis sub-phases to spikelet survival. In this study we tested whether dividing spike growth and development into sub-phases can reveal a critical sub-phase which is important for spikelet mortality. Miralles et al. (2000) reported that the period between triple mound to HD is important for yield in barley and extending this period might effectively increase spikelet fertility. Our analysis showed that the AP-TIP sub-phase is the most critical period for spikelet survival, where more than two-thirds of the reduction in spikelet number occurred, regardless of the environment in which the plants were grown. This finding further narrows down the critical spikelet survival phase to a very precise interval which is contained within the longer phase reported by Miralles et al. (2000). Our results therefore support the notion that the period before HD is the most crucial for spikelet abortion (Kernich et al., 1996). The causes for spikelet abortion are unclear, but it is likely that the duration from AP-TIP is not sufficient to allow most of the newly initiated spikelet primordia to become fertile. The early phases of SE also coincide with spikelet development, which possibly drains resources away from developing and growing spikes. The duration between AP to HD in barley is sensitive to photoperiod. In some cases, main culm spikelet primordia were aborted when plants were grown under long days, and this is likely due to the shortening of the spikelet development period under long photoperiods (Kernich et al., 1996). Our study was also conducted under long day conditions, suggesting that photoperiod contributed to the higher spikelet abortion observed between the AP and TIP stages of development.

2.5.3 Importance of the main culm spike in improving yield

The main culm spike in barley, which is formed earlier than the secondary spikes, was the greatest contributor to single-plant grain yield and is therefore a worthwhile target to improve spikelet survival. The importance of the main culm spike in single-plant grain yield lies in producing more and heavier grains compared to spikes from side tillers in spring barley (Cottrell et al., 1985). Due to wheat breeding programs over the last 20 years, SNS and grain number per spikelet improved more than 30% through specific genetic gains in grain number

on the main spike (Sanchez-Garcia et al., 2013). In our study, we tested the contribution of the main culm spike using distinct parameters and found that yield from the main culm spike, measured as MSHI or the ratio of MSHI to HI, is clearly higher in six-rowed barley than in two-rowed barley. This observation is very likely due to the higher GNS, higher GWS and lower number of spikes and tillers per plant in six-rowed barley, resulting in a relatively higher MSHI. From an agronomical point of view, both GNS and GWS were highly correlated with GNP and GWP in all growing conditions. Thus, improving single-plant grain yield through targeted improvements of the main culm spike will be an important future goal.

2.5.4 Start and duration of SE and its correlation with yield and yield components

One objective of the present study was to correlate i) the duration from AP to the end of SE (the late reproductive phase) and ii) the duration of the estimated onset to the end of SE (based on leaf height, Karsai et al. (2011), with yield components. A previously reported method to estimate the onset of SE made use of the appearance of the first node on the stem (Borras et al., 2009). However, Karsai et al. (2011) noted that this method leads to an inflated interval between first node appearance and onset of SE in spring barley. In our study, we calculated that the estimated onset of SE occurred approximately 3-4 days earlier than the average occurrence of AP across all row-types and different growing conditions. Positive correlations were found between the duration of SE and all traits, including spikelet survival (**Table 2-3**). The importance of SE for spikelet survival and grain yield has been studied in small grain cereals (Fischer, 2007;Miralles and Slafer, 2007). Kernich et al. (1996) reported that spikelet survival is negatively affected under long day conditions due to a shortened SE period, and this was also a likely consideration in our study. As shown in **Table 2-3**, the duration between AP to the end of SE had better correlations than the estimated onset to the end of the SE duration with spikelet survival and yield traits. Manipulating vegetative and reproductive phases independently, particularly by extending the reproductive phase, is likely to increase the number of fertile florets by increasing assimilate acquisition by the spikes in

barley and wheat (Slafer and Rawson, 1994;Kernich et al., 1996;Miralles et al., 2000). It seems that this particular interval is most critical because survival of the initiated spikelets is determined during this period, which in turn represents the final grain number. Despite the importance of the duration of this phase (i.e. AP to TIP), it has received little attention in the literature, probably because this type of study requires high-quality microscopic dissections of spikes.

2.6 Summary and Conclusion

Our study of barley accessions grown in different environments revealed that: (i) Maximum yield potential in both row-types was observed at the AP stage of development, and the difference in spikelet survival between two-rowed and six-rowed barely is significant. (ii) Spikelet abortion occurred at a rate of approximately 30% in two-rowed barley and 42% in six-rowed barley. The majority of spikelet abortion occurred during the AP-TIP phase, which coincides with stem elongation. More than two-thirds (~72%) of all aborted spikelets were aborted during this phase, emphasizing the importance of this phase for improving barley yield. (iii) The duration of the interval from AP to the end of SE is a better indicator than the estimated onset to the end of SE duration for spikelet survival and grain yield in barley. (iv) The main culm spike is a major contributor to single-plant yield, particularly in six-rowed barley. (v) All of the growing conditions used in this study were suitable for studying agronomical and developmental traits such as flowering time, phase durations and spikelet survival. (vi) Results from this study also indicated that spikelet survival in barley is highly genetically controlled and an in-depth analysis of this trait is a worthwhile target for increasing yield in barley.

2.7 Acknowledgements

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3.0 CHAPTER THREE: Barley Leaf Area and Leaf Growth Rates are Maximized During the Pre-Anthesis Phase

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3.1 Abstract

Abstract: Leaf developmental traits are an important component of crop breeding in small-grain cereals. Surprisingly, little is known about the genetic basis for the differences in barley (*Hordeum vulgare* L.) leaf development. The two barley row-type classes, i.e., two- and six-rowed, show clear-cut differences in leaf development. To quantify these differences and to measure the genetic component of the phenotypic variance for the leaf developmental differences in both row-type classes we investigated 32 representative spring barley accessions (14 two- and 18 six-rowed accessions) under three independent growth conditions. Leaf mass area is lower in plants grown under greenhouse (GH) conditions due to fewer, smaller, and lighter leaf blades per main culm compared to pot- and soil-grown field plants. Larger and heavier leaf blades of six-rowed barley correlate with higher main culm spike grain yield, spike dry weight, and harvest index; however, smaller leaf area (LA) in two-rowed barley can be attributed to more spikes, tillers, and biological yield (aboveground parts). In general, leaf growth rate was significantly higher between awn primordium and tipping stages. Moderate to very high broad-sense heritabilities (0.67–0.90) were found under all growth conditions, indicating that these traits are predominantly genetically controlled. In addition, our data suggests that GH conditions are suitable for studying leaf developmental traits. Our results also demonstrated that LA impacts single plant yield and can be

reconsidered in future breeding programs. *Six-rowed spike 1* (*Vrs1*) is the major determinate of barley row-types, the differences in leaf development between two- and six-rowed barleys may be attributed to the regulation of Vrs1 in these two classes, which needs further testing.

Keywords: *Hordeum vulgare* L; Leaf dry weight; *Vrs1*; Two-rowed; Six-rowed

3.2 Introduction

Leaf traits and leaf architecture are important for crop adaptation to environmental conditions. Leaf area (LA) is considered to be an indicator of crop growth, development, and plant health, and has a strong relationship with leaf dry weight (LDW) in wheat and barley (Aase, 1978). LA and LDW are major factors that affect the growth rate through leaf thickness and/or density (Witkowski and Lamont, 1991). Leaf mass area (LMA) reflects the relationship between them and varies greatly between species due to nutrient and moisture availability, light intensity, and temperature (Witkowski and Lamont, 1991;Poorter et al., 2009). LMA is considered to be a key trait in plant growth (Lambers and Poorter, 1992), plant breeding (Westoby et al., 2002), ecology, agronomy (Poorter et al., 2009), and influences crops' responses to different growth conditions through changes in LA or/and LDW. For example, Witkowski and Lamont (1991) reported that leaves are smaller and heavier (higher LMA) under nutrient/moisture stress conditions. The variation in LA and related traits of 8 two-rowed barleys was previously attributed to growth habits, which are dependent on vernalization requirements and photoperiod (Van Oosterom and Acevedo, 1993). Thus, studying the variation of LA and related traits in both barley row-type classes at specific developmental stages independent of growth habit could help to understand the genetic constitution of these traits. Leaf growth rate traits reflect the responses of winter barley to the environment and their relationship with phyllochron (Tesarová and Nátr, 1990). Moreover, leaf traits are important for competing against pests, for example, rapid early growth, droopy leaves, high LMA, leaf size, and leaf number can markedly reduce weed growth in rice and wheat (Coleman et al., 2001;Zhao et al., 2006).

During the 1960s and 1970s, several studies proposed to enhance grain yield potential by changing individual traits in cereal breeding programs following an "ideotype" concept where LA was one of the targeted traits (Jennings, 1964;Donald, 1968). Leaf attributes for ideotype breeding were based on successful rice breeding programs that produced smaller, narrower, shorter, and more erect leaves to adapt with wide-range of environments (Jennings, 1964;Peng et al., 2008). However, during twenty years of cereal breeding, many difficulties and challenges were encountered in selecting for leaf traits. In barley breeding programs, progress in demonstrating that leaf traits improve yield was slow (Rasmusson, 1987). Rasmusson (1987) reported that the major challenge for obtaining smaller leaves with larger spikes in wheat and barley was due to insufficient heritability of LA.

Many studies have highlighted that large LA is a valuable trait in breeding programs for improving yield (Yoshida, 1972;Rasmusson, 1987;Van Oosterom and Acevedo, 1993;Bertholdsson, 2013;Fenta et al., 2014). For example, genetic material from Indonesian *tropical japonica* rice landraces was used to create broader leaves in a breeding program for New Plant Type (NPT) to improve grain yields (Khush, 1995;Fujita et al., 2013). Moreover, another study reported that large LA (flag leaf) in two contrasting barley populations resulted in higher grain yield because of higher photosynthetic rates under field conditions (Berdahl et al., 1972). LA and its position in the canopy have an effect on the relationship between cereal growth, yield, and photosynthesis (Gallagher and Biscoe, 1978) and maximizing photosynthetic rate could be achieved by expanding LA in rice and wheat (Driever et al., 2014;Jiang et al., 2015). Manipulating LA to increase grain yield would be beneficial for future breeding programs (Richards, 1983;Rasmusson, 1987); however, LA manipulation in barley did not succeed because of low heritability which did not permit effective genetic manipulation.

Evaluation of crop growth rates across a wide-range of environments has been studied and related to LA index and radiation use efficiency (RUE), such as in evaluating barley under

drought stress conditions (Jamieson et al., 1995). This relationship is considered to be a key factor for determining crop yield and biomass due to a favorable canopy architecture associated with increase in leaf photosynthetic capacity (photosynthetic rate per unit LA) particularly in wheat and barley dwarfing genotypes (Morgan et al., 1990;Miralles and Slafer, 1997). However, RUE is crop-dependent, highly influenced by environments and simulated models, therefore the genetic progress for this trait is difficult to attain (Reynolds et al., 2000;Kemanian et al., 2004) because it seems more complex to be improved than other leaf traits.

The barley spike is composed of rachis nodes and each node possesses three spikelets (one central and two lateral spikelets) (Forster et al., 2007b). In six-rowed barley, the three spikelets are fertile (one central and two lateral), whereas only the central spikelet is fertile in two-rowed barley (Alqudah and Schnurbusch, 2014). Two-rowed wild barley (*H. vulgare* ssp. *spontaneum*) is the progenitor of cultivated barley (*H. vulgare* L. ssp. *vulgare*) (Badr et al., 2000) and six-rowed barley was domesticated thereafter (Zohary and M, 2000). Thus, the barley spike can appear in two major forms: two-rowed and six-rowed. Differences between two- and six-rowed barley have been extensively investigated in the context of spike-related traits (Alqudah and Schnurbusch, 2014). The barley row-type is predominantly regulated by the *SIX-ROWED SPIKE 1* (*Vrs1*) gene (Komatsuda et al., 2007), and it was found that loss of function *Vrs1* leads to fully developed, fertile lateral spikelets in the six-rowed barleys; whereas wild-type, functional *Vrs1* results in infertile lateral spikelets and a two-rowed phenotype.

To better understand the barley leaf developmental traits, also to quantify and characterize the leaf developmental differences (LA and related traits) between two- and six-rowed barleys, we examined a representative set of 32 spring barley accessions (14 two- and 18 six-rowed) under greenhouse (GH) and field conditions (pot and soil) during pre-anthesis developmental stages. In addition, we also intended to identify the extent to which leaf traits are genetically

controlled under these conditions; and compare leaf performance traits and their correlation with single plant grain yield. Understanding leaf growth rates during pre-anthesis developmental stages could give important cues for the biological mechanism underlying leaf development. Studying leaf developmental traits under GH and field conditions also provides a broad overview on the genetic components of leaf phenotypic variation between barley row-type classes.

3.3 Materials and methods

3.3.1 Plant materials

Thirty-two diverse barley accessions were grown under three independent growing conditions during 2014 growing season at the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben, Germany (51° 49′ 23″ N, 11° 17′ 13″ E, altitude 112 m). GH was used as a controlled condition with planting into pots whereas the other two growing conditions were conducted under the field condition; (i) field planting into pots and (ii) field planting into soil. Weather information is provided in **Table 3-1** for field and GH growing conditions. In this study we used 32 diverse spring barley accession (14 two-rowed and 18 six-rowed; **Table 3-2**) from different geographical origins. Most of the two-rowed barleys were from Europe, while most of the six-rowed barleys were from Americas and East Asia. More information about the germplasm status and origins of accessions are presented in **Table 2-2**. Detailed information is available from the IPK genbank website http://gbis.ipk-gatersleben.de/GBIS_I/

Table 3-1: Monthly average temperature (°C), precipitation (mm), relative humidity (%), and global solar radiation in the field and greenhouse during the 2012 growing season at IPK.

Month	Field				Greenhouse‡	
	Temperature °C	Rainfall (mm)	Relative humidity (%)	Global solar radiation (W/m²)	Temperature °C	Relative humidity %
April	8.8	17.4	75.8	157	9.3	72.9
May	15.0	48.7	73.0	161	14.3	71.7
Jun	15.6	72.4	80.3	175	18.8	77.2
July	18.1	93.4	78.7	194	19.7	75.9
August	18.7	38.1	75.3	194	20.0	72.6

‡Greenhouse-grown plants were maintained for 10 days at 20°C for germination, for 28 days at 4°C for vernalization, for 7 days at 14°C for hardening, and under normal greenhouse growth conditions at 20 ±1°C until harvest. The planting date for all growth conditions was April 1st 2012.

Table 3-2: Spring barley accessions according to row-type, name, germplasm status, and origin.

No.	Name	Germplasm status	Origin	Name	Germplasm status	Origin
	Six-rowed			Two-rowed		
1	BCC1453	Cultivar	Finland	BCC1497	Landrace	Kyrgyzstan
2	HOR2835	Landrace	Iran	BCC1541	Cultivar	Yugoslavia
3	BCC1494	Landrace	Kazakhstan	BCC869	Cultivar	Mexico
4	BCC579	Cultivar	India	HOR8006	Landrace	Turkey
5	BCC219	Landrace	Tajikistan	Barke	Cultivar	Germany
6	BCC447	Cultivar	China	BCC1566	Landrace	Greece
7	BCC719	Cultivar	Korea	BCC1589	Landrace	Italy
8	Morex	Cultivar	USA	Triumph	Cultivar	Germany
9	BCC814	Breeder line	USA	BCC801	Cultivar	Canada
10	BCC818	Cultivar	USA	Proctor	Cultivar	UK
11	BCC718	Cultivar	Korea	BCC1370	Cultivar	France
12	BCC551	Cultivar	India	BCC1371	Cultivar	France
13	BCC577	Cultivar	India	BCC903	Landrace	Afghanistan
14	BCC888	Cultivar	Canada	Weeah	Cultivar	Australia
15	BCC942	Cultivar	USA			
16	BCC875	Cultivar	USA			
17	BCC921	Cultivar	Colombia			
18	BCC868	Breeder line	Mexico			

3.3.2 Growth environment and experimental procedure

Thirty seeds for each of the 32 spring barley accessions were sown on 1st of April 2012 for all growing conditions. Under GH growing conditions, seeds germinated under controlled conditions (long-day (LD), 16/8 h day/night and ~20/~16 °C day/night) for 10 days. All seedlings were vernalized for 28 days at ~4 °C to be consistent with plants grown under field conditions and to promote plants for flowering and producing seeds. Seedlings were transferred to a hardening period (7 days) for gradual acclimatization (12h/12h and ~14 °C

/~12 °C, day/night respectively). One plant per 0.5 liter pot (9 cm x 9 cm diameter and height) was grown under GH conditions (LD, 16/8 h and ~20 /~16 °C) with potting medium structure substrate (Substrat 2, Klasmann-Deilmann GmbH, 49744 Geeste, Germany) with 14:16:18 / Nitrogen (N): Phosphorous (P): Potassium (K) and pH 6.5). Manual irrigation was performed daily as required, and 1.5 g (17:11:10 / N: P: K) fertilizer was added to each pot. Supplemental light (~300μmolm^{-2} s^{-1} PAR = 159 W/m^2) extended natural light via low intensity incandescent lamps (Philips son-t agro 400 w). Pots were randomized three times per week to minimize border and temperature gradient effects on growth and development.

Two field (open field) growing conditions were used in this study: (i) pot-grown field plants and (ii) soil-grown field plants. In pot-grown field planting conditions, one plant per pot was grown in each 0.5 liter pot which had the same potting substrate and fertilizer as mentioned in GH growing condition. For soil-grown planting conditions, 30 plants per accession (10 plants per row; 50 cm long with 20 cm between rows (100 plants/m^2) were directly grown in silty loam soil (14:78:85:1:7 / P: K: Magnesium (Mg): Boron (B): Iron (Fe) and pH 7). We selected this planting density to be consistent with GH and pot-grown field conditions. Fertilizer was evenly distributed (15 grams of 17:11:10 / N: P: K) to each row. In field-grown plants (pots and soil), each accession was randomly replicated in three rows. Rows were manually irrigated when required and to be in consistent with GH conditions. Under all growing conditions, the plants were grown as single plant stand with a border to eliminate light and temperature-gradient effects on growth and development. Weeds were controlled manually in all growing conditions.

3.3.3 Data recording and experimental design

Only completely unfolded and fully developed leaf blades from the main culm were counted and harvested by hand to measure leaf fresh weight (LFW, g) immediately (Sartorius, Germany). We identified the main culm as the strongest and developmentally most advanced culm. Main culm leaf blade area, LA (mm^2) was measured immediately by Li-COR area

meter. Leaf blades were oven dried at 40°C for 10 days (Heraeus, Germany) to measure main culm LDW (mg). LMA per main culm (mg/mm^2) was calculated at stage x as:

Equation 3-1: Leaf mass per area (LMA)

$$\boldsymbol{LMA} = \frac{\mathrm{LDWx}}{\mathrm{LAx}},$$

Growth rate for LFW per thermal time unit (LFWGR) were calculated at stage x as follows:

Equation 3-2: Leaf fresh weight growth rate (LFWGR)

$$\boldsymbol{LFWGR} = \frac{\mathrm{LFWx}}{°\mathrm{C} * \mathrm{Dx}}$$

where LFWGR is leaf blade fresh weight growth rate (g/°C*D), LFW is leaf blade fresh weight (g) and °C*D is the required thermal time at stage x. The same method was used to calculate LDW growth rate (LDWGR) and leaf blade area growth rate (LAGR, mm^2/°C*D). Leaf blade dry matter content (LDMC, mg/g) is the oven-dry weight (mg) of the leaf blade divided by its fresh weight (g) (Perez-Harguindeguy et al., 2013). Thermal time or growing degree-days (°C*D, GDD) was calculated as the mean of the daily maximum and minimum air temperature using 0°C as a base temperature.

Data was recorded at five major developmental stages: awn primordium (AP, maximum yield potential; Alqudah and Schnurbusch (2014), tipping (TIP, Z49, top awns apparent), heading (HD, Z55, half spike emerged), anther extrusion (AE, Z65, anthers apparent), and harvest (Zadoks et al., 1974); Details about these stages are reported by Alqudah and Schnurbusch (2014). Data was recorded when at least 50% of the main culm spikes in each accession reached each stage. Three biological replicates per accession were randomly selected from the center of the row to avoid border effect for data collection at the first four developmental stages, and six plants were used to collect data at harvest. Manual plant dissections were required to determine AP via microscopy (Stereo Microscope Stemi 2000-C with KL 1500 LCD; Axio Vision, 4.8.2, ZEISS Germany).

Six biological replicates per accession were randomly hand harvested to collect biological yield (BY; determined by weighing the total air-dried aboveground parts). Single-plant grain yield and yield components were measured by counting the number of grains per main spike, tillers and spikes number per plant. Main spike dry weight MSDW and total grain weight per main spike after hand threshing were measured. Harvest index (HI) per plant was measured as the ratio of grain weight per plant to BY per plant multiplied by one hundred.

3.3.4 Data analyses

Growing conditions were arranged in a completely randomized design with three replicates and each growing condition includes border plants which were not sampled. Analysis of variance (ANOVA) was conducted to compare spike row-type classes between growing conditions, and to compare row-type across growing conditions using SAS for Windows ver. 9.3 (SAS Institute Inc., Cary, NC, USA) at $P \leq 0.05$). Means were separated according to the Fisher's Least Significant Difference (LSD) at 0.05 levels of probability. Pearson's correlation coefficient was calculated for phenotyped traits using PROC CORR (SAS, 2013) at $P \leq 0.05$. Variance components were estimated by considering the genotype x growing condition. Broad-sense heritability (H^2) was estimated overall growing conditions according to Snedecor and Cochran (1980) using PROC VARCOMP (SAS, 2013)

Equation 3-3: Broad-sense heritability (H^2)

$$H^2 = \frac{\sigma^2 g}{(\sigma^2 g + \sigma^2 g \times gw/e + \sigma^2 e/re)}$$

where σ^2 g is genotypes variance; σ^2 g.gw is the variance of the interaction genotype × growing condition, r is replicates, and e the error.

Principal Component Analysis (PCA) based on phenotypic correlations of accession means values for each selected trait under each growth condition was calculated using GENSTAT for Windows version 16 (VSN International, Hemel Hempstead, UK). A two-dimensional PCA was calculated as multivariate analysis to interpret and summaries phenotypic

clusters/variations among growth conditions and/or row-type classes of barley by accessions by LA, LDW, and LMA at different growth stages (GenStat, 2013). PCA is an indicator ordination tool for obtaining multivariate data that can be explored visually in a two-dimensional PCA correlation. Coefficients of variation (CV) for individual trait were calculated as a percentage of standard deviation to the trait mean by GenStat (2013). CV was calculated to compare individual leaf trait variations within growth conditions between accessions.

3.4 Results

3.4.1 Correlation analysis between thermal time and leaf trait

Correlations between leaf traits and thermal time at developmental stages were generally higher under field conditions (pots and soil; **Table 3-3**). For leaf number per main culm, the correlation was stronger in the GH at the AP stage (r = 0.34 and 0.69 for two- and six-rowed, respectively) thereafter the field-grown plants (pots and field) showed the strongest correlation such as at HD stage $r \geq 0.45$ for both row-type classes from pot- and soil-grown field plants while it was negative for GH-grown plant. The strong correlation for GH-grown plants at AP could be attributed to vernalization period for GH seedlings (4 weeks) which leads to produce more leaf primordia thereby developed more leaves and delayed AP stage. There was no clear trend between other leaf traits (i.e. LFW, LDW, LA and LMA) and GDD under different growing conditions. However, in most cases, plants grown under field conditions (pots and soil) showed higher correlations than GH-grown plants. For example, the correlation for LA at TIP stage in six-rowed types was around r = 0.79 and 0.70 for pots and soil, respectively; whereas the correlation was negative in GH-grown plants (**Table 3-3**) for both row-type classes at the same stage. In some cases, GH plants had stronger correlations for LDW than field-grown plants. In two-rowed barleys at AE stage, the correlation with LDW for GH-grown plants was r = 0.65; whereas it was r = 0.30 and 0.39 for pot- and soil-grown field plants, respectively. Taken together, the impact of thermal time on leaf traits (i.e.

number of leaf, LFW, LDW, LA and LMA) was not evident enough and this may be attributed to phenotypic variation in thermal time between accessions to reach the stages.

Table 3-3: Correlations coefficients between thermal time (GDD) at different developmental stages with leaf traits.

Yield components	Growing condition	Thermal time/Growing degree-day (GDD)							
		Awn Primordium		Tipping		Heading		Anther Extrusion	
		Two-rowed	Six-rowed	Two-rowed	Six-rowed	Two-rowed	Six-rowed	Two-rowed	Six-rowed
Leaf number per main culm	**GH**	0.34	0.69	-0.21	-0.27	-0.27	-0.20	0.06	-0.17
	Pots	0.10	-0.23	0.45	0.46	0.56	0.45	-0.19	0.56
	Soil	0.07	0.20	0.65	0.48	0.63	0.48	0.35	0.42
Leaf fresh weight per main culm (g, LFW)	**GH**	0.67	0.68	0.70	0.64	0.36	0.32	0.36	0.18
	Pots	0.70	0.59	0.69	0.75	0.52	0.77	0.22	0.71
	Soil	0.40	0.47	0.49	0.74	0.43	0.24	0.32	0.23
Leaf dry weight per main culm (mg, LDW)	**GH**	0.52	0.65	0.68	0.55	0.68	0.48	0.65	0.46
	Pots	0.76	0.81	0.75	0.62	0.55	0.77	0.30	0.76
	Soil	0.52	0.49	0.46	0.70	0.71	0.48	0.39	0.29
Leaf area per main culm (mm, LA)	**GH**	0.40	0.37	0.23	-0.22	0.30	0.20	0.42	-0.09
	Pots	0.59	0.60	0.67	0.79	0.40	0.84	0.43	0.75
	Soil	0.40	0.27	0.52	0.70	0.53	0.48	0.72	0.42
Leaf mass area per main culm ($mg.mm^{-2}$, LMA)	**GH**	0.30	0.25	0.28	-0.32	-0.32	-0.24	-0.24	-0.42
	Pots	0.49	0.72	0.28	0.11	0.31	-0.01	0.82	0.40
	Soil	0.46	0.50	0.05	-0.22	-0.11	0.20	-0.47	0.02

Two-rowed (n=14 accession x 3 reps) and six-rowed (n=18 accession x 3 reps) barley were grown under greenhouse and field (pots and soil) growing conditions.

3.4.2 Analysis of phenotypic leaf variation

PCA analysis identified phenotypic groups within two- and six-rowed barley collections and/or growing conditions by using LA, LDW and LMA data at the HD stage. A two-dimensional scatter plot is presented to show the row-type clusters based on leaf traits (**Figure 3-1A**), the clusters of row-type based on growing conditions (**Figure 3-1B**) and the clusters of row-type within growing conditions (**Figure 3-1C**). The first PC-1 (LA, LDW or LMA) accounted for 48.97% of phenotypic variation and clearly separated two- and six-rowed accessions with few exceptions (**Figure 3-1A**). LA and LDW had the major contributions in this separation and the mixed dots (accessions) between row-types are from six-rowed barleys in the GH. The second PC-2 (growing conditions) accounted for 34.14% of the observed phenotypic variation (**Figure 3-1A**). Accessions from different growing conditions were mixed within each row-type and cannot be clustered based on leaf traits only, indicating intra-

class variation, while growing conditions explained leaf traits variation within each row-type (**Figure 3-1B and -C**). Herein, we cannot rule out the effect of germplasm diversity (geographical origins) and/or germplasm status (cultivar, landrace and line) on phenotypic diversity.

To compare individual leaf trait variations within growing conditions between accessions, we examined coefficients of variation (CV, **Supplementary Table 3-1**). For all measured traits, CV values under GH conditions were always higher than those of field-grown plants (pots and soil). Generally, CV values increased for all measured traits after AP under all growing conditions. LA under GH conditions resulted in intermediate to very high CV values over developmental stages, CV from 27% to 80% and 28% to 41% in two- and six-rowed barley, respectively. Low to intermediate CV values over developmental stages in field-grown plants (pot and soil) ranged from 14% to 35% and 16% to 31% in two- and six-rowed barley, respectively. Other leaf traits followed the trend, clearly suggesting that GH conditions (i.e. controlled temperature, light etc.) maximized phenotypic leaf trait expression between accessions compared to field conditions. The obtained and high CV values for our GH conditions indicate that they are appropriate for studying phenotypic/genetic variation of leaf traits.

The analysis of variance components among row-type classes and growing conditions at developmental stages highlighted that the genetic variance is the largest components (**Figure 3-2A**). Importantly, we found the broad-sense heritability values across growing conditions ranged from high to very high (H^2 = 0.66-0.90) at all developmental stages (**Figure 3-2B**). These values, most notably, suggest that leaf traits in both row-type classes are mainly heritable under all growing conditions at all developmental stages.

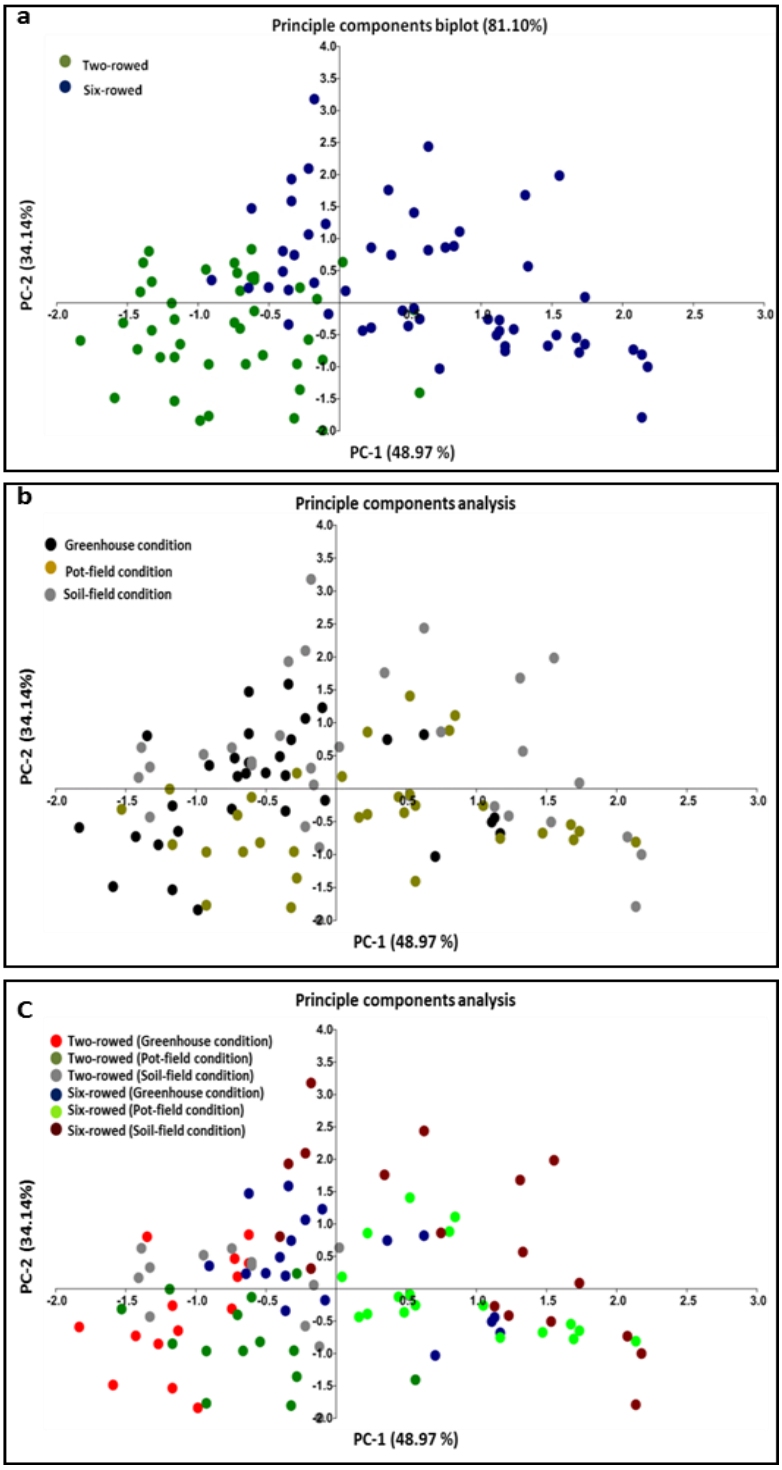

Figure 3-1: Principal component analysis (PCA) based on phenotypic data by leaf area (LA), leaf dry weight (LDW) and leaf mass area (LMA) per main culm at heading stage.

PCA for row-types, green and blue color denotes two- and six-rowed barley, respectively (a), and for growth conditions, black, greenish-brown and gray color denotes for greenhouse (GH), pot- and soil-field, respectively (b), and row-types within growth conditions (c); the light-red and dark-blue circles indicate two- and six-rowed plants, respectively, under GH conditions; the green and light green circles indicate two- and six-rowed plants, respectively, under pot-field conditions; and the gray and dark-red circles indicate two- and six-rowed plants, respectively, under soil-field conditions. The number of dots (n = 54, 18 x 3 growth conditions) for six-rowed and (n = 42, 14 x 3 growth conditions) for two-rowed barley.

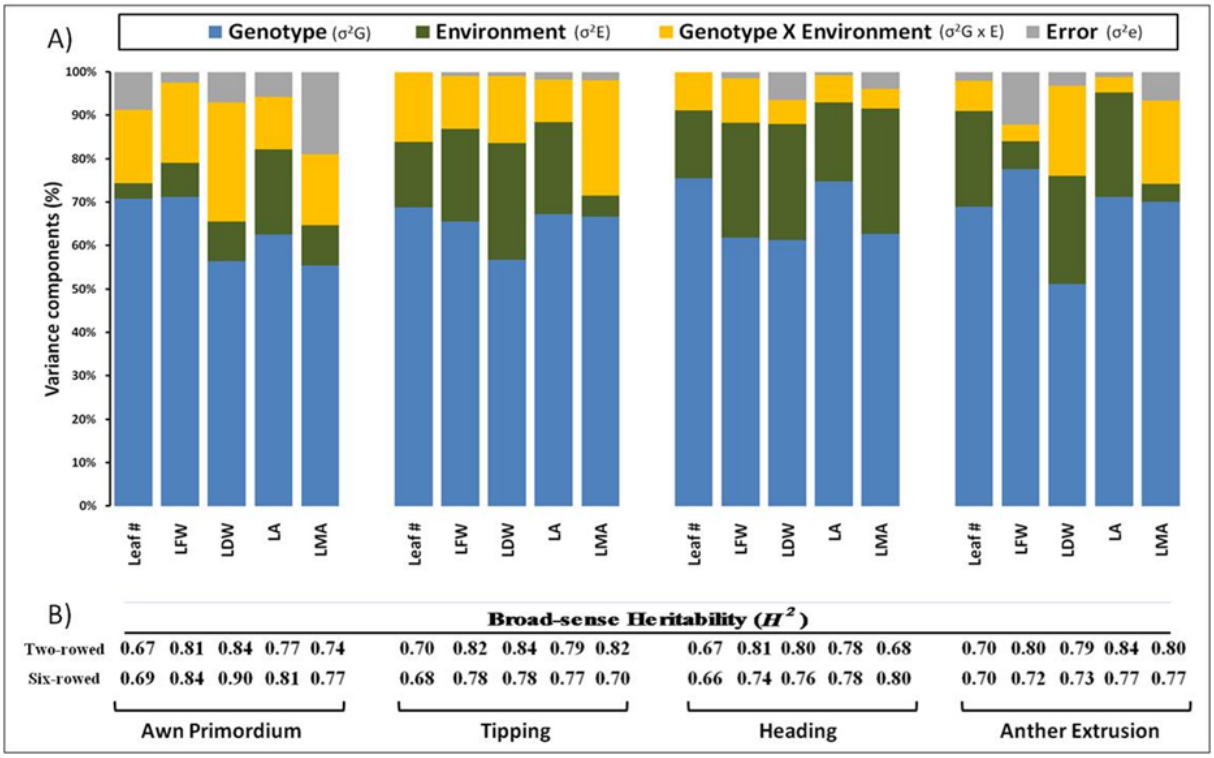

Figure 3-2: Variance components of leaf traits at different developmental stages in two- and six-rowed barley together overall growing conditions (A). Broad-sense heritability of leaf traits at different developmental stages overall growing conditions in two- and six-rowed barley separately (B).

3.4.3 Leaf traits in both row-type classes under different growth conditions at four developmental stages

Comparisons of leaf blade number per main culm at all developmental stages in two- and six-rowed barley yielded no significant difference was observed across all growth conditions (**Figure 3A**). However, significant differences ($P \leq 0.05$) between growth conditions were found in leaf blade number per main culm for both row-type classes at all developmental stages (**Supplementary Figure 1A**). GH-grown plants had significantly more leaves per main culm in both barley classes at all developmental stages compared to field-grown plants (pots and soil) due to a longer time required to reach AP (vegetative period) (**Supplementary Figure 1A**). For LA per main culm, we consistently found significantly higher LA in six-rowed barley at all stages (**Figure 3B**). With the exception of AP, field-grown plants

exhibited the largest LA, followed by pot-grown field plants and GH-grown plants (independent of row-type) (**Supplementary Figure 1B**). LDW followed the LA trend for all development stages, growth conditions, and row-type classes (**Figure 3C and Supplementary Figure 1C**). The LMA was significantly different between two- and six-rowed barley ($P \leq 0.05$) at AP, TIP, and HD stages independent of growth conditions, but not at AE (**Figure 3D**). Field-grown plants consistently showed significantly higher LA and LDW than GH-grown plants. However, GH conditions produced the broadest phenotypic variation for leaf traits between accessions, which is important for revealing the genetic variation of these traits. Interestingly, we found that six-rowed barley plants had greater LA and LDW, but lower LMA compared to two-rowed barley plants under all growth conditions. The found natural variation for LA and related traits between row-type classes suggests that the effect of major genes controlling row-type in barley may have pleiotropic effects on these traits. However, additional genetic evidence is required to elucidate this effect.

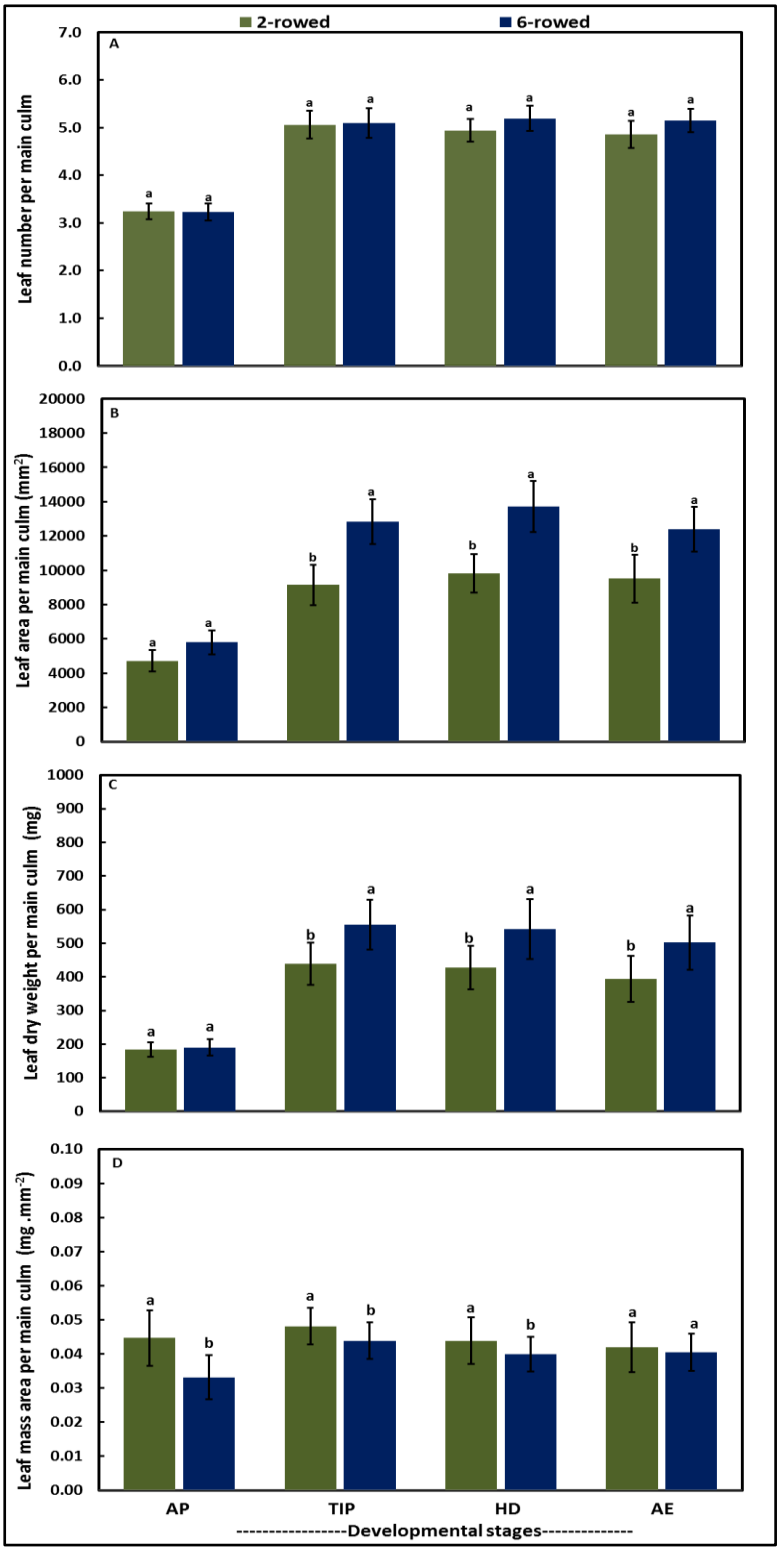

Figure 3-3: Leaf traits per main culm at different developmental stages for two- and six-rowed barley averaged across all growth conditions.

A) Leaf number; B) leaf area; C) leaf dry weight; and D) leaf mass area. The same letters at each developmental stage are not significantly different at $P \leq 0.05$ according to the Least Significant Difference. Bars indicate standard deviation [n = 42 (14 x 3) and 54 (18 x 3) for two- and six-rowed barley, respectively]. AP: awn primordium, Alqudah and Schnurbusch (2014); TIP: tipping, Z49; HD: heading, Z55; AE: anther extrusion, Z65, Zadoks et al. (1974)

3.4.4 Leaf growth rate in both row-type classes under different growing conditions at four developmental stages

For leaf growth rates per main culm, significant differences among row-type classes and growth conditions were found ($P \leq 0.05$; **Figure 4 and Supplementary Figure 2**). Regardless of growth conditions, six-rowed barley always exhibited a higher leaf fresh weight growth rate (LFWGR) than two-rowed plants at all developmental stages and sub-phases (**Figure 4A**). A significant difference existed between growth conditions; field-grown plants (pots and soil) showed significantly higher LFWGR than GH-grown plants at all developmental stages and sub-phases (**Supplementary Figure 2A**). Six-rowed barley had significantly higher leaf dry weight growth rate (LDWGR) and LA growth rate (LAGR) at most developmental stages and sub-phases compared to two-rowed barley ($P \leq 0.05$; **Figure 4B and C**). For leaf dry matter content (LDMC), two-rowed plants generally exhibited significantly higher LDMC ($P \leq 0.05$) than six-rowed barley plants, except at the TIP stage and between the AP-to-TIP phase (**Figure 4D**). Field-grown plants (pots and soil) showed significantly higher LDMC than GH-grown plants at all developmental stages and sub-phases, except at the AE stage and TIP-to-HD phase (**Supplementary Figure 2D**). Growth rates for all measured traits were significantly higher in field-grown plants than GH-grown plants, indicating that the field conditions promoted these traits. Notably, GH-conditions resulted in the highest phenotypic variation for leaf growth rates between accessions. We consistently found that six-rowed barley had significantly higher leaf growth rates than two-rowed barley. Moreover, independent of row-type and growth conditions, growth rates were greatest between the AP to TIP phase, illustrating that this developmental phase has the most rapid leaf biomass increase during the barley life cycle.

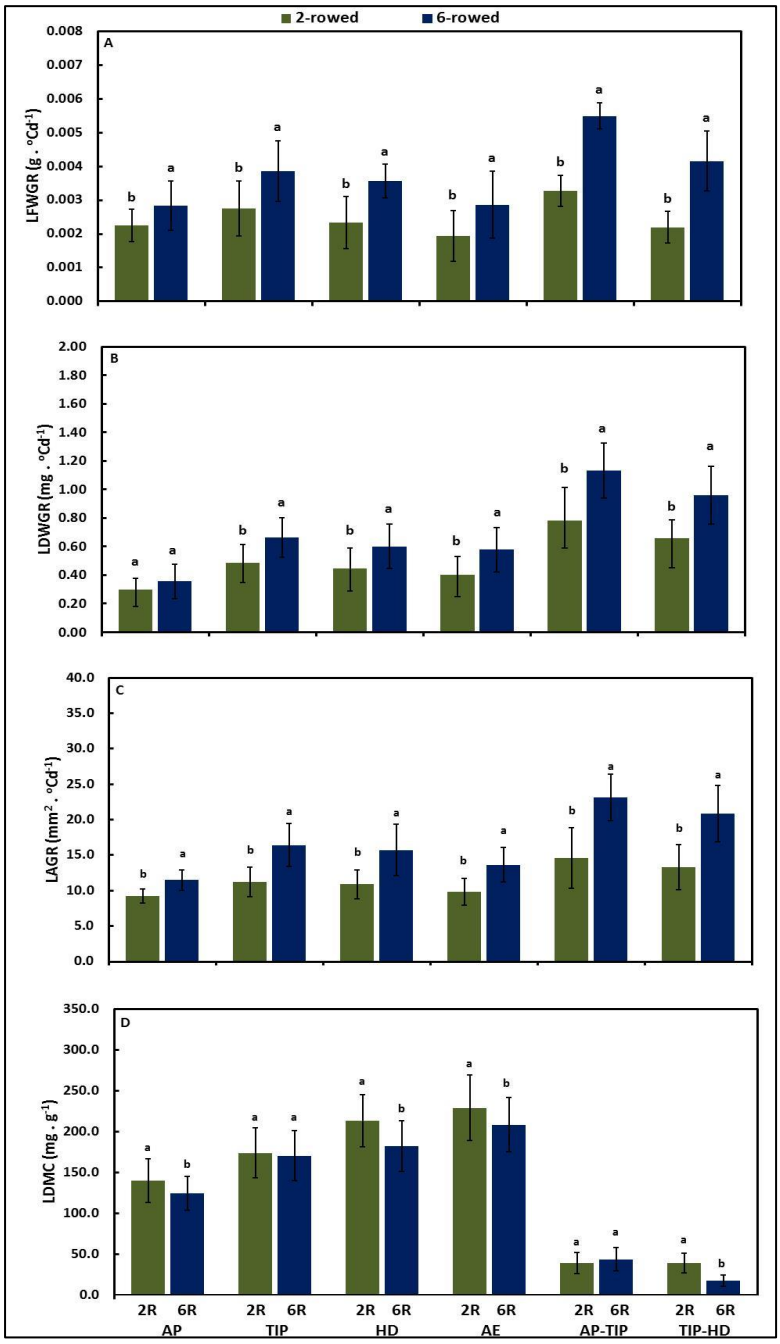

Figure 3-4: Leaf growth rate per main culm at different stages and phases for two- and six-rowed barley averaged across all growth conditions.

A) Leaf fresh weight growth rate (LFWGR); B) leaf dry weight growth rate (LDWGR); C) leaf area growth rate (LAGR); and D) leaf dry matter content (LDMC). The same letters at each developmental stage are not significantly different at $P \leq 0.05$ according to the Least Significant Difference. Bars indicate standard deviation [n = 42 (14 x 3) and 54 (18 x 3) for two- and six-rowed barley, respectively]. AP: awn primordium, Alqudah and Schnurbusch (2014); TIP: tipping, Z49; HD: heading, Z55; AE: anther extrusion, Z65, Zadoks et al. (1974). 2R is two-rowed and 6R is six-rowed.

3.4.5 Correlation analysis between leaf area (LA), single plant yield, and yield components

Correlation coefficients at developmental stages between LA (i.e. LA of the main culm) and main culm spike grain yield, main spike dry weight (MSDW), tillers and spikes per plant, BY, and HI were generally higher under field conditions (pots and soil; **Table 3-4**). Correlations at HD and AE stages were higher than for AP and TIP stages (**Table 3-4**). In both row-type classes under all growing conditions, we report positive correlations between LA per main culm with main culm spike grain yield (grain number and weight) and MSDW at all developmental stages (**Table 3-4**). The strongest correlations were found in field-grown six-rowed barleys at the AE stage. For HI, we always found a negative correlation with LA in two-rowed barley in all growing conditions (**Table 3-4**). For tiller number, spike number per plant, and BY, correlation analysis showed two-rowed barley at AE stage under GH conditions exhibited the strongest correlation between LA with tiller and spike number per plant (r = 0.61 and 0.51, respectively; **Table 3-4**), while LA from two-rowed barley in field-grown plants (pot and soil) highly correlated with BY at the AE stage (r = 0.65). Unlike in two-rowed barley, six-rowed barley showed consistent positive association between LA of the main culm and HI, whereas LA in six-rowed barley correlated negatively with BY, tiller and spike number per plant under all growing conditions. The correlation results indicate that two-rowed types produced more BY, tillers and spikes per plant; whereas six-rowed barleys produced more grains (per spike). In general, larger LA in six-rowed barley correlated with main spike grain yield, MSDW, and HI, while smaller LA in two-rowed barley influenced tiller and spike number per plant and BY. LA for field-grown plants (pots and soil) correlated better with yield and yield components compared with those grown in GH conditions.

Table 3-4: Correlations coefficients between leaf blade area per main culm (mm2) at different developmental stages with single-plant yield and yield components.

Yield components	Growing condition	Leaf blade area per main culm (mm^2)							
		Awn Primordium		Tipping		Heading		Anther Extrusion	
		Two-rowed	Six-rowed	Two-rowed	Six-rowed	Two-rowed	Six-rowed	Two-rowed	Six-rowed
Grain number per main culm spike at harvest	GH	0.13	0.20	0.14	0.28	0.37*	0.52*	0.43*	0.62*
	Pots	0.57*	0.55*	0.45*	0.56*	0.53*	0.64*	0.60*	0.74*
	Soil	0.21	0.38*	0.51*	0.61*	0.53*	0.62*	0.58*	0.65*
Grain weight per main culm spike at harvest (g)	GH	0.12	0.13	0.26	0.30	0.23	0.40*	0.33	0.46*
	Pots	0.53*	0.40*	0.40*	0.49*	0.35*	0.50*	0.50*	0.55*
	Soil	0.28	0.34	0.42*	0.62*	0.47*	0.53*	0.55*	0.68*
Main culm spike dry weight at heading (g)	GH	0.16	0.10	0.35*	0.34	0.31	0.24	0.33	0.36*
	Pots	0.10	0.36*	0.37*	0.79*	0.43*	0.57*	0.65*	0.83*
	Soil	0.31	0.10	0.56*	0.69*	0.43*	0.54*	0.74*	0.74*
Tillers per plant	GH	0.31	-0.07	0.01	-0.17	0.47*	-0.33	0.61*	-0.36*
	Pots	0.23	-0.26	0.20	-0.10	0.15	0.00	0.45*	-0.09
	Soil	0.03	-0.17	0.28	-0.24	0.39*	-0.38*	0.45*	-0.31
Spikes per plant	GH	0.25	-0.06	0.30	-0.54*	0.43*	-0.34	0.51*	-0.27
	Pots	0.22	-0.30	0.20	-0.23	0.38*	-0.22	0.47*	0.00
	Soil	0.16	-0.22	0.29	-0.22	0.33	-0.40*	0.40*	-0.31
Biological yield (g)	GH	0.00	-0.38*	0.27	-0.32	0.29	-0.21	0.39*	-0.11
	Pots	0.46*	-0.08	0.47*	-0.07	0.36*	-0.13	0.47*	-0.31
	Soil	0.20	-0.29	0.42*	-0.29	0.52*	-0.27	0.65*	-0.52*
Harvest Index (%)	GH	-0.26	0.38*	-0.14	0.37*	-0.29	0.12	-0.17	0.20
	Pots	-0.35*	0.29	-0.22	0.16	-0.39*	0.21	-0.17	0.28
	Soil	-0.22	-0.06	-0.12	0.22	-0.31	0.54*	-0.30	0.41*

Two-rowed (n=14 accession x 3 reps) and six-rowed (n=18 accession x 3 reps) barley were grown under greenhouse and field (pots and soil) growing conditions.
*Correlation values exceeding +/- 0.35

3.5 Discussion:

This study focused on the importance of variation between two- and six-rowed barley in LA and its relationship on single plant grain yield and yield components. The study also demonstrates that six-rowed barley had larger and heavier leaf blades compared to two-rowed barley. Moreover, we identified AP to TIP as being the most critical sub-phase for leaf growth and development. We also investigated the heritability of leaf traits (i.e. genetic basis) in barley row-type classes under various growth conditions and throughout developmental stages.

3.5.1 The importance of leaf area in improving single-plant yield

In this study, we found significant variation in LA and related traits during growth and development stages between barley row-types. Using LA of single leaf (flag or penultimate) for improving the yield of barley has been previously suggested (Berdahl et al., 1972;Rasmusson, 1987); however, no study has explored the specific contribution of main culm LA during pre-anthesis stages to yield based upon row-type classes. Our analysis showed that larger LA (six-rowed) correlate to higher main culm spike grain yield, spike dry weight and harvest index; while smaller LA (two-rowed) correlated to more spikes, tillers, and biological yield. We attempt to clarify the importance of large and small LA in improving yield which may help breeders in future breeding programs.

3.5.1.1 Large leaf area

In this study we showed that larger LA per main culm in six-rowed barley positively associates with a higher grain yield per main spike (**Table 3-4**), thereby increasing HI. LA is known to improve grain yield in breeding programs (Richards, 1983;Rasmusson, 1987;Peng et al., 2008). Broader leaves were important components in New Plant Type (NPT) breeding program to improve rice grain yields (Khush, 1995;Fujita et al., 2013). Rasmusson (1987) suggested that larger LA produces larger spikes and kernels, and in two bi-parental barley populations (small vs. large LA), higher grain weights and higher yield was achieved by higher photosynthetic rates per unit LA (Berdahl et al., 1972). A similar explanation for higher single plant grain yield in six-rowed barley is likely to be the cause in this study. Here, a high correlation between larger LA per main culm in six-rowed barley with MSDW at the AE (flowering) stage was apparent. Yoshida (1972) found a close relationship between LA at flowering time and grain number and grain yield at harvest. We similarly propose that larger LA leads to more dry matter accumulation before the AE stage from higher photosynthetic rates, which in turn increases spike dry matter. This could be one reason for producing heavier

single spike grain weight in six-rowed barley and higher HI. As presented in Figure 3, larger LA at the HD stage resulted in the highest amount of LDW in six-rowed barley, which is a result that may be attributed to the previous reasoning (high photosynthetic rate in large LA) and confirmed in past literature (Aase, 1978). Our observation highlights the importance of large LA in six-rowed barley, but differs from the ideotype concept of Donald (1968). Therefore, based on our observed correlations, improving single-plant grain yield through larger LA in six-rowed barley might be important for future barley breeding programs in the context of increasing spike grain yield, spike dry weight, and harvest index.

3.5.1.2 Small leaf area

We found that smaller LA in two-rowed barley was associated with more tillers and spikes per plant, which are findings that are in agreement with those of Berdahl et al. (1972). Producing smaller and narrower LA in dense stands can theoretically improve crops grain yield in cereals (Donald, 1968). As shown in **Figures 3-3 and 3-4**, smaller LA in two-rowed barley results in higher LMA and LDMC, suggesting that the leaves are thicker than those of six-rowed barley. LMA is a trait that responds to stress, as it reflects the amount of dry matter a plant accumulates through reduced LA (Witkowski and Lamont, 1991). Smaller LA, which results from a longer vegetative duration, could be one important trait for improving drought and cold tolerance depending on growth habits (Van Oosterom and Acevedo, 1993). Curtis et al. (2012) reported that leaves with higher LMA were thicker, narrower, and protected well against rapid fluctuations in temperature, while leaves with low LMA senesced earlier. Thus, two-rowed barley may adapt better to stress conditions than six-rowed barley based on leaf performance traits, such as LMA. Although LMA strongly varied under different conditions, such as water stress (Poorter et al., 2009), it may still be a useful trait to select for in a breeding program in order to produces lines with improved stress tolerance. Moreover, in a canopy situation, narrower leaves utilize sunlight more efficiently due to decreased shading between tillers and neighboring plants, thereby increasing light perception. Taken together,

the opposing relationship between LA and yield-related traits, such as BY and HI, in two- and six-rowed barley, respectively, is undoubtedly an interesting plant architectural feature and deserves further attention.

3.5.2 Maximized leaf growth rate in the two row-type classes

Our study provides a first set of leaf growth rate parameters at four developmental stages and in three growing conditions for two major barley row-type classes. Previous research focused on the importance of the leaf appearance/emergence rate (phyllochron) in barley and wheat instead of leaf growth rate parameters (Kirby et al., 1985;Kirby, 1995). An initial lag phase up to AP stage existed for all parameters studied (**Figure 3-5**). Leaf weight and LA significantly increased with the onset of the late reproductive phase and reached a plateau at HD stage (**Figure 3-5**). Increased leaf weight and area resulted from a higher number of developed leaves during more advanced developmental stages. As presented in **Table 3-1**, LA is critical for biomass accumulation processes (LDW) and is indicative of increased photosynthetic rates (Berdahl et al., 1972). This, in turn, may explain increased LFWGR and LDMGR. Moreover, we identified the period from AP to TIP as the most important sub-phase for leaf growth rate in barley (**Figure 3-3**). This period is characterized by 50% LA and biomass accumulation, but decreases with advancing age (after HD) due to leaf senescence. This concords with Alqudah and Schnurbusch (2014), who found that the AP to TIP sub-phase is the most critical period for spikelet survival with approximately ~70% of total spikelet/floret abortion. Importantly, the AP to TIP period temporally coincides with stem elongation and maximal leaf growth rates, further corroborating that within-plant competition (stem/leave vs. spike) may be a major trigger for observed spkikelet/floret decline (Alqudah and Schnurbusch 2013). These findings illuminate opportunities to better understand internal networks of competing barley organs and might open up novel research in terms of source-sink relationships (Sadras and Denison, 2009).

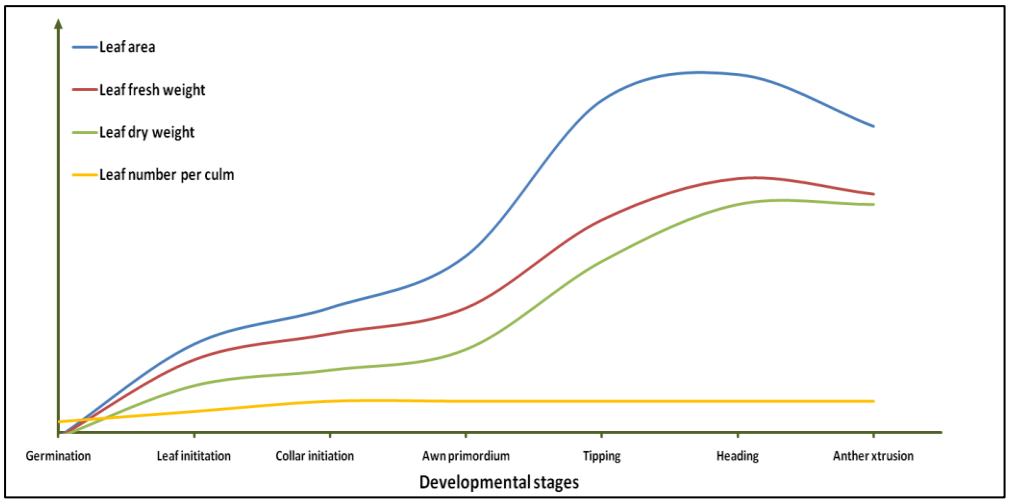

Figure 3-5: General trend of leaf traits in barley at developmental stages.
Data was collected from nine biological replicates of 32 barley accessions (n=32 accession x 3 reps x 3 growth conditions).

3.5.3 Genetic background and variance analysis

One major objective was to estimate variance components and broad-sense heritability (H^2) values for different leaf traits under variable growth conditions. Interestingly, all heritability values across growth conditions obtained for leaf traits were above 0.65 in small LA (two-rowed) and large LA (six-rowed), suggesting that these traits are predominantly genetically controlled (**Figure 4-2**). Rebetzke et al. (2004) reported that heritability values for estimated LA per plant in wheat (30 genotypes) and barley (3 genotypes) at an early stage (4 leaves stage) of development under different conditions (GH and field) was approximately 0.90. In a study conducted by Rasmusson (1987), LA heritability only ranged from 0.24-0.37 in three barley populations. One reason for the discrepancy between our findings and those of Rasmusson (1987) may be that we evaluated leaf traits in a diverse collection (different regions and genetic background) under two environmental conditions (GH and field) and at different developmental stages. Evidently the genetic component is the largest component in our diverse collection, and since environmental effects are small, genetic regulation plays a major role in the observed variation. Moreover, it is possible that single plant leaf measurements are more reliable than leaf measurements conducted in dense stands (i.e., canopy or plot situation) (Fasoula and Fasoula, 1996). For example, LA components in alfalfa

plants significantly differ as a direct result from stand density and light competition (Baldissera et al., 2014). Similar factors may be in play for barley plants grown under light competition in a field-plot situation, thus creating variable environmental factors (light competition and shading) that cause shoot and leaf growth variation. Therefore, estimating the genetic components of leaf traits is more accessible under controlled conditions using single plants (Fasoula and Fasoula, 1996).

Our results show that measured leaf traits are mainly genetically determined and less affected by environment. For instance, LMA was affected by many factors (within species or/and environments) for which a large part of the variation was still unaccounted (Poorter et al., 2009). In the literature, the heritability value for LMA is low and is considered as a complex trait, and understanding the genetic factors underlying this trait has been unsuccessful to date. In the present study, we found that LMA was predominantly genetically controlled ($H^2 \geq$ 0.68), which was likely due to our analysis of this trait under different environments in a diverse collection and at different developmental stages (i.e., experimental design). We also examined whether growth conditions and/or row-type classes affected leaf traits. Leaf traits were generally higher for field-grown plants than for GH-grown plants, which might be due to greater space for root growth and nutrient availability. Results from the present study showed that leaf traits are influenced by growth conditions to a greater extent than phase duration or spike-related traits (Alqudah and Schnurbusch, 2014). In the GH, we tried to minimize environmental effects by randomizing pots several times a week; yet, we still postulate that field conditions at several locations/seasons are important for further leaf trait validation work. Certainly, leaf traits are similarly affected by pot size and/or substrate content in the GH, but CV values under GH conditions were always higher than those of field-grown plants clearly suggesting that single-plants in the GH maximized phenotypic leaf trait expression between accessions. and therefore are more appropriate for studying phenotypic/genetic variation of leaf traits (Fasoula and Fasoula, 1996). Based upon the PCA analysis, leaf traits

(PC-1, 48.97%) were major distinguishing features of row-type classes. However, we cannot rule out the effect of germplasm diversity (geographical origins) on phenotypic variation.

In this study, we found significant differences in leaf traits between two- and six-rowed barley, especially LA. Phenotypic differences in LA between row-type classes showing a high heritability value provide an unexplored opportunity to better understand the genetic of LA in barley. Whether the phenotypic differences between row-type classes in LA and other traits may be related to the action of the predominant row-type gene *SIX-ROWED SPIKE 1* (*Vrs1*) is not yet clear. Future research is required to elucidate these relationships in more detail.

3.6 Conclusion

We found substantial differences for leaf performance traits between two-and six-rowed barley in our study. This impacts future barley breeding programs related to improving both single-plant grain yield and environmental stress adaptation. Further work is needed to understand genetic factors related to optimum LA, which increases grain yield and environmental adaptation.

3.7 Acknowledgements

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Author Contributions

AMA carried out all experimental work and analyzed the data. TS conceived the project, designed experiments, and supervised the experimental work. Both authors contributed to writing the article.

Conflicts of Interest

The authors declare no conflict of interest.

4.0 CHAPTER FOUR: Genetic Dissection of Photoperiod Response Based on GWAS of Pre-Anthesis Phase Duration in Spring Barley

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4.1 Abstract

Heading time is a complex trait, and natural variation in photoperiod responses is a major factor controlling time to heading, adaptation and grain yield. In barley, previous heading time studies have been mainly conducted under field conditions to measure total days to heading. We followed a novel approach and studied the natural variation of time to heading in a world-wide spring barley collection (218 accessions), comprising of 95 photoperiod-sensitive (*Ppd-H1*) and 123 accessions with reduced photoperiod sensitivity (*ppd-H1*) to long-day (LD) through dissecting pre-anthesis development into four major stages and sub-phases. The study was conducted under greenhouse (GH) conditions (LD; 16/8 h; ~20/ ~16 ^{o}C day/night). Genotyping was performed using a genome-wide high density 9K single nucleotide polymorphisms (SNPs) chip which assayed 7842 SNPs. We used the barley physical map to identify candidate genes underlying genome-wide association scans (GWAS). GWAS for pre-anthesis stages/sub-phases in each photoperiod group provided great power for partitioning genetic effects on floral initiation and heading time. In addition to major genes

known to regulate heading time under field conditions, several novel QTL with medium to high effects, including new QTL having major effects on developmental stages/sub-phases were found to be associated in this study. For example, highly associated SNPs tagged the physical regions around *HvCO1* (barley *CONSTANS1*) and *BFL* (*BARLEY FLORICAULA*/*LEAFY*) genes. Based upon our GWAS analysis, we propose a new genetic network model for each photoperiod group, which includes several newly identified genes, such as several *HvCO-like* genes, belonging to different heading time pathways in barley.

4.2 Introduction

Heading time is an important trait for barley (*Hordeum vulgare* L.) adapting to particular environmental cues and hence for maximizing grain yield. Its complex genetic architecture was considered as one of the major breeding goals during last century. The capacity to regulate heading time provides crop plants with the opportunity to successfully complete their life cycle under a wide range of environments, which exceed the distribution range of their wild relatives (Andres and Coupland, 2012). The optimal time to flower is crucial for high crop grain yields (Purugganan and Fuller, 2009). For decades, studies on heading/flowering time solely focused on the total number of days until heading/flowering and its effect on grain yield in response to environmental cues. However, the time prior to anthesis (i.e. pre-anthesis developmental phases) in barley consists of vegetative, early and late reproductive phases (Sreenivasulu and Schnurbusch, 2012;Alqudah and Schnurbusch, 2014). Grain yield and yield potential are significantly influenced by the reproductive, pre-anthesis phase durations (Borràs et al., 2009), which were shown to be genetically controlled (Alqudah and Schnurbusch, 2014). Despite the importance of this earlier phase of development, most of what we know about the genetic control of pre-anthesis phases is purely based on traditional quantitative trait locus (QTL) analysis, wherein often vegetative and reproductive phases were not clearly separable (Borràs-Gelonch et al., 2010;Borràs-Gelonch et al., 2011). Alqudah and

Schnurbusch (2014) proposed an amended approach for dissecting the longest pre-anthesis phase (late-reproductive phase) into three sub-phases: awn primordium (AP) to tipping (TIP); TIP to heading (HD); HD to anther extrusion (AE). This refined approach, based upon clearly defined developmental pre-anthesis stages, might shed more light on the causal genetic factors responsible for the variation in developmental stages/sub-phases in response to photoperiod in barley.

Most studies in barley aimed to unravel the genetics of heading time and the underlying specific genes in response to photoperiod, vernalization and/or earliness per se. The first two factors change heading time in response to environmental conditions, while the last factor determines heading time independent of photoperiod and temperature (Bullrich et al., 2002). In barley, a long-day (LD) crop, the *PSEUDO-RESPONSE REGULATOR* (*HvPRR37*) gene, also known as *PHOTOPERIOD RESPONSE LOCUS1* (*Ppd-H1*), is the central heading time gene regulated in responses to LD, at which recessive alleles (*ppd-H1*) reduce the response to LD (Turner et al., 2005). Variation at *Ppd-H1* affects heading time of accessions originating from different geographical regions. Spring barley accessions originating from Middle East, e.g. tend to carry photoperiod responsive *Ppd*$^{-}$*H1* alleles, causing early heading under LD, while the delay of heading time in Northern European accessions of spring barley is due to reduced photoperiod sensitivity, *ppd-H1*, alleles (Turner et al., 2005;Andres and Coupland, 2012). Therefore, strength of photoperiod response is a key factor to understand the natural genetic variation and pathway of heading time in barley.

In addition to the *HvPRR*37 gene, which is located on the short arm of chromosome 2H (Turner et al., 2005), further genes of the heading time pathway have been identified in barley. *Ppd-H2*, responsive to short-day (SD) is located on 1HL, for which *HvFT3* has been proposed as a candidate gene (Faure et al., 2007). Five *FLOWERING LOCUS T* (*FT*-like; *HvFT1-HvFT5*) genes were found in barley, and these genes play various roles during plant development through their photoperiod response, of which *HvFT1* has a major role in the

transition from the vegetative-to-reproductive phase as an important source of variation in heading time (Faure et al., 2007). Moreover, the *CONSTANS*-like (*CO*) gene family is known to regulate flowering time through the photoperiod pathway in *Arabidopsis* (a LD plant) and rice (SD plant). In barley,Griffiths et al. (2003);Cockram et al. (2012) identified numerous homologs of *CO*-like genes (*HvCO1* to *HvCO18*) but their roles in the barley heading time pathway are still unclear. *GIGANTEA (GI)-CO-FT* is considered as a conserved central interaction partner in plant photoperiod pathway under LD, in *Arabidopsis* (Higgins et al., 2010); however, the function of *HvGI* in the barley photoperiod pathway is still unclear. CCT domain gene families (CO, CO-LIKE, TIMING OF CAB1 (TOC1)), i.e. *CO*-like and *PRR*, have an important role in controlling heading time; in addition to these families (Cockram et al., 2012) introduced uncharacterized genes carrying single CCT domains called *CCT MOTIF FAMILY* (*CMF*) genes. *PHYTOCHROME* (*HvPhy*) and *CIRCADIAN CLOCK ASSOCIATED* (*HvCCA*) genes clearly affect barley heading time pathway through interaction with other genes, such as *HvPhyC* which induces early heading by up-regulating *HvFT1* and bypassing *HvCO1* under LD (Nishida et al., 2013). In *Arabidopsis*, *LATE ELONGATED HYPOCOTYL* (*LHY*) and *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) suppress *FT* expression independent of the *CO* causing delayed flowering (Fujiwara et al., 2008). Similarly, *SHORT VEGETATIVE PHASE*-like (*SVP*-like) genes such as *VEGETATIVE TO REPRODUCTIVE TRANSITION gene2* (*HvVRT2*) in barley delayed heading time by inhibiting spike development under LD (Trevaskis et al., 2007). *RICE FLORICAULA/LEAFY* (i.e. *RFL*; syn. *ABERRANT PANICLE ORGANIZATION2*, *APO2*) is the homolog of *Arabidopsis LEAFY* and plays important roles in regulating the transition from vegetative to reproductive phase, maintenance of inflorescence meristem, floral organ identity/determinacy and flowering time in rice (Rao et al., 2008;Higgins et al., 2010;Ikeda-Kawakatsu et al., 2012). In *Arabidopsis*, *LFY* acts downstream of *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*, (*SOC1*) (Higgins et al., 2010); whereas *RFL* functions upstream of *OsSOC1* and reduced expression of

RFL delayed flowering time in rice (Rao et al., 2008). With regard to genes that are involved in responses to vernalization, *Vrn-H1* located on 5HL (promotes transition from the vegetative to the reproductive phase) is dominant in spring barley (Hemming et al., 2008), while *Vrn-H2* (*HvZCCT*) located on 4HL delays heading in plants that have not been vernalized (Karsai et al., 2005); it similarly delays heading time under LD (Casao et al., 2011a). *Vrn-H3* (syn. *HvFT1*), a central integrator of different heading time pathways, had been identified on 7HS (Yan et al., 2006). In addition, many independent *EARLINESS PER SE* (*EPS*) and *EARLY MATURITY* (*EAM*) loci have been identified in barley: *eps2S* (*eam6*) on 2HS, *eps3L* (*eam10*) on 3HL, *eps4L* on 4HL, *eps5L* on 5HL, *eps6L.1* and *eps6L.2* on 6HL, *eps7S* on 7HS and *eps7L* on 7HL (Laurie et al., 1995). The precise position of these genes in coherent barley heading time pathway is not yet understood.

High-throughput genotyping platforms recently developed in barley provide sufficient marker coverage to perform genome-wide association scans (GWAS) (Waugh et al., 2009). GWAS is a powerful tool for mapping complex plant traits, with unprecedented genetic resolution for gene identification in large-genome crops such as barley and wheat. GWAS can identify genes responsible for natural phenotypic variation through screening a large, diverse collection of accessions with high density genetic markers to find causal genes as a result of historical recombination (Waugh et al., 2009). In barley, GWAS has been used to identify single nucleotide polymorphism (SNP) markers associated with heading time (Comadran et al., 2012;Pasam, 2012). However, information on the genetic variation of pre-anthesis stages/sub-phases as key components of barley adaptation and grain yield is still lacking.

The aim of this study is to detect QTL underlying natural variation of pre-anthesis stages/sub-phases based upon differences in photoperiod response (*Ppd-H1*/*ppd-H1*) through dissecting time to heading into sub-phases in a world-wide spring barley collection. To achieve this objective, we phenotyped more than 3,000 plants at four developmental stages (AP, TIP, HD and AE) under controlled GH conditions, derived from 95 photoperiod responsive (*Ppd-H1*)

and 123 accessions with reduced photoperiod sensitivity (i.e. *ppd-H1*), respectively. Distinction of these two photoperiod groups in our GWAS analysis allowed us to control population structure, while using a 9K SNP chip provided us with an unprecedented genetic resolution for studying the natural variation of time to heading. In combination with accurate phenotyping of pre-anthesis stages into sub-phases (i.e. sowing to AP, includes vegetative and early reproductive phases; AP-TIP; TIP-HD and HD-AE) within each photoperiod group, natural genetic variation of the time to heading could be genetically dissected resulting in the identification of novel QTL that were anchored to the barley physical map (e.g. several associations around *HvCO*-like genes). Clearly, novel rich genomic regions with highly associated SNPs were detected, which have not been detected before. This paper proposes a new heading-time model for barley with specific reference to allelic combinations to photoperiod-response groups.

4.3 Materials and methods

4.3.1 The collection, genotyping and population structure

A collection of 218 world-wide spring barley accessions was used in this study. This collection includes 125 two- and 93 six-rowed accessions; i.e. 149 cultivars, 57 landraces and 18 breeding lines. The barley spike possesses three spikelets (one central and two lateral spikelets) per rachis internode. In six-rowed barley, all three spikelets are fertile, while the two lateral spikelets are sterile in two-rowed barley (Bonnett, 1935). The origins of these accessions were from Europe (EU, 108), West Asia and North Africa (WANA, 45), East Asia (EA, 36) and Americas (AM, 29). This collection has been described by Haseneyer *et al.* (Haseneyer et al., 2010) and more information is available under the following link: http://barley.ipk-gatersleben.de/ebdb.php3. In this study the collection was divided into two groups: *Ppd-H1*and *ppd-H1*accessions. These groups consist of two- and six-rowed cultivars, landraces and lines from different origins, more information about row-types and origins is provided in **Table 4-1**.

Table 4-1: Spike row-type and origins of spring barleys accessions with photoperiod-sensitive (*Ppd-H1)* and reduced photoperiod sensitivity (*ppd-H1*).

Origin‡	Photoperiod-sensitive (*Ppd-H1*)		Reduced photoperiod sensitivity (*ppd-H1*)		Total
	Two-rowed	Six-rowed	Two-rowed	Six-rowed	
WANA	12	21	11	1	45
EU	10	6	80	12	108
EA	0	28	2	6	36
AM	6	12	4	7	29
Total	**28**	**67**	**97**	**26**	**218**
	95		**123**		

‡ WANA: West Asia and North Africa, EU: Europe, EA: East Asia, AM, Americas.

The collection was genotyped using the 9K iSelect SNP chip from Illumina, which was developed from RNA-seq data of 10 diverse barley cultivars (Comadran et al., 2012). Finally for our GWAS analyses, we focused on SNPs which had genetic and physical positions on the barley genome after quality control checking, filtering and evaluating 9K SNP (Comadran et al., 2012;Pasam et al., 2012;Mascher et al., 2013). In each group, only the SNPs with minor allele frequency (MAF) ≥ 5% were used for association analyses (4228 and 4050 SNPs for *Ppd-H1*and *ppd-H1*group, respectively). We used genetic marker positions anchored by physical map positions for SNP markers based on Barke x Morex RILs POPSEQ population (Mascher et al., 2013).

The population structure of this collection was determined by 6355 polymorphic SNPs (**Figure 4-1**). In this study, we divided this collection based on photoperiod response (*Ppd-H1*and *ppd-H1*) as major groups and geographical regions present as sub-groups. Principal component analysis (PCA) was also used to infer the population structure in this collection. PCA is an indicator ordination tool for obtaining clusters, which can be explored visually in a two dimensional using GenStat (GenStat, 2014).

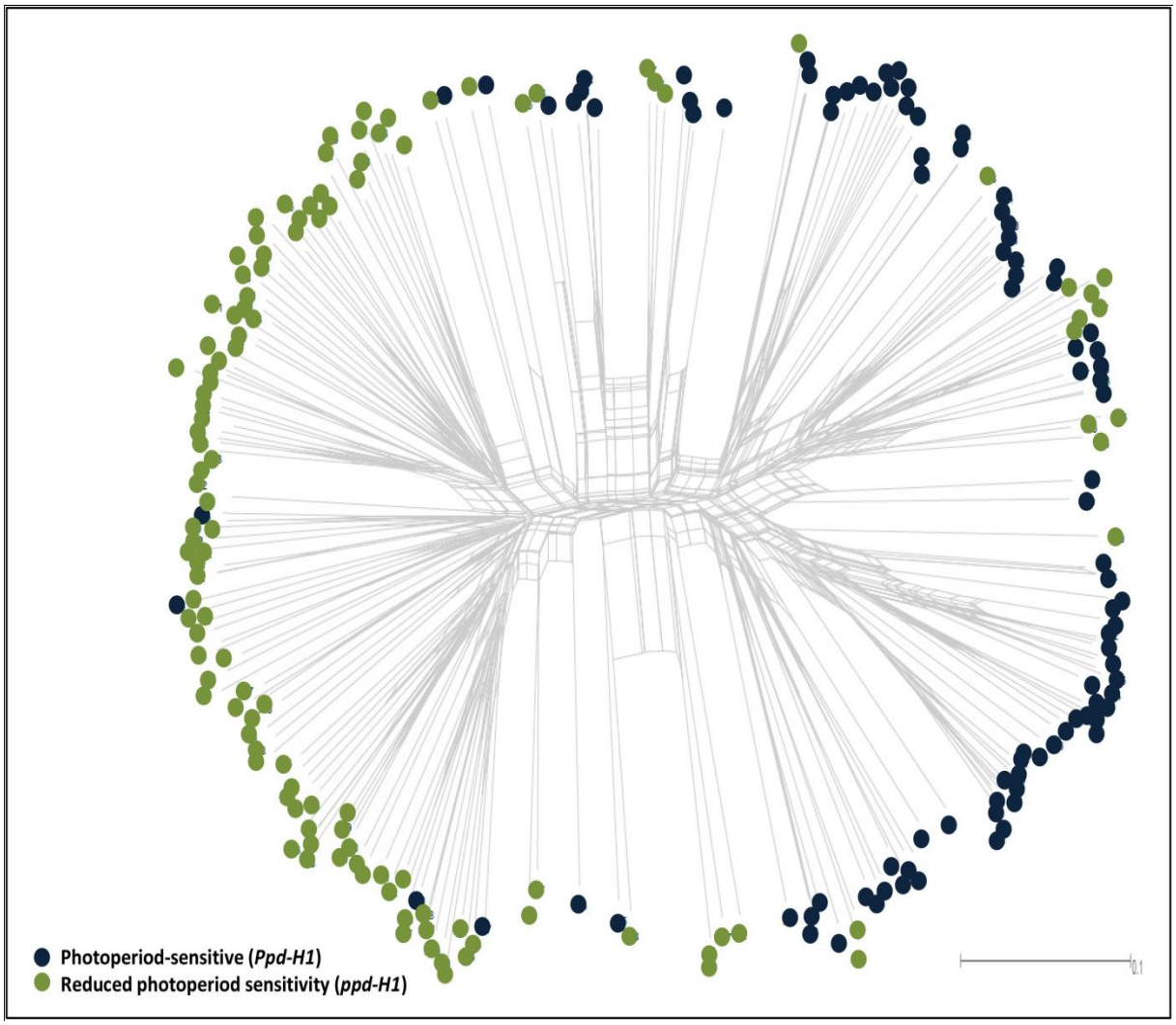

Figure 4-1: Population structure of 218 spring barley accessions based on 6355 SNPs information. 95 accessions showing photoperiod response (*Ppd-H1*) and 123 accessions with reduced photoperiod sensitivity (*ppd-H1*).

4.3.2 Phenotyping

Thirty-five seeds of each accession from the collection of 218 spring barley accessions were germinated for 10 days under controlled condition in GH (LD condition, 16/8 h day/night and ~20/ ~16 ^{o}C day/night). Seedlings were transferred to vernalization chamber (SD condition, 10/14 h and ~4 ^{o}C) for a period (28 days) when they reached 2-3 leaves stage. Afterwards seedlings were kept in an acclimation chamber for a period of 7 days (16/8 h and ~14 /~12 ^{o}C). Finally, strongest 30 seedlings of each accession were transplanted into 0.5 liter pots (one plant per pot; nine centimeter pot diameter and nine centimeter height) under GH condition. Plants were grown in a substrate containing peatmoss with 14: 16: 18 / Nitrogen (N):

Phosphorous (P): Potassium (K). To avoid any mineral deficiency each pot was additionally fertilized with 1.5 gram of solid fertilizer (that constitute minerals 17:11:10 / N: P: K). Plants were irrigated daily and supplemental light (~300 mE $m^{-2}s^{-1}$ PAR) was used to extend the natural light with low intensity incandescent lamps. Pots were randomized three times per week to reduce border and temperature-gradient effects on plant growth and development. The time for each stage was recorded when at least 50% of the main culm spikes in each accession had reached at the stage: awn primordium stage, AP (Z31-33, maximum yield potential; Alqudah and Schnurbusch (2014)); tipping stage, TIP (Z49, first awns visible on main culm); heading time stage, HD (Z55, half main culm spike emerged from flag leaf sheath); anther extrusion stage, AE (Z65, half of main culm spike with anthers; Zadoks et al. (1974)); more information on the stages can be obtained in 4- 2 and (Alqudah and Schnurbusch, 2014). To identify the exact timing for the AP stage, regular dissection (three times per week) from immature barley main culm spikes was performed by microscopic dissection and image capture using Stereo Microscope Stemi 2000-C with KL 1500 LCD; Axio Vision, 4.8.2, ZEISS, Germany.

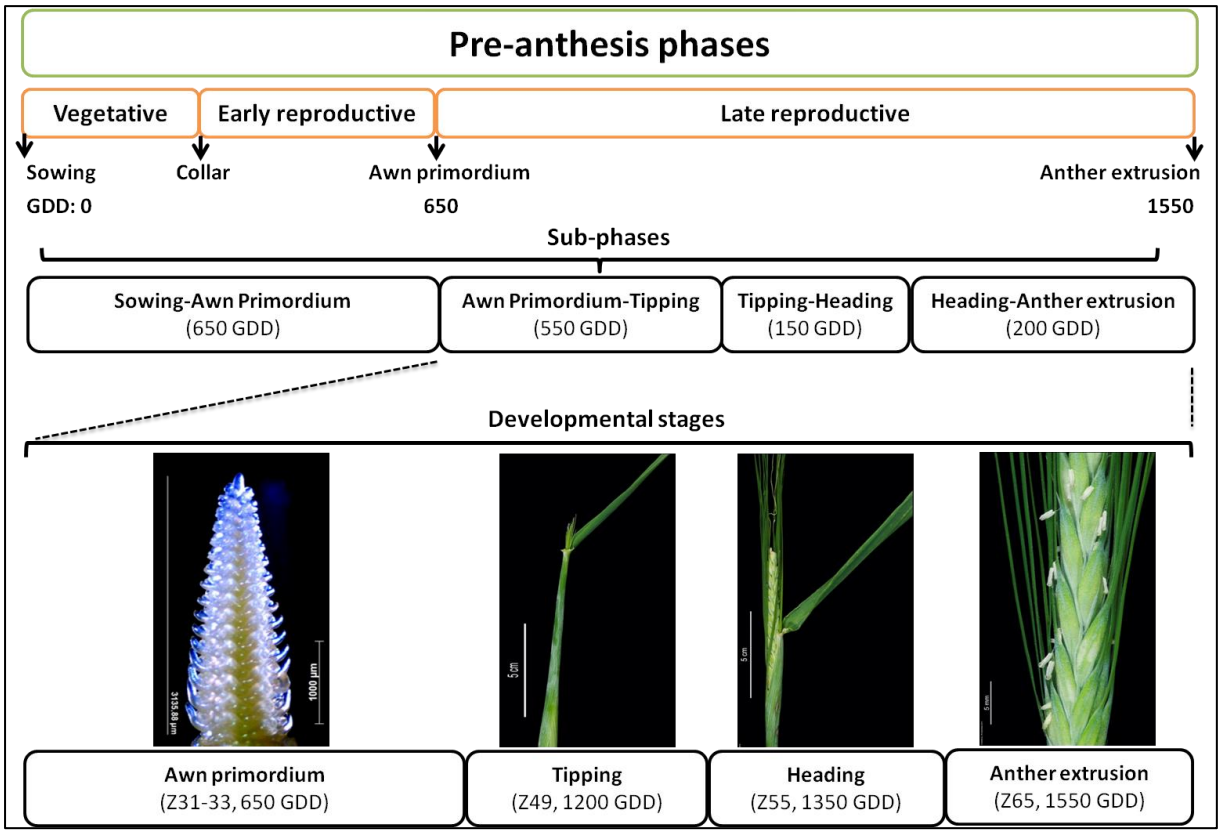

Figure 4-2: General figure of barley pre-anthesis phases.
The figure includes the beginning developmental point from sowing to each stage (e.g. time to tipping is 1200 GDD; i.e. from sowing time to tipping stage) and the differences between stages (phase; e.g. tipping to heading phase (150 GDD); i.e. GDD for heading stage (1350 GDD) minus tipping stage (1200 GDD)). GDD is the average GDD in the whole collection (i.e. including both photoperiod groups). This figure also describes the developmental stages and sub-phases which form the late reproductive phase as described in (Alqudah and Schnurbusch, 2014).

Thermal time ($^{o}C.D^{-1}$) or growing degree-days (GDD, base temperature was 0 ^{o}C) from sowing to reach each stage was recorded to measure the required days/thermal temperature for each stage and the duration between the stages (**Table 4-S1**). The phenotypic analyses of pre-anthesis sub-phases (i.e. dissection work) had to be performed in eight batches due to limited GH space and feasibility of workload. Each batch contained thirty accessions plus two check lines (Morex and Barke); hence, all 218 accessions were grown from September 2011 to April 2012 in eight consecutive batches for dissection and phenotypic, stage-specific data collection under controlled GH conditions. Each batch of accessions had a completely randomized design with 30 plants per accession. At least three biological replications at each developmental stage were recorded for analysis. The collected data were analyzed using SAS software version 9.3 at probability level $P \leq 0.05$. *Student's t-test* was used to compare between

*Ppd-H1*and *ppd-H1* groups. REML (Residual Maximum Likelihood) was used to analyze phenotypic data by SAS software (SAS, 2013). Best Linear Unbiased Estimates (BLUEs) were used to estimate phenotypic means for each trait in individual accession and estimated means were used for association analysis. Broad-sense heritability for traits in each group was calculated across growing times as the ratio between the genetic variance and the phenotypic variance which includes genotypic by growing times (environment) interaction variance and error variance components using PROC VARCOMP (SAS, 2013).

4.3.3 Genome-wide association study (GWAS) analysis

GWAS of two groups: 95 *Ppd-H1* and 123 *ppd-H1* accessions were identified using their corresponding genotype datasets according to the G/T SNP22 (Turner et al., 2005). The association of phenotypic traits (BLUEs) and each single marker was analyzed by mixed linear model (MLM) implemented using GenStat 16 (GenStat, 2014). Eigenanalysis with Single Trait Association Analysis (Single Environment) was used as correction for population structure in MLM for accounting relatedness to avoid false positives in GWAS as described previously (Pritchard et al., 2000;Tondelli et al., 2013). A threshold of *P-value* 0.01 was used in all traits detecting significant SNPs with $-\log_{10}$ *P-values* ≥2. Such set of significant SNPs (i.e. exceeding the $-\log_{10}$ *P-values* ≥2) underwent another test on robustness using the false discovery rate (FDR) at 0.05 (Storey and Tibshirani, 2003). FDR analysis provides highly significant associations (*P-values* ≥FDR) among which significant *P-values* from ≥2 and lower ≤FDR; this technique has been widely applied to GWAS analyses, see the review by van den Oord (2008). Allele effects were estimated as relative to the performance of "Mansholt zweizeilig" and "Isaria" cultivars for *Ppd-H1*and *ppd-H1* groups, respectively (Tondelli et al., 2013). To validate our association results, we analyzed all stages under GH condition for comparing the significant loci (known genes) with association from the same collection under field conditions (Pasam, 2012;Pasam et al., 2012). Genetic maps were drawn using MapChart 2.2 Windows (Voorrips, 2002) usin those SNP markers exceeding FDR

threshold to determine highly associated QTL within confidence interval ±5 cM. Known heading time genes (bold and italicized in **Figure 4-6 and 4-7**) have been genetically anchored and located according to the Barke x Morex RILs (POPSEQ) sequence contigs; more information about these genes, their accession numbers and genetic chromosome positions are available in **Table 4-S2**.

4.4 Results

4.4.1 Population structure of a world-wide spring barley collection

The population structure of this collection was determined using polymorphic SNP data from the 9K array. To this end, we divided the collection into two groups based on the presence of a single diagnostic SNP in *HvPRR37*, thereby separating photoperiod responsive (i.e. photoperiod-sensitive, *Ppd-H1*) accessions from those with reduced photoperiod sensitivity (i.e. *ppd-H1*; Turner et al. (2005); Sharma *et al. in preparation*; **Figure 4-1**). The relationships among spring barley accessions were inferred using principle component analysis (PCA). The genetic variation between groups was explained by PCA and the collection was clearly separated into *Ppd-H1* and *ppd-H1* spring barleys based on heading time data (**Figure 4-S1**). PCA-1 explained 28.1% of variation and separated the *ppd-H1*-group from the *Ppd-H1*-group, with few exceptions, clearly showing greater genetic variation among accessions from the *Ppd-H1*-group compared to *ppd-H1*-carrying accessions (**Figure 4-S1**). Interestingly, the genetic variation at heading time could be further subdivided based on geographic origins (**Figure 4-S2**). The European (EU) accessions clustered from the remaining regions with few exceptions (**Figure 4-S2**). Although a significant proportion of the collection clustered separately based on photoperiod response, row-type classes formed another determinant within the geographic and photoperiod groups. Notably, most of the accessions in the *Ppd-H1*-group are six-rowed barleys from West Asia and North Africa (WANA) and East Asia (EA), while most of the accessions in the *ppd-H1*-group are two-rowed barleys from EU (**Table 4-1**). Generally, these results suggest that the spring barley collection (218 accessions)

is separable into two major groups based on the response to photoperiod and reduced photoperiod sensitivity (*Ppd-H1*/*ppd-H1*) at heading time.

4.4.2 Natural phenotypic variation in pre-anthesis developmental stages and sub-phases

It is difficult to understand the full complexity of the time to heading in cereals by only studying the period from sowing until heading/flowering. Our analyses aimed at examining associations for particular pre-anthesis stages or sub-phases, to maximize the likelihood of finding new associations. Hence, we developmentally dissected the pre-anthesis time of barley into four stages (AP, TIP, HD and AE) and four sub-phases (sowing-AP, AP-TIP, TIP-HD and HD-AE; **Figure 4-2**). The first investigated pre-anthesis stage was AP. Plants at this stage had already passed the vegetative-to-reproductive transition and finished early spike differentiation. The time from sowing to AP represents approximately 40% of the entire time to AE in barley and on average it took 650 growing degree-days (GDD, **Figure 4-2**). Late reproductive development in barley can be further sub-divided into three sub-phases, of which AP-TIP is the longest phase with an average of 550 GDD (**Figure 4-2**).

Comparisons of thermal time to reach different developmental stages in *Ppd-H1*and *ppd-H1* spring barley groups under GH condition yielded highly significant variation between the groups ($P \leq 0.05$; **Figure 4-3 and Table 4-S1**). The duration from sowing to reach each developmental stage was significantly longer in the *ppd-H1*group at all stages except AP stage; i.e. at TIP, HD and AE the differences were +302, +406 and +373 GDD, respectively, in favor of the *ppd-H1* group (**Figure 4-3**). Separate analysis of thermal time to reach developmental stages between geographical regions in *Ppd-H1* and *ppd-H1* spring barley groups can also explain the genetic variation (**Figure 4-4A and B**). In the *Ppd-H1* group, there was no clear trend of results between geographical regions (**Figure 4-4A**), whilst the genetic variation between geographical regions were much clearer in the *ppd-H1* group (**Figure 4-4B**). In particular EU accessions of the *ppd-H1* group had the longest durations

from sowing to different pre-anthesis stages (TIP, HD and AE stages) except AP stage (**Figure 4-4B**).

Durations between sub-phases can explain the significant variation between the two photoperiod groups. The duration between AP-TIP was the longest late-reproductive sub-phase in both groups resulting in 690 GDD for the *ppd-H1*-carrying accessions but only 400 GDD for the *Ppd-H1* group (**Figure 4-3**). The duration between TIP-HD and HD-AE was not significantly different between both groups. Generally, the AP-TIP sub-phase is the most important developmental period related to the observed genetic variation for the time to heading between these groups. In terms of origin, EU accessions had the longest duration between AP-TIP and TIP-HD sub-phases in both groups (**Figure 4-4A and B**). The genetic variation in pre-anthesis stages between geographical regions became clearer in the *ppd-H1* group. Thus, the results of our dissection of the pre-anthesis stages provide a promising route to explain the genetic variation between the photoperiod groups (*Ppd-H1* and *ppd-H1*) in barley.

Notably, the broad-sense heritability values for pre-anthesis developmental stages and sub-phases in each photoperiod group (*Ppd-H1*and *ppd-H1*) were above 0.88, indicating that the traits related to pre-anthesis phase duration are highly heritable (**Table 4-2**). Due to the very high heritability values associated with pre-anthesis stages we are able to detect particular QTL for each stage and sub-phase within each photoperiod group.

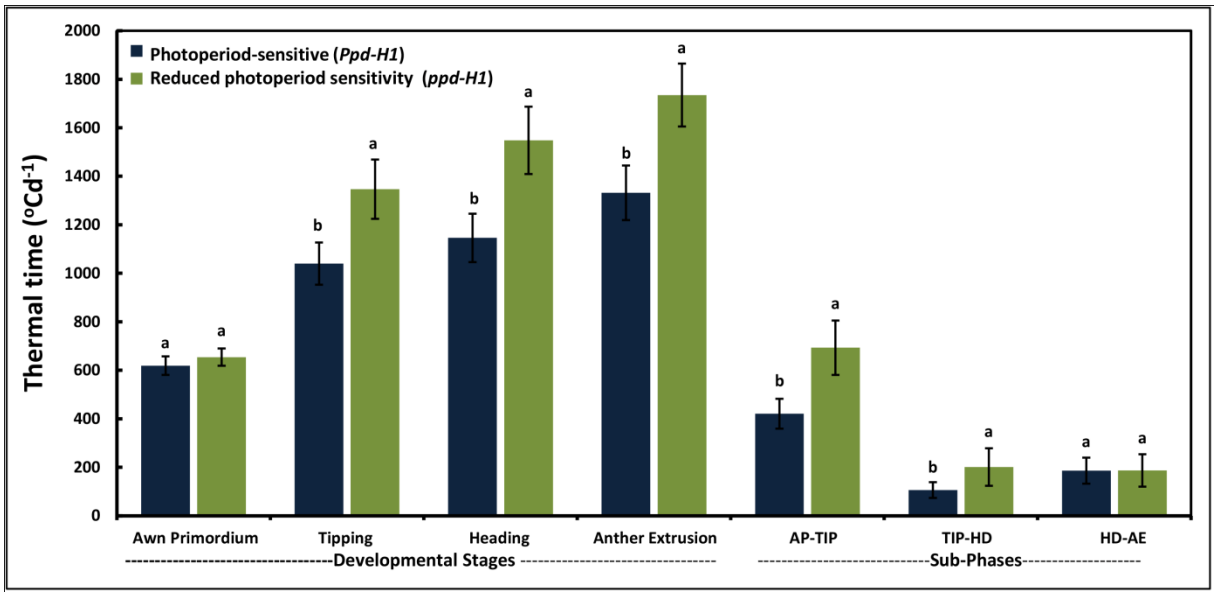

Figure 4-3: Thermal time for different developmental stages and sub-phases in photoperiod-sensitive (*Ppd-H1*) and reduced photoperiod sensitivity (*ppd-H1*).
Thermal time from sowing to the beginning of awn primordium (AP), tipping (TIP), heading (HD) and anther extrusion (AE) stages and thermal time of the duration of sub-phases. Letters differentiate between photoperiod-sensitive (*Ppd-H1*) and reduced photoperiod sensitivity (*ppd-H1*). The same letters are not significantly different at $P \leq 0.05$. Bars indicate standard deviation (n = 95 and 123 for *Ppd-H1* and *ppd-H1* barleys, respectively).

Table 4-2: Estimation of broad-sense heritability (H^2) for developmental stages and sub-phases measured as thermal time $^{o}C.D^{-1}$ (GDD) in the association mapping groups.

Stage/phase†	Photoperiod-sensitive (*Ppd-H1*)	Reduced photoperiod sensitivity (*ppd-H1*)
Awn primordium (AP)	0.92	0.90
Tipping (TIP)	0.91	0.92
Heading (HD)	0.90	0.92
Anther extrusion (AE)	0.91	0.93
AP-TIP	0.89	0.91
TIP-HD	0.92	0.90
HD-AE	0.90	0.88

†AP: awn primordium (Alqudah and Schnurbusch, 2014); TIP: tipping, Z49; HD: heading, Z55; AE: anther extrusion, Z65 (Zadoks et al., 1974).
H^2: broad-sense heritability for each group overall growing times based on accessions mean. n = 95 and 123 for barleys with photoperiod-sensitive and reduced photoperiod sensitivity, respectively.
Developmental stages calculated based on thermal time $^{o}C.D^{-1}$ (GDD).

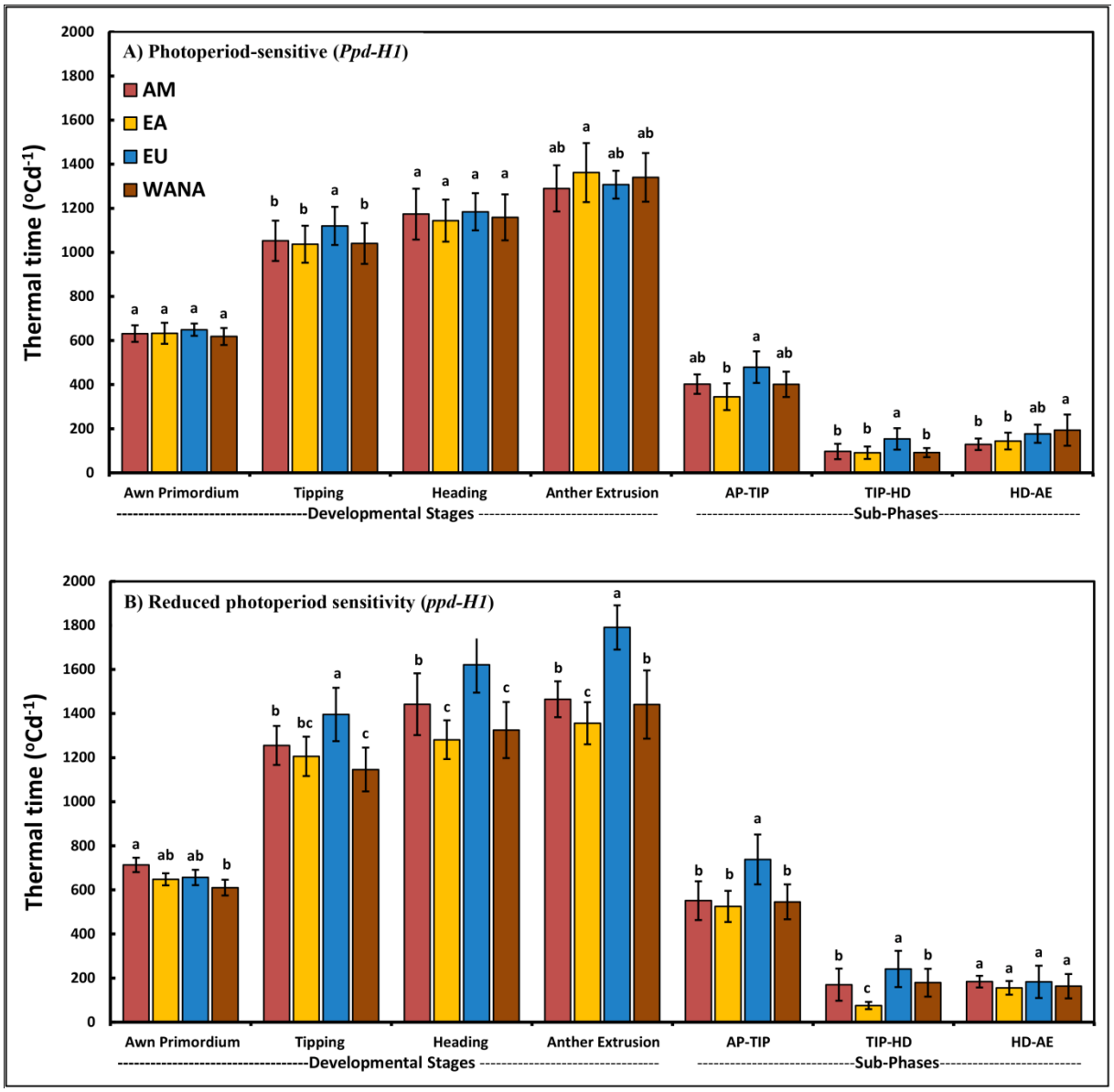

Figure 4-4: Thermal time for different developmental stages and sub-phases based on accessions' origins within photoperiod-sensitive (*Ppd-H1*) and reduced photoperiod sensitivity (*ppd-H1*).

Thermal time from sowing to the beginning of awn primordium (AP), tipping (TIP), heading (HD) and anther extrusion (AE) stages and thermal time of the duration of sub-phases. Letters differentiate between origins within photoperiod-sensitive (*Ppd-H1*) and reduced photoperiod sensitivity (*ppd-H1*). The same letters are not significantly different at $P \leq 0.05$. Bars indicate standard deviation. A). Number of *Ppd-H1*-carrying accessions for WANA = 33, EU = 16, EA = 28 and AM = 18. B). Number of accessions with reduced photoperiod sensitivity (*ppd-H1*) for WANA = 12, EU = 92, EA = 8 and AM = 11.

4.4.3 Identification of natural genetic variation for pre-anthesis development using GWAS

4.4.3.1 Strategies for validating and improving GWAS analyses

We performed GWAS for all developmental stages under GH conditions on the collection to compare it with previous results of the same collection obtained from multiple field evaluations (Pasam, 2012;Pasam et al., 2012). In our study, the major locus for HD (*Ppd-H1*)

appeared to be identical to the previously known locus, for instance at HD stage (**Figure 4-S3**), clearly indicating that field and GH data are comparable. Compared to the previous study (Pasam, 2012), the power of our GWAS to detect associated loci was increased, likely as a result of more controlled growing conditions (e.g. GH, higher heritability) and the higher number of SNP markers (9K). These results re-confirm that GH conditions are appropriate for studying pre-anthesis phase durations (Alqudah and Schnurbusch, 2014).

GWAS was conducted for each photoperiod group (*Ppd-H1* and *ppd-H1*) independently using SNPs derived from the 9K array (**Figure 4-S4 and 4-S5**). Association analyses between each SNP and thermal time between developmental stages/sub-phase were performed using mixed models to generate Manhattan plots (**Figure 4-S4 and 4-S5**). Although the number of significant SNPs were higher in the *Ppd-H1* group (i.e. SNP marker, $-\log_{10} >2$, *P*-value=0.01; **Figure 4-S6**), the association signals and map resolution were much clearer in the *ppd-H1* group (123 accessions) than in the *Ppd-H1* group (95 accessions) likely as a result of accession number. Because of the high number of shared QTL (defined as confidence interval ±5 cM) between developmental stages (**Figure 4-5**) and sub-phases (**Table 4-S3**), we reduced the number of spurious associations by only considering those SNPs, which exceeded the false discovery rate (FDR; **Figure 4-S7**). To increase the precision-power of our GWAS analysis (genetic SNP positions), we used the latest version of the barley physical map (Mascher et al., 2013). This map provided great power to locate physical/genetic positions of known heading time gene(s) and/or QTL(s) for each developmental stage/sub-phase.

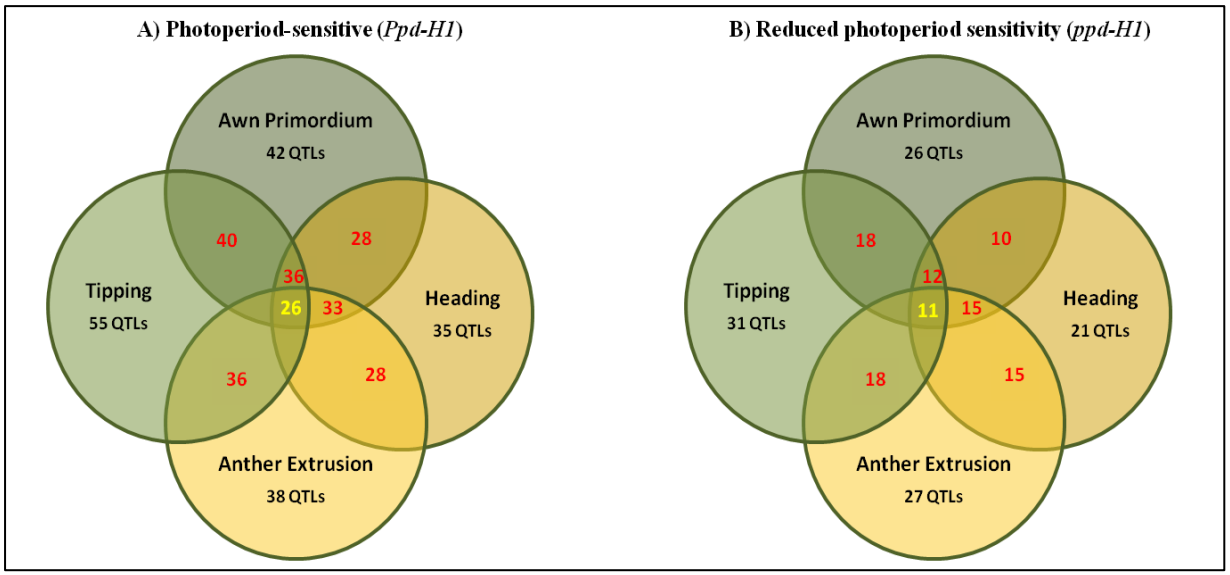

Figure 4-5: Number of QTLs (within confidence interval ±5 cM) overlapping between developmental stages in groups carrying A) photoperiod-sensitive (*Ppd-H1*) and B) reduced photoperiod sensitivity (*ppd-H1*).
Number in red denotes the number of shared QTLs between each pair of developmental stages. Number in yellow denotes the number of shared QTLs between all developmental stages. QTLs exceeding significance level ($-\log_{10}$ (*P-value* = 0.01)) are considered as significantly associated.

4.4.3.2 Identification of marker-trait association within the photoperiod-sensitive (*Ppd-H1*) group

All 95 accessions in this group possess functional *Ppd-H1* alleles and so display a strong response to long day (LD) condition. GWAS analysis in this group detected in total thirty significantly associated chromosomal regions, of which eleven are group-specific and only occur here, indicating that *Ppd-H1*-carrying accessions exhibit a complex genetic architecture for pre-anthesis development. Another specific feature of this group is the high number of significant associations for the time to AP (>FDR for AP, i.e. ten regions on four chromosomes; **Figure 4-6**), suggesting that there is ample natural genetic variation in the duration for this first sub-phase among accessions. Many of these chromosomal regions very precisely co-localized with known heading time genes (e.g. 7HS 31.8-34-3, *Vrn-H3/HvFT1* having the most significant effect until AP; or 2HS 26.8-31.0 cM, *Ppd-H1*/*HvPRR37*; **Figure 4-6**) and novel candidate gene regions (i.e. 1HS 41.1-48.2 cM, incl. *HcCMF10*; 2HS 38.2-41.9 cM, *HvCO18*; 5HS 43.7-51.6 cM, *HvCO3*, *HvTFL1*(barley *TERMINAL FLOWER 1*),

HvCMF13; 7HS 11.8-13.9). Many *CO*-like genes are among the chromosomal regions controlling early development until AP, suggesting that these family members promote the vegetative-to-reproductive phase transition and early spike development. All of these *CO*-like genes also showed highly significant associations with later developmental stages and sub-phases (i.e. TIP, HD, AP-TIP etc.) except for the two chromosomal regions on 4H (51.1-54.6 cM, *HvCO16*, *HvPRR59*, *HvPhyB*, *HvPRR73*; **Figure 4-6**) and 6HL (67.9-69.3 cM, *HvCO14*, *HvCO2*, *HvCO11*), which were only detectable during later developmental stages/sub-phases in this group. Other interesting associations were found in the centromeric region of 2H (*HvFT4*, *HvCEN*, *HvCO4*, *HD6-2H*), for the barley ortholog of *RICE FLORICAULA/LEAFY* (*BFL*) on 2HL (107.3 cM, *BFL*; **Figure 4-6**) and on chromosomal 5HL (*Vrn-H1*, *HvPhyC*) all affecting early (AP and TIP) and later development in this group. For one region on 7HS (20.8-24.2 cM) showing significant associations with time to AP and TIP, we were not able to locate known flowering time genes.

It was previously shown that the duration between AP-TIP is positively related with single-plant yield and yield components in barley (Alqudah and Schnurbusch, 2014). For this second sub-phase (i.e. AP-TIP), we found in total eighteen out of thirty significant associations (i.e. ~60% from the total identified QTL) within the photoperiod-sensitive (*Ppd-H1*) group (**Figure 4-6**), suggesting the existence of abundant genetic variation for breeding. Among these regions, the highly associated SNPs around *HvCO14*, *HvCO2* and *HvCO11* (6HL, 67.9-69.3 cM; **Figure 4-6**) showed the strongest phenotypic effect on the time from AP to TIP (-80 GDD) and occurred exclusively in this group. GWAS analysis of the time to TIP identified thirty significant associations (**Figure 4-6**). The time to TIP can be seen as an integrator of the two sub-phases sowing-AP and AP-TIP. Hence, it is not surprising that many associations overlap with AP, AP-TIP and later stages and sub-phases, indicating that many of these loci affect more than one stage or sub-phase. Nevertheless, we found three chromosomal regions in this group, which had major phenotypic effects on the time to TIP: these were highly

associated SNPs around the *Ppd-H2* locus on 1HL (87.8-93.1 cM; **Figure 4-6**), and two regions on chromosomal 5H, containing *HvCO3, HvTFL1, HvCMF13* (5HS, 43.7-51.6 cM) and the *Vrn-H1, HvPhyC* locus (113.8-125.8 cM). The two late sub-phases, i.e. TIP-HD and HD-AE, are relatively short compared with the other two earlier sub-phases (**Figure 4-3**); but we were still able to find twenty-five and thirteen significantly associated chromosomal regions, respectively (**Figure 4-6**). As expected, the large majority of these loci preferentially co-located with regions being associated with other developmental stages and sub-phases, again reinforcing the fact that a high degree of pleiotropic gene action exists between several stages and sub-phases.

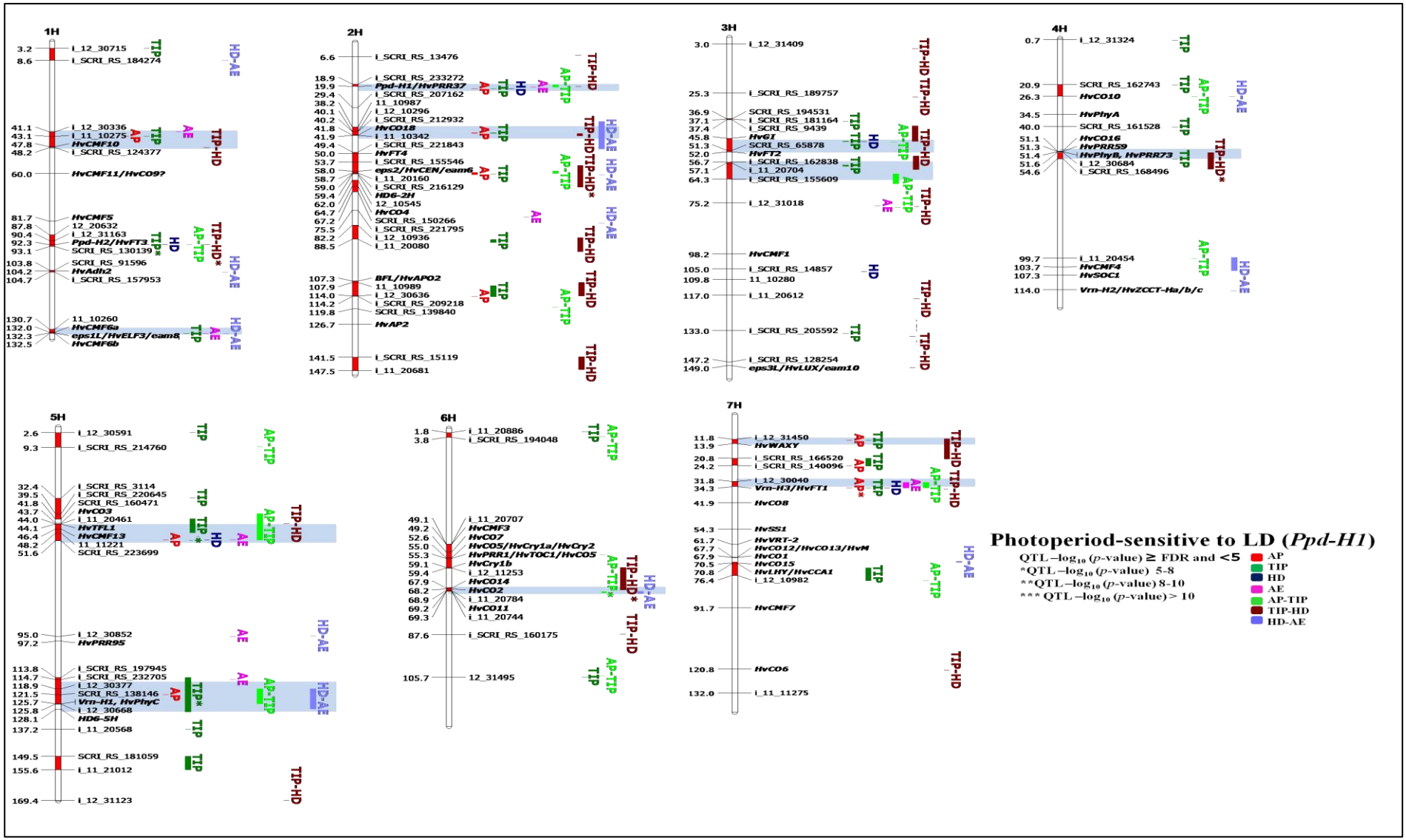
1H
3.2 i_12_30715
8.6 i_SCRI_RS_184274
41.1 i_12_30336
43.1 i_11_10275
47.8 HvCMF10
48.2 i_SCRI_RS_124377
60.0 HvCMF11/HvCO9?
81.7 HvCMF5
87.8 12_20632
90.4 i_12_31163
92.3 Ppd-H2/HvFT3
93.1 SCRI_RS_130139
103.8 SCRI_RS_91596
104.2 HvAdh2
104.7 i_SCRI_RS_157953
130.7 11_10260
132.0 HvCMF6a
132.3 eps1L/HvELF3/eam8
132.5 HvCMF6b
2H
6.6 i_SCRI_RS_13476
18.9 i_SCRI_RS_233272
19.9 Ppd-H1/HvPRR37
29.4 i_SCRI_RS_207162
38.2 11_10987
40.1 i_12_10296
40.2 i_SCRI_RS_212932
41.8 HvCO18
41.9 i_11_10342
49.4 i_SCRI_RS_221843
50.0 HvFT4
53.7 i_SCRI_RS_155546
58.0 eps2/HvCEN/eam6
58.7 i_11_20160
59.0 i_SCRI_RS_216129
59.4 HD6-2H
62.0 12_10545
64.7 HvCO4
67.2 SCRI_RS_150266
75.5 i_SCRI_RS_221795
82.2 i_12_10936
88.5 i_11_20080
107.3 BFL/HvAPO2
107.9 11_10989
114.0 i_12_30636
114.2 i_SCRI_RS_209218
119.8 SCRI_RS_139840
126.7 HvAP2
141.5 i_SCRI_RS_15119
147.5 i_11_20681
3H
3.0 i_12_31409
25.3 i_SCRI_RS_189757
36.9 SCRI_RS_194531
37.1 i_SCRI_RS_181164
37.4 i_SCRI_RS_9439
45.8 HvG1
51.3 SCRI_RS_65878
52.0 HvFT2
56.7 i_SCRI_RS_162838
57.1 i_11_20704
64.3 i_SCRI_RS_155609
75.2 i_12_31018
98.2 HvCMF1
105.0 i_SCRI_RS_14857
109.8 11_10280
117.0 i_11_20612
133.0 i_SCRI_RS_205592
147.2 i_SCRI_RS_128254
149.0 eps3L/HvLUX/eam10
4H
0.7 i_12_31324
20.9 SCRI_RS_162743
26.3 HvCO10
34.5 HvPhyA
40.0 SCRI_RS_161528
51.1 HvCO16
51.3 HvPRR59
51.4 HvPhyB, HvPRR73
51.6 i_12_30684
54.6 i_SCRI_RS_168496
99.7 i_11_20454
103.7 HvCMF4
107.3 HvSOC1
114.0 Vrn-H2/HvZCCT-Ha/b/c
5H
2.6 i_12_30591
9.3 i_SCRI_RS_214760
32.4 i_SCRI_RS_3114
39.5 i_SCRI_RS_220645
41.8 SCRI_RS_160471
43.7 HvCO3
44.0 i_11_20461
44.1 HvTFL1
46.4 HvCMF13
48.2 11_11221
51.6 SCRI_RS_223699
95.0 i_12_30852
97.2 HvPRR95
113.8 i_SCRI_RS_197945
114.7 i_SCRI_RS_232705
118.9 i_12_30377
121.5 SCRI_RS_138146
125.7 Vrn-H1, HvPhyC
125.8 i_12_30668
128.1 HD6-5H
137.2 i_11_20568
149.5 SCRI_RS_181059
155.6 i_11_21012
169.4 i_12_31123
6H
1.8 i_11_20886
3.8 i_SCRI_RS_194048
49.1 i_11_20707
49.2 HvCMF3
52.6 HvCO7
55.0 HvCO5/HvCry1a/HvCry2
55.3 HvPRR1/HvTOC1/HvCO5
59.1 HvCry1b
59.4 i_12_11253
67.9 HvCO14
68.2 HvCO2
68.9 i_11_20784
69.2 HvCO11
69.3 i_11_20744
87.6 i_SCRI_RS_160175
105.7 12_31495
7H
11.8 i_12_31450
13.9 HvWAXY
20.8 i_SCRI_RS_166520
24.2 i_SCRI_RS_140096
31.8 i_12_30040
34.3 Vrn-H3/HvFT1
41.9 HvCO8
54.3 HvSS1
61.7 HvVRT-2
67.7 HvCO12/HvCO13/HvM
67.9 HvCO1
70.5 HvCO15
70.8 HvLHY/HvCCA1
76.4 i_12_10982
91.7 HvCMF7
120.8 HvCO6
132.0 i_11_11275
Photoperiod-sensitive to LD (Ppd-H1)
QTL –log10 (p-value) ≥ FDR and <5
*QTL –log10 (p-value) 5-8
**QTL –log10 (p-value) 8-10
*** QTL –log10 (p-value) > 10
AP
TIP
HD
AE
AP-TIP
TIP-HD
HD-AE

Figure 4-6: Genetically anchored position of highly associated QTLs at all barley developmental stages and sub-phases in the photoperiod-sensitive (*Ppd-H1*) group using 9K SNP markers.

Bold and italicized gene names indicate genetically anchored positions of known heading time genes in the Barke x Morex RILs. Associated chromosomal regions are highlighted with different colors according to stages and sub-phases. Red chromosomal areas indicate the range of significantly associated QTLs (within confidence interval ±5 cM) which are exceeding FDR level of each developmental stage or sub-phase. Highlighted chromosomal regions in light blue denote group-specific associations.

4.4.3.3 Identification of marker-trait association within the *ppd-H1*-carrying group

All 123 accessions in this group carry the *ppd-H1* allele with reduced photoperiod sensitivity and thus reach most stages/sub-phases significantly later under LD (**Figure 4-3**). GWAS analysis in this group identified in total at least twenty significantly associated chromosomal regions of which only six were group-specific, indicating that *ppd-H1*-accessions display a much less complex genetic architecture for pre-anthesis development (**Figure 4-7**). Among those regions, most highly associated SNPs around *HvCO1* (7HS, 67.6-73.4 cM; **Figure 4-7**) resulted in the major phenotypic effect in this group. However, *HvCO1* resides in a chromosomal region, which physically contains three other *CO*-like family members (*HvCO12*, *HvCO13/HvM* and *HvCO15*) and the circadian clock-related genes, *HvLHY*/*HvCCA1*. Nevertheless, specific associations could be found for several physically anchored and co-located SNPs for each of the genes therefore making it possible to attribute phenotypic effects to individual genes. Hence, the most significant effects were found for SNPs co-locating with *HvCO1* for the time between AP-TIP***, time to TIP***, HD*** and AE* (**Figure 4-7**) thereby shortening duration, i.e. promoting heading, by -126, -148, -172 and -153 GDD, respectively. The physically close *HvLHY*, *HvCCA1* region, however, showed up in both groups (**Figure 4-6 and 4-7**) a consistent heading time-inhibiting effect (i.e. here in the *ppd-H1* group at TIP +128 and +105 GDD for *HvLHY*, *HvCCA1*, respectively), suggesting that these two tightly linked chromosomal regions on 7HS function reciprocally on *HvFT1* expression. Another very consistent but group-specific effect was obtained from SNPs on 6HS (16.0 cM; **Figure 4-7**), reducing time to TIP* and HD* by -151 and -173 GDD, respectively. For the last two specific regions in this group with highly significant associations we did not succeed to locate any known candidate genes (3HL, 122.6-126.7 cM; 5HL, 83.5-85.6 cM). All of these four group-specific regions have in common that they primarily affect later developmental stages and/or sub-phases (**Figure 4-7**). Importantly, in this group there was only one region associated with SNPs around *HvFT1* on 7HS (34.3-43.8

cM; **Figure 4-7**), which showed significant associations with time to AP (i.e. first sub-phase). All other detected regions affected later developmental stages or sub-phases (e.g. thirteen for AP-TIP, ten for TIP, three for TIP-HD, six for HD; **Figure 4-7**), suggesting that genetic variation for later developmental stages/sub-phases is more relevant in *ppd-H1*-carrying accessions.

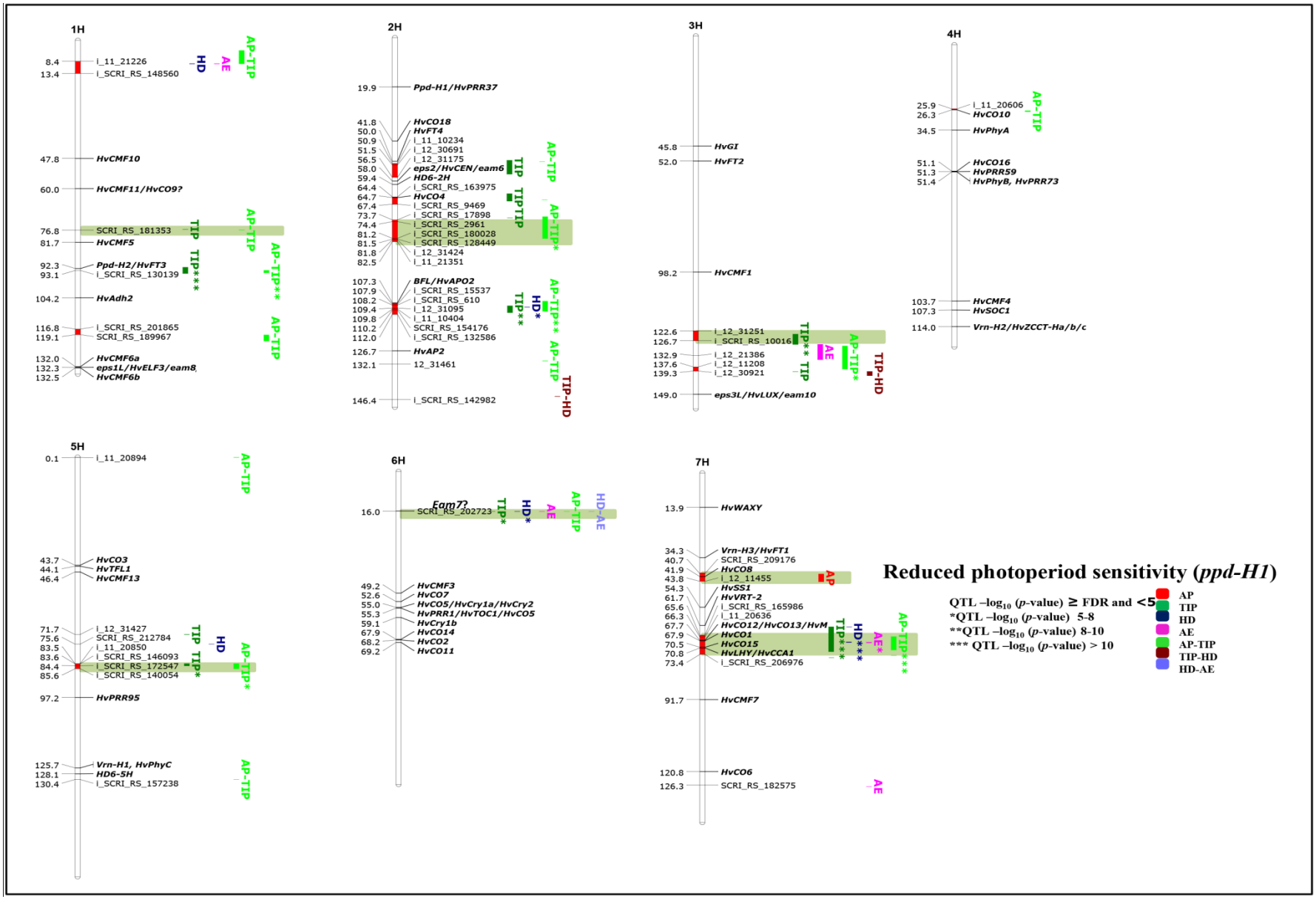
1H
8.4 i_11_21226
13.4 i_SCRI_RS_148560
47.8 HvCMF10
60.0 HvCMF11/HvCO9?
76.8 SCRI_RS_181353
81.7 HvCMF5
92.3 Ppd-H2/HvFT3
93.1 i_SCRI_RS_130139
104.2 HvAdh2
116.8 i_SCRI_RS_201865
119.1 SCRI_RS_189967
132.0 HvCMF6a
132.3 eps1L/HvELF3/eam8
132.5 HvCMF6b
2H
19.9 Ppd-H1/HvPRR37
41.8 HvCO18
50.0 HvFT4
50.9 i_11_10234
51.5 i_12_30691
56.5 i_12_31175
58.0 eps2/HvCEN/eam6
59.4 HD6-2H
64.4 i_SCRI_RS_163975
64.7 HvCO4
67.4 i_SCRI_RS_9469
73.7 i_SCRI_RS_17898
74.4 i_SCRI_RS_2961
81.2 i_SCRI_RS_180028
81.5 i_SCRI_RS_128449
81.8 i_12_31424
82.5 i_11_21351
107.3 BFL/HvAPO2
107.9 i_SCRI_RS_15537
108.2 i_SCRI_RS_610
109.4 i_12_31095
109.8 i_11_10404
110.2 SCRI_RS_154176
112.0 i_SCRI_RS_132586
126.7 HvAP2
132.1 12_31461
146.4 i_SCRI_RS_142982
3H
45.8 HvGI
52.0 HvFT2
98.2 HvCMF1
122.6 i_12_31251
126.7 i_SCRI_RS_10016
132.9 i_12_21386
137.6 i_12_11208
139.3 i_12_30921
149.0 eps3L/HvLUX/eam10
4H
25.9 i_11_20606
26.3 HvCO10
34.5 HvPhyA
51.1 HvCO16
51.3 HvPRR59
51.4 HvPhyB, HvPRR73
103.7 HvCMF4
107.3 HvSOC1
114.0 Vrn-H2/HvZCCT-Ha/b/c
5H
0.1 i_11_20894
43.7 HvCO3
44.1 HvTFL1
46.4 HvCMF13
71.7 i_12_31427
75.6 SCRI_RS_212784
83.5 i_11_20850
83.6 i_SCRI_RS_146093
84.4 i_SCRI_RS_172547
85.6 i_SCRI_RS_140054
97.2 HvPRR95
125.7 Vrn-H1, HvPhyC
128.1 HD6-5H
130.4 i_SCRI_RS_157238
6H
16.0 Eam7? SCRI_RS_202723
49.2 HvCMF3
52.6 HvCO7
55.0 HvCO5/HvCry1a/HvCry2
55.3 HvPRR1/HvTOC1/HvCO5
59.1 HvCry1b
67.9 HvCO14
68.2 HvCO2
69.2 HvCO11
7H
13.9 HvWAXY
34.3 Vrn-H3/HvFT1
40.7 SCRI_RS_209176
41.9 HvCO8
43.8 i_12_11455
54.3 HvSS1
61.7 HvVRT-2
65.6 i_SCRI_RS_165986
66.3 i_11_20636
67.7 HvCO12/HvCO13/HvM
67.9 HvCO1
70.5 HvCO15
70.8 HvLHY/HvCCA1
73.4 i_SCRI_RS_206976
91.7 HvCMF7
120.8 HvCO6
126.3 SCRI_RS_182575
Reduced photoperiod sensitivity (ppd-H1)
QTL –log10 (p-value) ≥ FDR and <5
*QTL –log10 (p-value) 5-8
**QTL –log10 (p-value) 8-10
*** QTL –log10 (p-value) > 10
AP
TIP
HD
AE
AP-TIP
TIP-HD
HD-AE

Figure 4-7: Genetically anchored position of highly associated QTLs at all barley developmental stages and sub-phases in the group carrying reduced photoperiod sensitivity (*ppd-H1*) using 9K SNP markers.

Bold and italicized gene names indicate genetically anchored positions of known heading time genes in the Barke x Morex RILs. Associated chromosomal regions are highlighted with different colors according to the stages and sub-phases. Red chromosomal areas indicate the range of significantly associated QTLs (within confidence interval ±5 cM) which are exceeding FDR level of each developmental stage or sub-phase. Highlighted chromosomal regions in light green denote group-specific associations.

4.5 Discussion

The new approach of combining phenotypic dissection of pre-anthesis development with a high-density marker scan provided an unprecedented opportunity to better understand the genetic basis of time to heading in barley, representing a cereal crop species of worldwide importance. Splitting the mapping population based on photoperiod response (*Ppd-H1* and *ppd-H1*) for GWAS analysis revealed a comprehensive network of QTL that can be directly used to refine heading time pathways. Following this approach we were able to detect novel stage- and sub-phase-specific associations, which otherwise would not have been found by simply scoring heading time.

4.5.1 A refined strategy for studying time to heading

It is essential to clearly define pre-anthesis phases and explain the importance of this refined approach for future studies. Previous works defined pre-heading phases in barley as leaf/spikelet initiation phase (from sowing to the onset of jointing) and stem elongation phase (Borràs-Gelonch et al., 2010;Borràs-Gelonch et al., 2011), which most likely lead to an overlap between vegetative and reproductive phases. Alqudah and Schnurbusch (2014) proposed a new approach for studying pre-anthesis spike development, which enables a more precise detection of specific stages and sub-phases. This approach appears advantageous because it precisely defines pre-anthesis phases to developmental intervals, and hence allows the study of genetic factors controlling pre-anthesis stage and sub-phase durations. We anticipate that follow-up work for studying time to heading in cereals will gain value-added information while following this dissection-approach.

4.5.2 The effectiveness of SNP array, population structure and GWAS

Obtained association signals and the power of GWAS were much more informative when the allelic status at *Ppd-H1* was considered for population structure. The results show that SNP array density (9K) and collection size were sufficient to identify highly significant marker-trait associations. We compared previously identified heading time-related associations/QTL

using the present spring barley collection studied with 957 SNPs (Pasam et al., 2012) and results from bi-parental populations (Borràs-Gelonch et al., 2010;Borràs-Gelonch et al., 2011). For heading time, we similarly detected all previously identified QTLs when using the larger SNP array (9K) but most importantly several new QTL underlying pre-anthesis spike development. Compared to previous studies, the power of our GWAS to detect associated loci was increased, likely as a result of better genome coverage and more SNPs. The power of GWAS can be strongly attributed to the *Ppd-H1*-based population structure, which had not been applied before to study natural variation in heading time for barley. This approach provides strong associations, especially when *Ppd-H1* alleles were less active thereby revealing the importance of *HvCO1* as potential candidate gene. Notably, among the 13 most highly associated SNP markers for this locus one *HvCO1*-specific marker (BK_03; **Figure 8 and Table 4-S2**) was detectable, clearly indicating that our GWAS analysis reached a high predictive capability. Moreover, specific gene-derived marker-trait associations were found in two other cases, thereby validating effects seen at *Vrn-H3/HvFT1* and *Ppd-H1* (**Table 4-S2**).

In this study, high broad-sense heritability (**Table 4-2**) and many shared QTL between developmental stages (**Figure 4-5 and Table 4-S3**) confirmed that these stages are genetically and pleiotropically controlled. This is most likely the result of precision-phenotyping under stable GH conditions, using pre-anthesis stages and sub-phases, natural population structure (differences in photoperiod response) and larger number of SNP markers, including their precise physical location. A combination of all of these findings empowered us to reveal new QTL/genes for natural variation in the time to heading for barley.

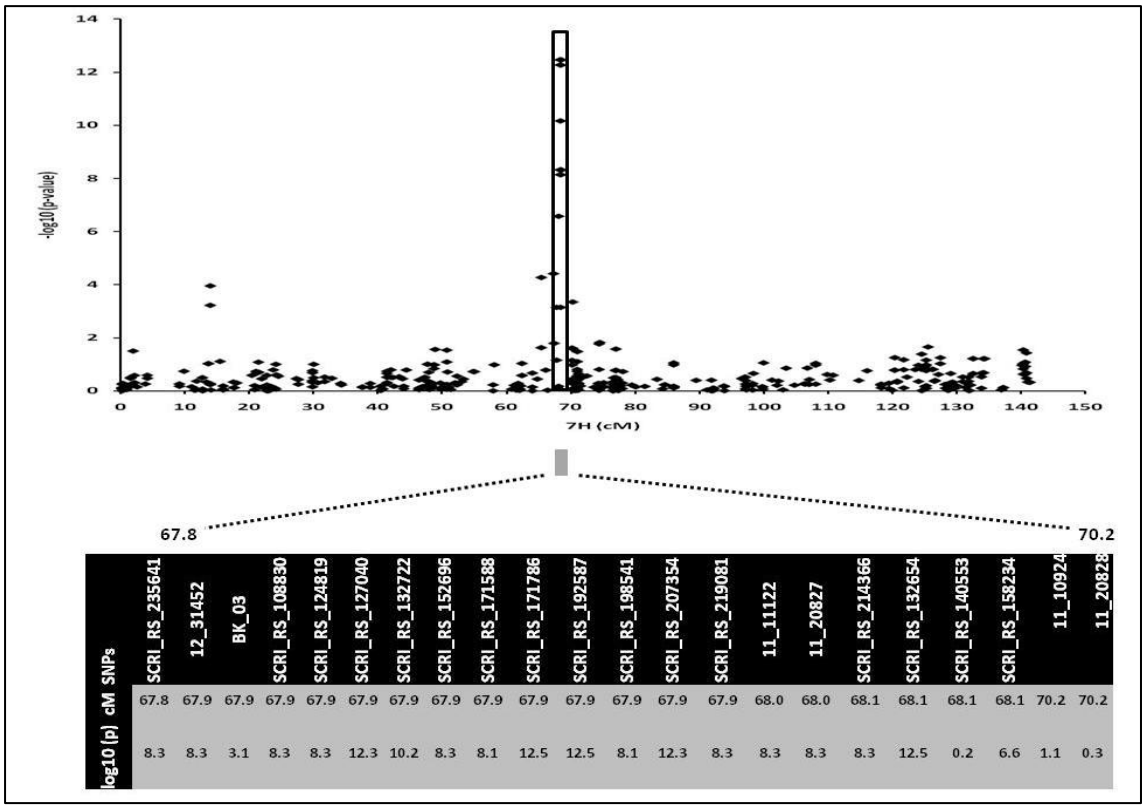

Figure 4-8: Regions of chromosome 7H showing association signals of the candidate gene (*HvCO1*) at the heading stage.

The top of the panel shows the region of the SNP marker peak ($-\log_{10}$ (*p-value*)). The lower panel zooms into a narrow region for the candidate region with the position and $-\log_{10}$ (*p-value*) of highly associated markers which are co-located with *HvCO1* (67.9 cM).

4.5.3 Heading time genetic network models

Our GWAS analysis revealed several genetically anchored genes being involved in the regulation of heading time. Based upon the detected candidate regions in combination with previous knowledge on these genes we developed genetic network models for the two photoperiod groups (**Figure 4-9**), including genes which play a role in both groups (center panel of **Figure 4-9**).

Previous studies clearly established that *Ppd-H2* induces *Vrn-H1* under LD conditions, which in turn up-regulates *HvFT1* expression thus promoting heading ((Casao et al., 2011a); **Figure 4-9**). Natural variation around these three loci consistently showed phenotypic effects in our world-wide spring barley collection regardless of photoperiod status but with *Ppd-H2* as the most important locus. Almost all developmental stages and sub-phases were significantly associated with the *Ppd-H2* chromosomal region within the *Ppd-H1* group, clearly indicating

that this locus is important in photoperiod-responsive and vernalized spring barleys under LD. It was shown that the earliness effect of *Ppd-H2* is repressed through *Vrn-H2* expression under LD (Casao et al., 2011a). However, we were not able to find significant associations at *Vrn-H2*, suggesting that this locus is either deleted, or present at very low frequency in our collection. Furthermore, significant associations in the *ppd-H1* group for TIP and AP-TIP fits well to the notion that *Ppd-H2* primarily affects early developmental stages or phase transition (Casao et al., 2011b) and has less relevance during later stages.

The spring allele at *Vrn-H1* is known to inhibit *Vrn-H2* and was associated with earlier heading (Hemming et al., 2008;Casao et al., 2011a) which is consistent with *Vrn-H1* promoter-GFP expression analysis (Alonso-Peral et al., 2011). Interestingly, we used vernalized spring barley but still observed significant associations at *VrnH1* in the *Ppd-H1* group, which may suggest rich allelic variation at *Vrn-H1* or/and *HvPhyC* in this group. It is known that *Vrn-H1* expression can vary due to deletions within its first intron (Fu et al., 2005;Cockram et al., 2007;Szucs et al., 2007;Oliver et al., 2013), suggesting that quantitative differences in phase duration could be related to differences in *Vrn-H1* transcript levels; however, further work is still required to ascertain the allelic effects of the *Vrn-H1, HvPhyC* locus. No allelic variation within the *ppd-H1*-carrying group occurs at *Vrn-H1, HvPhyC* rather suggesting selection for late-heading time alleles within this group. Whether *Vrn-H1* also promotes heading through induction of *CO*-like gene family members is yet unknown (**Figure 4-9**; arrow with "?"). Allelic variation around *BFL* appears as the first report for temperate cereals that this important phase-transition-gene is relevant in both photoperiod groups. Postulating a similar role for *BFL* in rice (Rao et al., 2008), heading time control is very likely promoted particularly in the earlier heading and *Ppd-H1* group through a combination of allelic variation found at *HvSOC1* (Rao et al., 2008), **Figure 4-9**). However, we did not find any associations for *HvSOC1* within the *ppd-H1* group; this might be explainable through the fixation of only one allele-type. In rice, higher transcript levels of *RFL* had been causal for

early flowering (Rao et al., 2008). Later heading time of the *ppd-H1* accessions suggests that *BFL* transcript levels may be lowered in this group, thereby resulting in an extended AP-TIP period. Thus, *BFL* appears to be an important gene affecting late sub-phase duration, especially in *ppd-H1*. Significant SNPs around *HvCEN* also became detectable in both groups, indicating that the heading time delaying effect of this gene (*Ppd-H1*: +60 GDD; *ppd-H1*: +138 GDD) has been manifested in our spring barely collection. However, Comadran et al. (2012) reported that natural protein variation at *HvCEN* is low and only revealed two functional haplotypes in cultivated barleys (i.e. p.Pro153Ala substitution). Assuming a very similar haplotype structure at *HvCEN* in our spring barley collection may be sufficient to explain obtained results. However, we cannot completely rule out the possibility that the observed effect has been derived from another physically linked unknown gene, which is in linkage disequilibrium with *HvCEN* in the large centromeric region of chromosome 2H (Comadran et al., 2012).

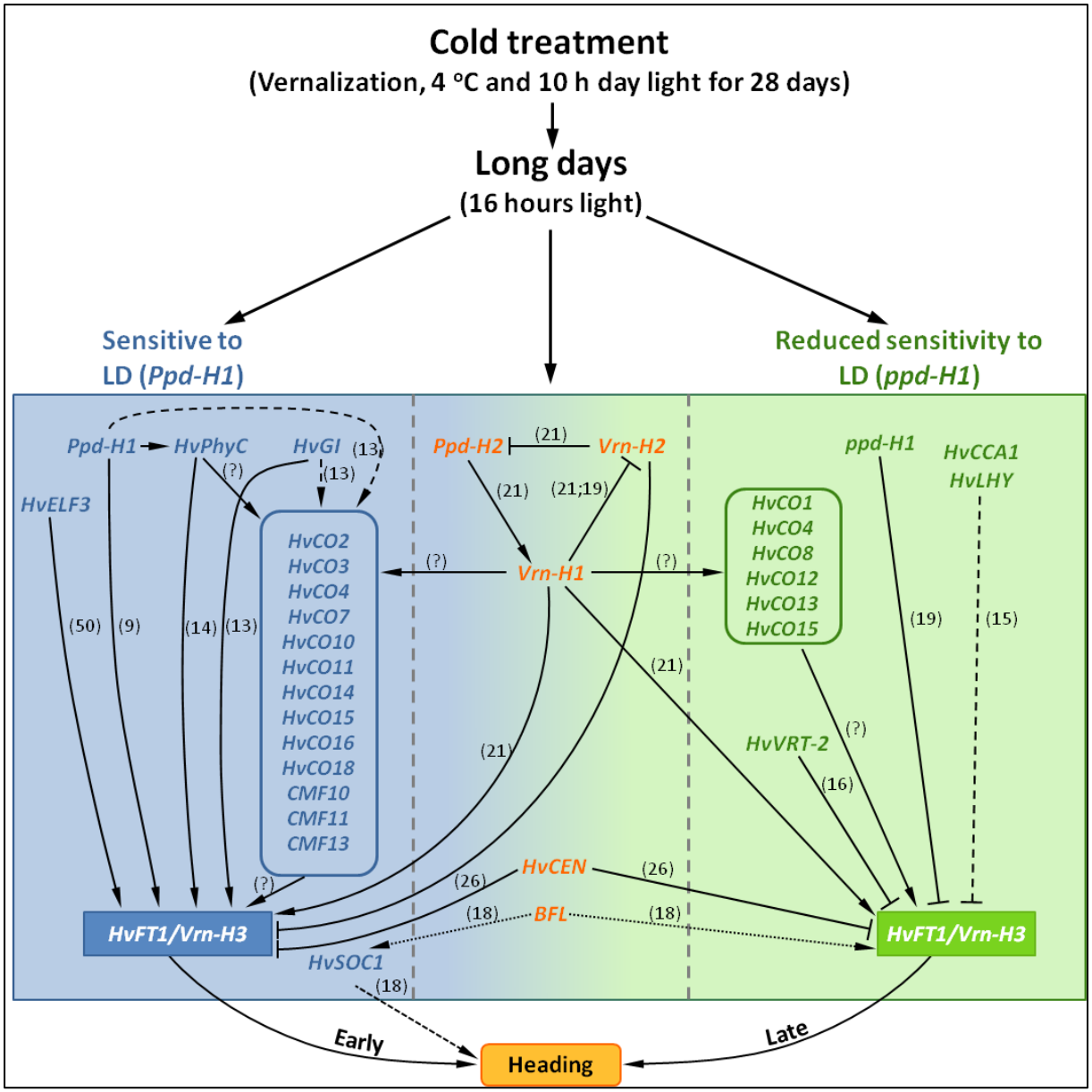

Figure 4-9: Model of heading-time regulation in both photoperiod groups (*Ppd-H1: ppd-H1*) under long day (LD) condition.

Arrow heads indicate promotion of heading; whereas flat arrow heads indicate delay of heading. Genes with known roles in the regulation of heading time in barley are shown by continuous lines. Known interaction from Arabidopsis is shown in dashed lines. Known interaction from rice is shown in round dotted lines. Ambiguous interaction is indicated by a question mark. Numbers in parenthesis show the reference to published interaction.

Barley plants carrying *Ppd-H1* alleles respond to LD conditions through up-regulation of *HvFT1* resulting in early heading (Turner et al., 2005). For this group we found significant association around the *Ppd-H1* locus (i.e. the *HvPRR37* gene) affecting all stages and sub-phases. This finding can mainly be explained through the presence of natural variants around *Ppd-H1* in our collection (Sharma *et al. in preparation*). Similarly, natural variation at *HvFT1*

consistently accounted for heading time effects in this group, further corroborating previous findings that natural copy number variation at *HvFT1* contributes towards accelerated heading time in barley (Nitcher et al., 2013). Overall, we found that the *Ppd-H1* group exhibited a complex genetic constitution for pre-anthesis spike development and final time to heading. One reason for the high degree of complexity in this group is the involvement of 11 genes belonging to the *CO*-like family, which co-located with at least one significant association in our analysis (**Figure 4-9**). *CO*-like genes usually regulate heading time by photoperiod in barley (Griffiths et al., 2003;Cockram et al., 2012). However, in the past it was not possible to specifically assign *CO*-family members to particular photoperiod groups, or to estimate their possible role in a stage or sub-phase dependent manner (Andres and Coupland, 2012). Following our strategy of genetically dissecting pre-anthesis development it becomes possible to specifically relate allelic variation around *CO*-like family members to specific photoperiod groups and developmental stages (**Figure 4-9**). One important example among *Ppd-H1* accessions is the *CO*-like gene cluster on 6H (*HvCO2, CO11, CO14*; 67.9-69.3 cM) which mainly affects phase durations after AP stage. More detailed SNP analyses suggest that the effect is more closely related to the *HvCO2-HvCO11* region; however, future functional work on individual genes is required to show whether one or more genes in this cluster contribute to variation in pre-anthesis development and time to heading. Similar conclusions can be drawn from gene clusters on chromosome 4H, which besides *HvCO16* also includes *HvPRR59*, *HvPhyB* and *HvPRR73* (see 4H: 51.1-51.4 cM), 5HS (including *HvCO3*, *HvTFL1*, *HvCMF13*; 43.7-51.6 cM) and 1HS (*HvCMF10*; 41.1-48.2 cM). Most interestingly, associations found for all of these regions exclusively occurred in the *Ppd-H1* group, indicating that these chromosomal regions harboring *CO*-like and *CMF* genes may play a major role in adaption for early heading time in barley. Another group-specific effect was found for SNPs around the *FT*-like homolog *HvFT2* and the circadian clock-related gene *HvGI* (3HS: 45.8-52.0 cM) both promoting time to heading. *HvGI* was detected with minor effect, supporting the notion by

Dunford et al. (2005b) that *HvGI* does not provide a major source of adaptive variation in photoperiod response. Nevertheless, we assume that *HvGI* may have an important role in this group by promoting pre-anthesis spike development through the conserved *GI-CO-FT* pathway (Higgins et al., 2010).

Taken together, these results suggest a central role of *HvCO-like* genes in the early heading time pathway under LD in photoperiod responsive spring barley accessions. We noted that some of the associations identified in this group are of minor effect with *P*-values just passing significant thresholds, which are more likely affected by variation in response to LD, gene interaction and/or geographical origin effect. Compared to previous studies, controlled GH conditions in conjunction with the novel pre-anthesis dissection approach and high-resolution physical map information draws a much more refined picture of natural adaption to photoperiod in barley.

Less active alleles of *HvPRR37* (i.e. *ppd-H1*) delay heading time under LD by reduced apex and spike development (Hemming et al., 2008), at which *ppd-H1* alleles mainly alter expression levels of *HvCO1* as a result of changed circadian timing (Turner et al., 2005). In this study, the highly significant effect of *HvCO1* on pre-anthesis spike development and time to heading was markedly associated only within the *ppd-H1* group. Although its genetic position strongly suggests that the strongest associations reside very close to *HvCO1* (supported by 13 SNP markers; see **Figure 8**), we cannot completely rule out that linkage with other *CO* genes in this region like *HvCO12*, *HvCO13/HvM* and *HvCO15* (**Figure 4-7**) contribute to this effect. Natural variation around this gene possibly provides the genetic basis for a large portion of the observed differences in pre-anthesis development within this late-heading group (*ppd-H1*). Following conclusions by Turner *et al.* (Turner et al., 2005), it can be anticipated that early-heading in this group is most likely coupled with higher *HvCO1* transcript abundance, thus promoting heading. To verify this hypothesis future work is needed to test structural and functional differences at this locus. Among other highly significant

group-specific regions the association for promoting heading on 6HS (16.0 cM) stood out. Previous work identified that the *eam7* mutant phenotype had been linked to a region on 6HS, now establishing this position as a candidate for this mutant (Stracke and Borrner, 1998). Moreover, we found three important chromosomal regions on 2H (107.3-112.0 cM), 3HL (122.6-126.7 cM) and 5HL (83.5-85.6 cM), which showed group-specific associations but did not co-locate with known candidate genes. The 3HL region is approximately 30 cM proximal to the known circadian clock-related *HvLUX* gene (Gawronski et al., 2014). More future work is required to validate this association.

The number of fewer but more significant associations identified in this group (*ppd-H1*) compared to (*Ppd-H1*) could be due to the fixation of heading time alleles or due to the larger number of accessions used in this group (123) and/or longer duration of developmental stages. Only highly significant association around *Ppd-H2*, *HvCO1* and *BFL* genes promote heading within this group. This suggests that within this group the genetic architecture of heading time is less complex and that three genes play a major role on heading time compared to several genes in the *Ppd-H1* group. Another argument could be that within the *ppd-H1*-group *Ppd-H2* might have taken over the effect of *PpdH-1*, and *HvCO1* substitutes the *CO*-family gene effect of other *CO* genes.

In summary, around 75% of the *ppd-H1* accessions in this study originate from EU. EU spring barley accessions mainly carry *ppd-H1* alleles (Turner et al., 2005), thus leading to elongated post-AP phase durations due to the reduced response to LD. The collection used in this study, however, includes world-wide spring barley accessions which possess different genetic backgrounds, and hence, can easily explain the detected natural variation of heading time within this group. These results reinforce the importance of *CO*-like genes, specifically *HvCO1*, in the late-heading time pathway (*ppd-H1*) under LD.

Functional validation of candidate associations found in this work will help to complete our knowledge about developmental stages/sub-phase and/or heading time genetic networks in the

future. Studying pre-anthesis development under GH and/or field conditions will greatly aid in the detection of causal variants in small grain cereals.

4.6 Acknowledgements

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5.0 CHAPTER FIVE: General Discussion

Pre-anthesis developmental phases are considered as one of the most important phases for yield potential. The most important processes for growth and development, such as spikelet/floret formation and their survival occur during this period. Apart from yield potential, the pre-anthesis development is also a key period for determining the plant architecture because tillers and leaves also show the maximal growth rate during this period. Hence, understanding the phenotypic variation and the genetic basis of phase durations and developmental events during pre-anthesis development is essential for breeding programs in terms of yield potential and/or adaptation. Because there are no ample evidences for variation in pre-anthesis phase durations, its underlying genetic control and the implication in yield-related traits, we studied pre-anthesis development comprehensively in this dissertation. Using GWAS for identification QTL underlying natural variation of phase durations has provided unprecedented value. This dissertation presents a framework for in-depth understanding of how these phases are related to agronomic traits.

5.1 Genetic control of the studied traits

Heritability is the degree to which a trait is genetically determined and calculated as the ratio of the genetic variances to the total phenotypic variance. This feature is important for stability or homeostatic parameters, i.e. individuals respond stable in response to environmental fluctuations (Waddington, 1942;Lacaze et al., 2009). High heritability for most of the analyzed traits in chapter 2 and 3, such as spikelet survival, yield- and leaf-related traits means that these traits are predominantly controlled by genetics under different environmental conditions. In another words, these traits are stable under GH and field condition which is important to introduce these traits into breeding programs. Yield stability under a wide range of conditions is the major feature of a landrace attributed to the heterogeneity or dynamic stability (Newton et al., 2010) and is characteristic for cultivar/breeding lines (Zeven, 1998), thus several breeding strategies have been attempted to improve high yield stability across

diverse environments through combining these features. High genetic variance in a diverse population as detected in the study (chapter 2 and 3) could be attributed to the allelic diversity of the genes controlling trait expression across environments. The same trend of heritability values were detected for phase duration in whole population under GH conditions where we found high genetic variation at most of heading time genes. The analysis of genetic variation at some important genes for interesting stable traits using association analysis is necessary to detect causative SNP(s) of phenotypic stability under different environmental conditions in whole diverse collection (218 accessions) or in a specific geographical region.

It is also plausible that some accessions show different phenotypes in response to environments (Price et al. (2003). Examples about high phenotypic variation in response to environmental changes were observed in LMA which is considered as one of the key traits for investigation of adaptive phenotypic across conditions (Poorter et al., 2009;Nicotra et al., 2010). Since we have a diverse population, the variation in phenotypes could be related to the existence of different levels of sensitive alleles which lead to the variation in response to environments (different expression levels of genes across environments). To verify this, future work is needed to investigate allelic diversity and expression profiles in our population under different environments.

Understanding interactions between genotype × environment is essential for detecting QTL underlying phenotypic variation. Lacaze et al. (2009) detected QTLs related to phenotypic variation which are co-located with genotype × environment interaction QTL. Interestingly, environmental conditions used in this study (chapter 2 and 3) suggested that detecting genotype × environment interaction QTL in some traits, such as LMA and tiller number, is important to reveal the causal SNP(s)/allele(s) of natural phenotypic variation by using large populations and under multiple environments in GWAS.

5.2 The importance of AP-TIP phase in vital processes

The beginning of this phase (i.e. AP stage) is highly important for yield because of the maximum spikelet number per spike that can be counted at this stage. The relationship between spikelet number and other plant organs is critical to decide how many spikelets can develop. For example, based on our data we found that SE phase started 5 days before AP stage, suggesting that the onset of SE is critical for spikelet development, and any spikelet initiated after this stage (i.e. SE onset) may not develop into a grain. The number of spikelet per spike at AP stage is also important; our observations allow us to suggest a competition among florets/spikelets within one spike i.e. more number of florets/spikelets per spike means more competition among them (e.g. two- vs. six-rowed spikes). Generally, low spikelet survival in six-rowed barley was most likely attributed to competition among spikelets within the same spike (Appleyard et al., 1982), which is also dependent on spikelet position and structure (Arisnabarreta and Miralles, 2006). Wheat spikelet possesses 8-10 floret primordia and only 2-5 survive probably as a result of competition among florets and/or spikelets (Kirby, 1988). However, there are differences between wheat and barley spike/spikelet architecture (one wheat spikelet consists of several florets, while one barley spikelet possesses one floret); one can assume that the competition among wheat spikelet/florets is more severe (Guo and Schnurbusch, unpublished data). However, the genetic factors controlling spikelet/floret development and survival are still unknown and more genetic evidences are required. The GWAS analysis for number of spikelet per spike at AP with phase duration, stem length, tiller number and LA might allow us to detect gene(s) which are co-localized with known gene(s) related to these traits. For example, it may be that some heading time genes have an important role in spikelet/floret development and survival. Moreover, the natural variation analysis at these genes could also explain the allelic diversity which can elucidate the differences between sub-population (e.g. row-type classes) and geographical regions in spikelet/floret development and survival.

One of the main results of this dissertation is the identification of the AP-TIP phase as the most vital phase in barley life cycle determining final yield and plant architecture. Leaves, tillers and spikelets are initiated prior to AP-TIP phase and we found that these grow rapidly during AP-TIP phase, which then might result in trade-off and/or competing relationship among them. We observed that two-third of spikelets were aborted during this phase, whereas most of SE and leaf development were synchronized with spikelet abortion. Several studies suggested that spikelet abortion is based on competition for resources. For instance, floret death in wheat was attributed to competition between floret development and SE for limited resources (Brooking and Kirby, 1981;Kirby, 1988) and the same reason may be applicable for barley as well. The data obtained for dry-matter and relative growth rate in the present study showed that AP-TIP phase is an important time period for dry-matter accumulation in the plant, with most of the accumulation in stem and leaves, and low levels in spike. This is in agreement with results from Kirby (1988) showing a two-fold higher growth rate for leaf and tillers compared to spikes during the reproductive phase (terminal spikelet to anthesis) in wheat. Most of the spikelet abortion occurred at the duration of highest growth rate (AP-TIP) which might be due to the competition for limited resources. It seems that spikelet/floret is a vulnerable part and cannot compete with other plant parts because it is only a sink tissue while leaf is a sink and the major source of photosynthesis. This empowers us to propose that more studies about the interactions of plant parts are essential to understand the source-sink network (Sadras and Denison, 2009). GWAS analysis for stem-, leaf- and spike-relative growth rate/dry-matter accumulation in whole population could provide genetic framework of QTL that controlling these traits. Thereafter, we would able to detect novel stage- and sub-phase-specific associations, which are important for future breeding program. Detection of shared associations between these traits with phase duration is also important for improving yield. Allelic diversity at genes regulating these traits (i.e. relative growth rate/dry-matter accumulation) based on row-type classes and/or phase duration could explain the adaptation

strategy to environments in barley. We assume that the early heading accessions (mostly from Middle East) are fast in growth, thereafter accumulate small amount of dry-matter to avoid hot-dry summer. While the EU accessions are more relaxed in growth and they accumulate more dry-matter then yield.

AP-TIP consists of white-, green- and yellow-anther stages (Kirby and Appleyard, 1987). In the present study, we did not dissect the spikes at these stages due to workload in dissection spike at stages of growth and development phase. Nevertheless, dissecting spikes at these stages during AP-TIP phase is crucial to understand the process of spikelet/floret growth, development and abortion; and therefore, genetic, physiological and biochemical analysis would also provide important results for understanding spikelet/floret abortion mechanisms. In addition, association analysis of these stages in the diverse spring barley collection will provide great genetic background to get better understanding of the underlying genetic factors.

The competition among plant organs such as stem and spike has been well studied in crop physiology and plant breeding points of view (see the review by Sadras and Denison (2009). The competition between spike and stem might be caused by the synchronization of the stem elongation with spike growth and development (Borras et al., 2009). Related to the whole plant, the spike from the main culm develops more spikelets than other spikes probably due to the competition among succeeding tillers. Lowering the competition of different plant organs for resources by extending the length of the critical developmental phase (AP-TIP) may lead to equal growth rates and may be a promising strategy for improving yields via enhancing spikelet survival thereafter grain yield.

The genetics of the competing structures and how the manipulation of the competition network components can improve yield are rarely studied (Sadras and Denison, 2009). For example, improving grain yield in cereals during last decades was attributed to plant height genes, which may play a role in decreasing the competition among spike and stem growth

rates. For instance in wheat, dwarfing gene (*REDUCE HEIGHT*, *Rht*) is positively associated with grain number by decreasing spike: stem (leaves and tillers) competition (Miralles et al., 1998), which leads to increased floret survival (Slafer et al., 2001). In barley, semi-dwarfing genes, including *SEMI-DWARF 1* (*sdw1* or *denso*), were more useful than dwarfing genes in breeding programs to improve agronomical traits (Haahr and Wettstein, 1976;Wang et al., 2014). Decreasing barley plant height was the main strategy for improving grain yield and HI by reducing lodging (Bezant et al., 1996), e.g. by using *sdw1*/*denso* in EU and EA (Hellewell et al., 2000). Recently, Wang et al. (2014) reported that a new plant height QTL on 7H showed positively associated effects on agronomical traits and grain yield. Semi-dwarfing genes are frequently found in barley landraces and/or cultivars (Mickelson and Rasmusson, 1994), thus it is worthwhile to study natural diversity of plant height genes in worldwide barley collection which includes cultivars, landraces and breeder's lines. This proposed study is important to find out the allelic diversity and their effects on heading date, grain yield and spikelet survival.

5.3 The importance of phytohormones in spikelet development

Since long time, the importance of hormones and metabolites were hypothesized to be involved in spikelet/floret development. Most of previous hormonal studies have been focused on relation of changes of endogenous hormone and grain development after anthesis (Wang et al., 1999). The effect of hormones (endo- and exogenous) on spikelet/floret development have been studied on small scale, for example, the effects of exogenous hormones (injected into the leaf sheath around the young spike) on wheat floret development and grain set (Wang *et al.* 2001). Wheat floret development promoted by injected cytokinins which in turn increased fertile florets number thereby grain set while injected 3-indole acetic acid (IAA), gibberellic acid (GA3) and abscisic acid (ABA) inhibited floret development through different pathways (Wang et al., 2001). This study demonstrated that injected IAA and ABA at most of developmental stages inhibited the development of florets in the spikelet

whereas injected GA3 at terminal spikelet stage lead to increase the fertile florets number but decreased grain set (Wang et al., 2001). Applying 6-benzylaminopurine 6-BA to wheat enhanced the floret development and grain setting at different position of spikelet (Zheng et al., 2014). In rice, gibberellic acid and kinetin (6-furfuryl amino purine) improved spikelet growth, development and grain yield on all branches, while IAA was identified to be important in the distal branches (Patel and Mohapatra, 1992).

Regarding to endogenous hormones, (Wang et al., 1999) found that the changes of endogenous hormone levels is affected by developmental stages (i.e. stage-dependent) during wheat floret development. Decreases the level of ABA and GA1 in spike at anther development phase may be the reason to maximize fertile florets and improvement of grain set in wheat (Cao et al., 2000). Auxin was shown to play a fundamental role in developing axillary meristems in maize; whereas applying auxin transport inhibitors lead to the formation of single instead of paired spikelets (Wu and McSteen, 2007). The membrane localized *PIN-FORMED* (*PIN*) proteins belong to the class of auxin transport proteins, expressed in different part of spikelet tissue in *Brachypodium distachyon*, e.g., highly expressed *Sister-of-PIN1* (*SoPIN1*) in the *Brachypodium* spikelet epidermis suggests that *BdSoPIN1* has functions in the localization of new primordia (O'Connor et al., 2014).

The importance of sugar as the initial regulator of apical dominance rather than auxin was described by Mason et al. (2014). These findings enable us to ask whether sugar and auxin have crucial roles in barley's spikelet development/survival (central vs lateral; top vs bottom vs middle). Injected cytokinins increased the soluble sugar and sucrose concentrations of the spike at anthesis which may be promoted the development of florets in the spikelet (Wang et al., 2001). Comprehensive studies of hormone and sugar analysis during critical phase (AP-TIP) will be essential to understand the spikelet growth, developmental and abortion pathways.

5.4 Natural diversity

Natural diversity in traits, which are frequently used by breeders, is an important source of genetic material that can be used directly to improve adaptation and yield performance. Interestingly, the germplasm used in our study could be divided into sub-populations based upon row-type classes (two-rowed, *Vrs1* vs. six-rowed, *vrs1*) and on photoperiod response (photoperiod-sensitive, *Ppd-H1* vs. reduced photoperiod sensitivity, *ppd-H1*), which illustrates the natural diversity among these accessions in the decisive traits. In terms of row-type classes, the fundamental differences between two- vs. six-rowed were observed in 32 diverse accessions for yield-related traits, LA and leaf growth-related traits. Two-rowed accession had more yield, small LA and more LMA compared to six-rowed, which means *Vrs1* gene could have a role in these traits. In other words, two-rowed (*Vrs1*) barley has an advanced adaptation in response to environments through producing small and thicker leaf then more yields. This speculation could be explained in terms of geographical regions, for example, most of EU accessions are two-rowed barley, which produce more yield using the above strategy (i.e. small and thicker leaf). This example about how we can use our fundamental results in further genetic diversity analysis such as allelic diversity using 218 worldwide accessions. Even though, the two-rowed accessions are less diverse than six-rowed accessions in the population which we used (Pasam et al., 2012), studying the genetic variation of important traits in this population using GWAS is worthwhile to detect the important SNP(s)/allele(s). Moreover, geographical origins of the accessions could also be used to explain the genetic diversity based upon their dispersal.

The importance of heading time in adaptation and yield determination is well studied and documented in the last decades. The *Ppd-H1* gene is the key regulator of barley heading time (Turner et al., 2005) and GWAS analyses in our study based on the *Ppd-H1* alleles showed many significant associations. GWAS results expressed how much *Ppd-H1* gene is important in adaptation, which can help breeders to improve spring barley adaptation and grain yield traits. Among pre-anthesis sub-phases, we found the AP-TIP phase has a major impact on

genetic variation for heading time and spikelet survival. The observations reported in chapter 2, 3 and 4, raised questions about the importance of variation in this sub-phase on some important traits in spring barley. We found that AP-TIP phase is longer in EU accessions compared to other geographical regions, suggesting that spring barley developed sophisticated ways to adapt specific environments and the adaptation was caused by human selection. We assume that the EU accessions (carrying *ppd-H1*) produce more grain yield because of longer duration of critical phase AP-TIP which leads to reduced competition therefore high spikelet survival. Taken together LA with relative growth rate, two-rowed barley in Europe (*Vrs1/ppd-H1*) has small LA, more accumulated dry-matter and long phase duration thus more spikelet survival and grain yield. In case of six-rowed accessions from Middle East (*Ppd-H1/vrs1*) they have large LA, fast growth, low accumulated dry-matter, short phase duration therefore low spikelet survival and grain yield. However, the farmer in Middle East still using six-rowed because it can produce more grain and straw yield with short duration i.e escape early drought stress (Samarah et al., 2009). Sharma et al. (in preparation) found that selection response to LD conditions at *Ppd-H1* happened after barley domestication, indicating that the changes in response to LD was more efficient in EU accessions, which lead to delaying heading time. The results support the assumption of Turner et al. (2005), that most of EU accessions had longer duration prior to heading and higher yield as a result of an attenuated response to LD conditions (*ppd-H1*). Andres and Coupland (2012) proposed that the spring barley accessions originated from Middle East owing *Ppd-H1* alleles are fast in heading time as a strategy of adaptation to environment and consequently, display a reduction in grain yield. The genetic evidence of natural variation in *Ppd-H1* alleles and their impact on grain yield related-traits using GWAS in worldwide population is important to elucidate the genetic diversity i.e. whether there are specific geographical alleles or not. The hypothesis of allelic diversity at important genes (e.g *Vrs1* and *Ppd-H1*) could be applied for other important traits such as LA and tiller number between photoperiod groups, row-type classes and/or among

geographical regions. The studies of natural diversity in important genes may clarify the roles of these genes in evolutionary studies, which may help in breeding programs that are seeking to modify agronomical important traits in cereals.

The natural diversity studies over the past years have increased the knowledge of understanding barley domestication through provision of advanced molecular genetics analyses of wild and cultivated barley at the genomic level. Three key barley domestication traits (row-type, hulled grain and brittle rachis) were fine mapped or cloned, such as row-type *Vrs1* was cloned by Komatsuda et al. (2007). However, discovering the significant difference between wild and cultivated barley in major heading time gene *Ppd-H1* and introducing it as a domestication gene (Sharma et al. in preparation), prompted us to ask whether other heading time genes play a role in domestication. The association analysis and proposed genetic network model based on photoperiod response (*Ppd-H1* / *ppd-H1*) in this study (chapter 4), suggested that some genes like *HvCO1* seems to be domestication related because it appeared strongly in *ppd-H1* accessions, which are mostly originated from EU; whereas it is less detectable in *Ppd-H1* accessions. Thus, re-sequencing of this gene in worldwide collections of cultivated and wild barleys is crucial to validate this hypothesis.

5.5 The features of this study

This study has several specific features which could explain why we achieved these results. Evaluating the traits in representative population under different growing conditions (chapter 2 and 3) provide great information regarding to genetic background and the association between the growing conditions. For advanced genetic study on the whole population, GH condition was used based on strong associations between growing conditions in quantitative traits. We propose this strategy to study the quantitative traits such as phase durations and spikelet survival, but the field condition is indispensable for further evaluation. The phenotypic dissection approach used in this study is an additional advantage to evaluate quantitative traits which revealed the importance of AP-TIP phase in vital processes and

provide high resolution genetic map. The data collection strategy showed an enormous importance of single plant approach to study quantitative traits. This approach enables the plants to maximize their phenotypic/genetic performance which is important to detect the responsible gene. By eliminating the plant-plant competition factor (which restrict plants performance) and reducing the variation between plants, led to increased heritability values. Breeders should consider this approach in evaluation and selection process (see the review by Fasoula and Fasoula (1997)). Based on our observations in chapter 2, 3 and 4, it's clear that the population used is highly diverse and provide great natural diversity results. Using this population (218 accessions) to study quantitative traits such as spikelet survival and leaf-related traits is important to understand the allelic diversity in these traits.

6.0 Outlook

The results of the dissertation demonstrate that pre-anthesis developmental phases have substantial value in improving grain yield, crop stature and adaptation to environmental conditions. The experimental design for evaluation and dissection of worldwide spring barley collection was highly beneficial to detect QTL for interesting traits by using high throughput SNP markers in GWAS. The developments in next-generation sequencing technology enable us to increase map resolution of QTL underlying candidate genes. The associated markers in regions of important QTL for some traits such as phase duration can be used for further validation by biparental mapping populations and/or doubled-haploid populations. It is worth to use these regions as a basis for marker development, which can be used for map-based cloning of candidate QTL and re-sequencing of these QTL in large populations. Once the candidates underlying QTL are identified they can be further validated by gene silencing approaches, like RNA interference (RNAi), and Transcription Activator-Like Effector Nucleases (TALENs) with preference to use it because of its specificity, ease of assembly and expansive target range (Wendt et al., 2013;Gurushidze et al., 2014).

It would be important to understand the vital mechanisms during AP-TIP phase by dissecting the stages of this phase (White-, green- and yellow-anther). Association analysis using the 9k chip can be applied to find QTL underlying these stages. Intra-plant organ competitional network analysis among spikelet/floret, spike, leaf, tiller and stem elongation seems to be necessary to analyze massive spikelet abortion during this phase. Because there are several studies about the role of hormone and sugar signaling in apical development (Mason et al., 2014;O'Connor et al., 2014) more future work is needed to discover the role of hormones/sugars in spike/spikelet/floret development during AP-TIP phase.

Interestingly, the diverse worldwide spring barley collection is a valuable tool to find new alleles for agronomic and adaptive traits. Re-sequencing of highly associated gene(s) such as *HvCO1* in phase duration in this population might be a way to find the significant alleles.

Thereafter, haplotype analysis and amino-acid changes based on alleles would be a possibility to find the differences between photoperiod group, row-types and/or origin of accessions. RNA expression analysis by quantitative real-time PCR would be important to detect differences in gene expression between these groups and/or origins at different developmental stages. For further genetic diversity analysis, the number of worldwide accessions should be increased by including wild/landrace accessions to validate our hypothesis regarding allelic diversity, haplotype analysis and domestication.

7.0 Summary

The pre-anthesis phase of temperate cereals consists of three major phases (vegetative, early- and late-reproductive) during which distinct biological and developmental processes take place. Among these phases, late-reproductive phase is the most crucial phase for spikelet/floret development and survival. With the help of the proposed phenotyping approach in this study, we identified the most decisive phase for spikelet/floret survival, i.e. awn primordium (AP) to tipping (TIP) stage, where spikelets/florets compete with other organs for limited resources. We demonstrated that the maximum yield potential in barley is at AP stage and six-rowed barley had significantly higher rate of spikelet abortion than two-rowed barley. In spite of these differences, spikelet abortion happened alongside with rapid growth of other organs such as stem elongation (SE) and leaf growth. During this phase (AP-TIP), more than 50% of leaf area and ~75% of SE occurred providing further evidence for the importance in organ growth and development in barley. Moreover, most of the targeted traits (spikelet number and survival, growth stages and leaf traits) were predominantly heritable traits and had an impact on single-plant yield. These findings were documented in different growing conditions and we found that GH conditions are appropriate for studying phenotypic/genetic variation of traits. Based upon these results, we suggest putting more attention on the crucial phase (AP-TIP), which seems to be the key duration for improving yield and has to be considered in future breeding programs.

Genome-wide association studies (GWAS) are a powerful tool for detecting and fine mapping of quantitative trait loci (QTL) underlying complex traits. In the present study, a 9k single nucleotide polymorphism (SNP) marker chip with known genetic marker positions was used in GWAS analyses combined with a population structure based on photoperiod response (*Ppd-H1* and *ppd-H1*) to long-day (LD) in barley. Combined with the phenotyping approach for stages in worldwide spring barley collection (218 accessions) under GH condition, we were able to analyze the natural variation of time to heading. Phenotypically, reduced

photoperiod sensitivity group (*ppd-H1*) had longer stage/sub-phase durations with most of accessions coming from Europe (EU). GWAS analysis of stages/sub-phases provided novel and reliable results. Therefore, we re-confirmed associations and genetic positions of major heading time genes from previous field experiments and we were able to detect several novel QTL belonging to developmental stages/sub-phases. Highly associated SNPs tagged the genetic regions of *CONSTANS*-like genes particularly *HvCO1*, which appeared to be the major regulator of heading time in reduced photoperiod sensitivity accessions (*ppd-H1*, late-heading) under LD. Several newly identified heading time genes were associated to stages/sub-phases in both photoperiod groups, for example *BFL* (*BARLEY FLORICAULA*/*LEAFY*) and *HvTFL1* (*TERMINAL FLOWER 1*). Our GWAS results allow expanding the knowledge about time to heading and provide new insights into the natural variation of pre-anthesis stages/sub-phases. Thus, we propose a new genetic network model in barley which includes highly associated candidate genes based on their response to photoperiod.

8.0 Zusammenfassung

Die Vorblüteentwicklung bei Getreide umfasst drei größere Entwicklungsphasen (vegetative, früh- und spät-reproduktive Entwicklungsphase), in der wichtige entwicklungsbiologische Prozesse ablaufen. Von den o.g. Phasen ist die spät-reproduktive Entwicklungsphase als besonders wichtig einzustufen hinsichtlich Entwicklung und Untergang von Ährchen- und Blütchenanlagen. Unter Zuhilfenahme des verwendeten Phänotypisierungsansatzes konnten wir in der vorliegenden Arbeit die entscheidende Entwicklungsphase für den Untergang bereits angelegter Ährchen bzw. Blütchen bestimmen: nämlich vom Awn Primordium (AP) bis zum Tipping (TIP) Stadium; eine Phase in der Ährchen/Blütchen insbesondere mit anderen Pflanzenorganen um knappe Nährstoffressourcen in Konkurrenz treten. Wir konnten zeigen, dass Gerstenpflanzen zum AP Stadium das maximale Ertragspotential zeigen, und dass sechszeilige Gersten einen signifikant höheren Ährchenabort zeigten als zweizeilige Gersten. Trotz dieses Unterschieds konnte generell festgestellt werden, dass der Ährchenabort i.d.R. zeitgleich mit schnellem Halm- und Blattwachstum einhergeht. Während dieser Phase (d.h. AP-TIP) wurden ca. 50% der Blattfläche und ca. 75% des Halmwachstums erzeugt. Des Weiteren konnte gezeigt werden, dass die relativ hohen Erblichkeiten der meisten Zielmerkmale (d.h. Ährchenanzahl sowie –untergang, Wachstumsstadien und Blattmerkmale) einen messbaren Effekt auf den Einzelpflanzenertrag hatten. Diese Ergebnisse sind unter verschiedenen Umweltbedingungen erzielt worden und zeigten deutlich, dass kontrollierte Gewächshausbedingungen auseichend für die Untersuchung der o.g. Merkmale sein können. Basierend auf dieser Datengrundlage schlagen wir daher vor, dass dieser wichtigen Entwicklungsphase (AP-TIP) der Gerste zukünftig mehr Aufmerksamkeit gezollt werden sollte; v.a. im Hinblick auf Ertragsverbesserungen und in Züchtungsprogrammen.

Genomweite Assoziationsstudien (GWAS) sind hervorragend geeignet zum Nachweis und zur Feinkartierung komplexer Eigenschaften mittels Quantitativer Merkmal-Loci (QTL). In der

vorliegenden GWAS Studie wurde ein 9k Single-Nucleotide-Polymorphism (SNP) Marker-Chip mit bekannten genetischen Markerpositionen in Kombination mit der photoperiodischen Reaktion (*Ppd-H1* und *ppd-H1*) unter Langtag(LD)-Bedingungen der Kulturgerste als Populationsstruktur verwendet. Mit Hilfe eines entwicklungs- und stadien-spezifischen Phänotypisierungsansatzes unter kontrollierten Gewächshausbedingungen in einer weltweiten Sommergersten Kollektion (218 Akzessionen) war es uns möglich die natürliche Variation für das Merkmal „Zeit bis zum Ährenschieben“ zu ermitteln. Akzessionen mit reduzierter Sensitivität gegenüber LD-Bedingungen (*ppd-H1*), die überwiegend aus Europa stammten, benötigten fast immer einen längeren Zeitraum, um Stadien und Entwicklungsphasen zu passieren. Diese entwicklungs- und stadien-spezifische GWAS-Analyse der Vorblüteentwicklung der Sommergerste erzeugte neue und zuverlässige Resultate. Gefundene Assoziationen bestätigten genetische Positionen wichtiger Majorgene für Ährenschieben unter Feldbedingungen sowie ermöglichten die Detektion neuer bisher nicht bekannter QTL für entwicklungs-spezifische Stadien und Phasenlängen. Hochsignifikante Marker-Assoziationen in der Nähe von *CONSTANS*(*CO*)-verwandten Genen wurden gefunden, insbesondere nahe *HvCO1*, welches sich als Majorregulator für Ährenschieben in der *ppd-H1* Gruppe mit verminderter photoperiodischer Reaktion unter LD-Bedingungen zeigte. Mehrere neue Gene für Ährenschieben wurden in beiden photoperiodischen Gruppen identifiziert, wie z.B. *BFL* (*BARLEY FLORICAULA*/*LEAFY*) und *HvTFL1* (*TERMINAL FLOWER 1*). Die GWAS-Resultate erlauben uns somit unser vorhandenes Wissen über die „Zeit zum Ährenschieben“ zu erweitern und ermöglichen daher neue Einblicke in die natürliche Variation dieses Merkmals einschließlich ihrer entwicklungsspezifischen Stadien und Phasenlängen. Schließlich präsentieren wir ein neues genetisches Netzwerk-Model für die „Zeit zum Ährenschieben“ in Sommergerste basierend auf signifikanten Assoziationen in beiden photoperiodischen Gruppen (*Ppd-H1* und *ppd-H1*).

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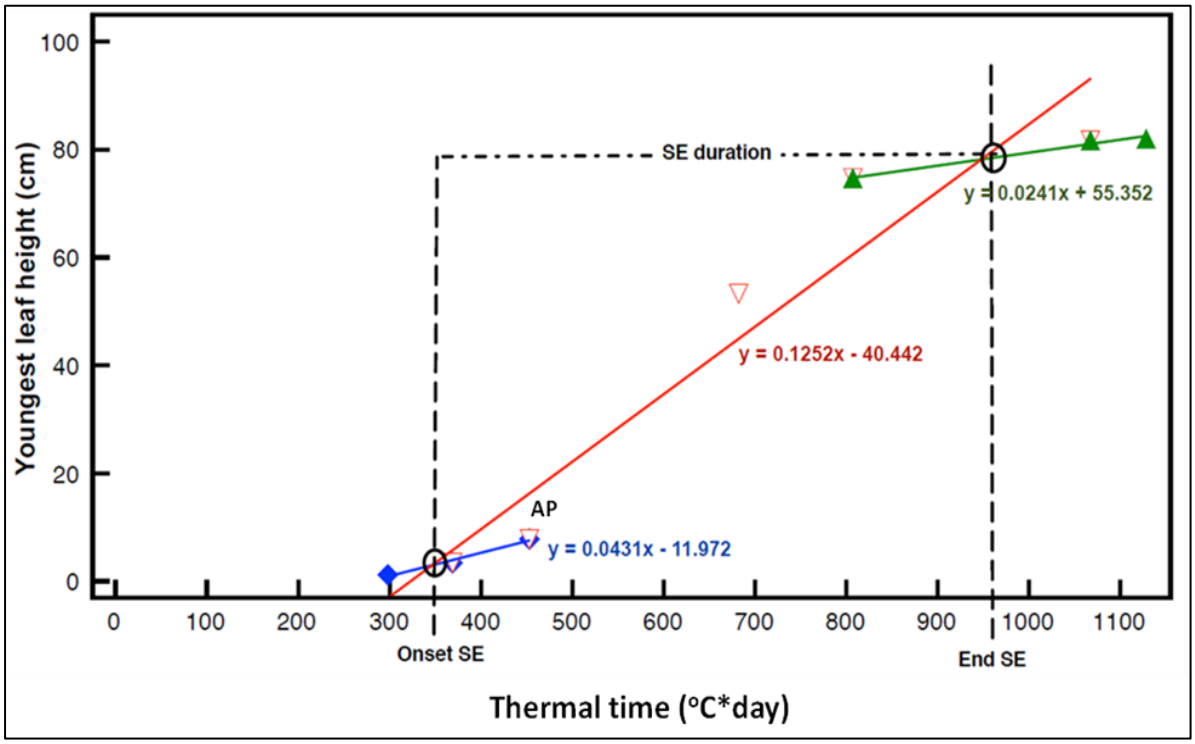
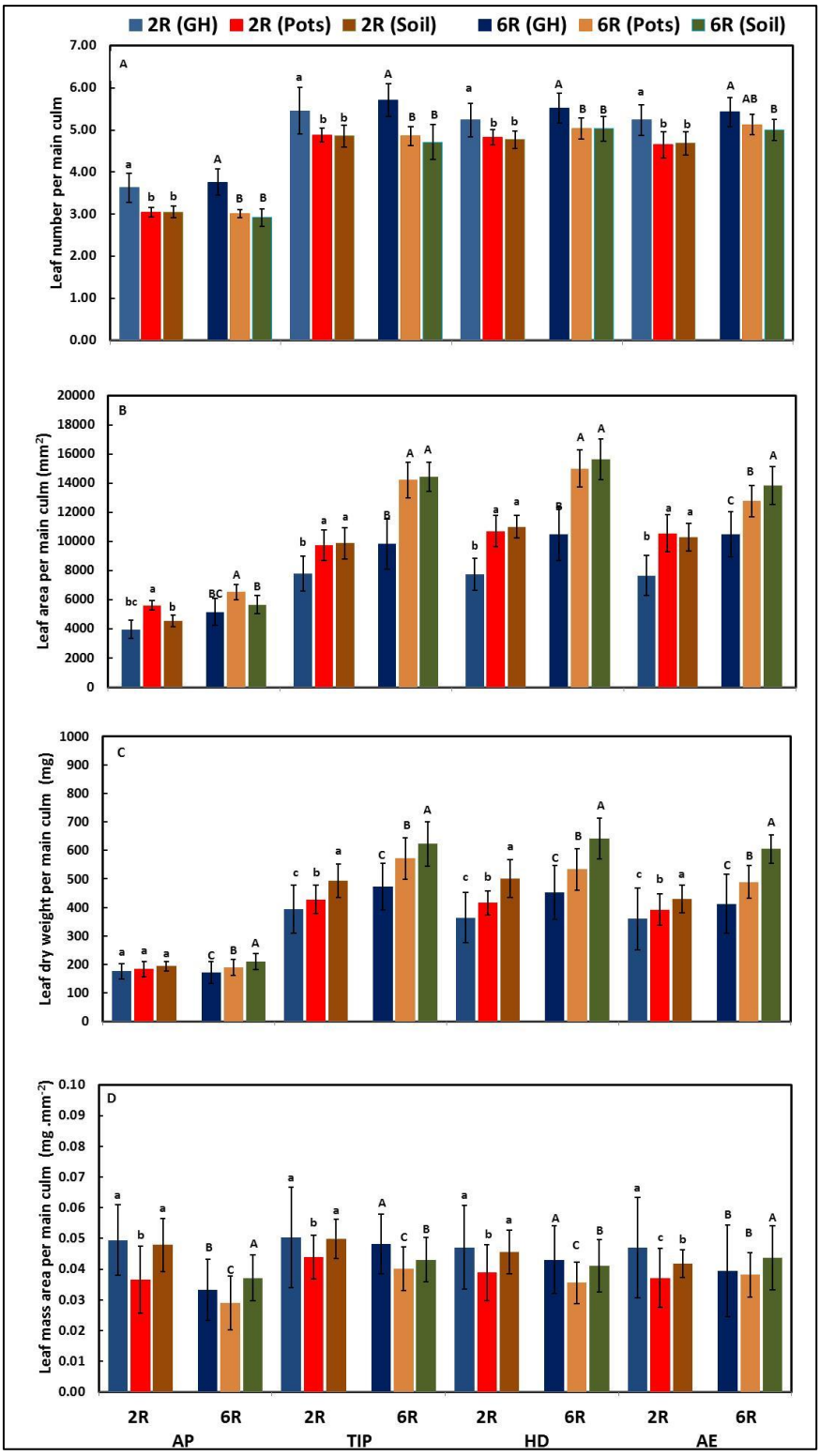
10.0 Supplementary Figures:

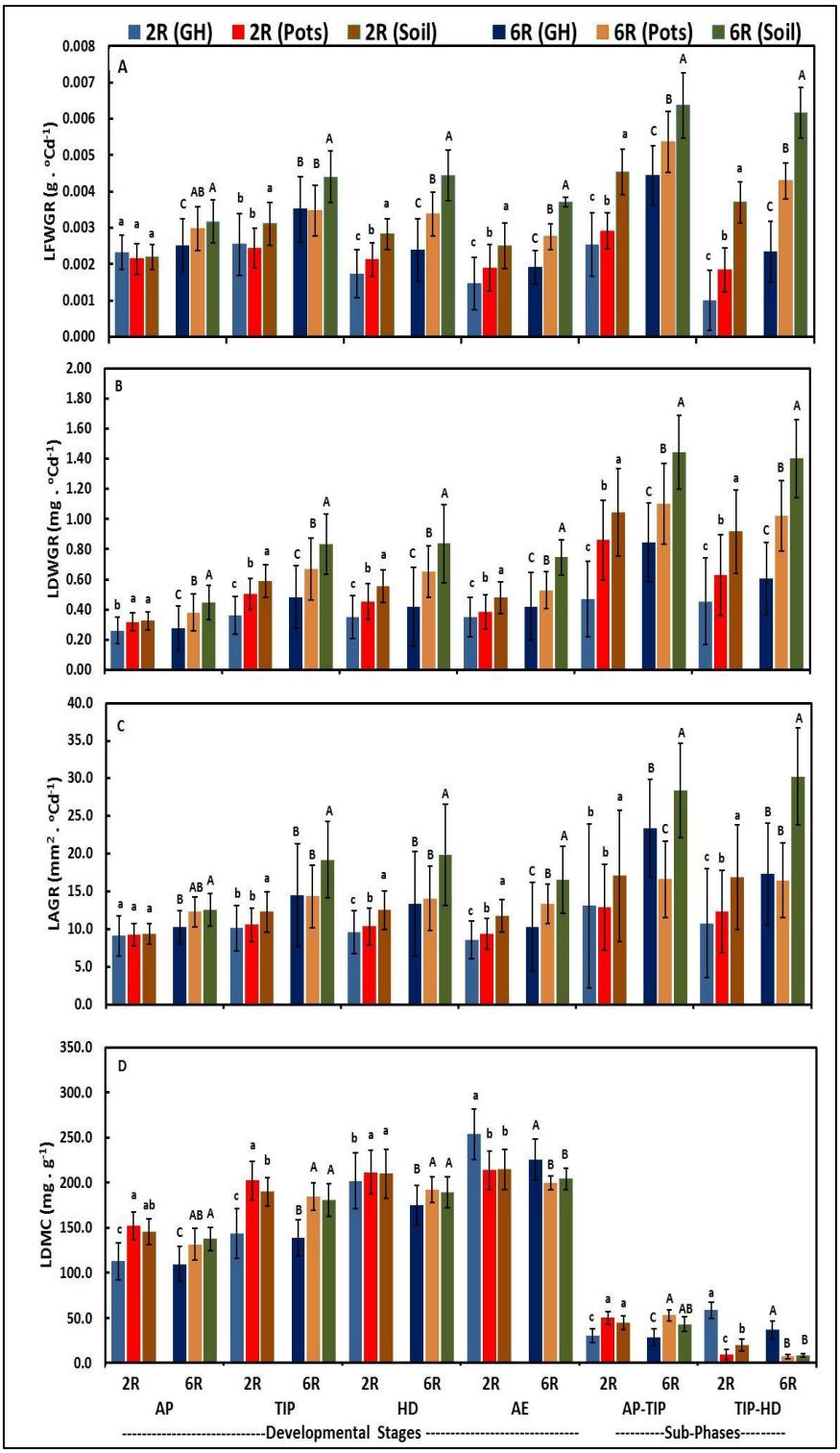


Supplementary Figure 2-1: Mathematical estimation of the onset and end of stem elongation (SE) by measuring the height of the youngest fully developed leaf on the main stem during the growing period (Karsai et al. 2011).



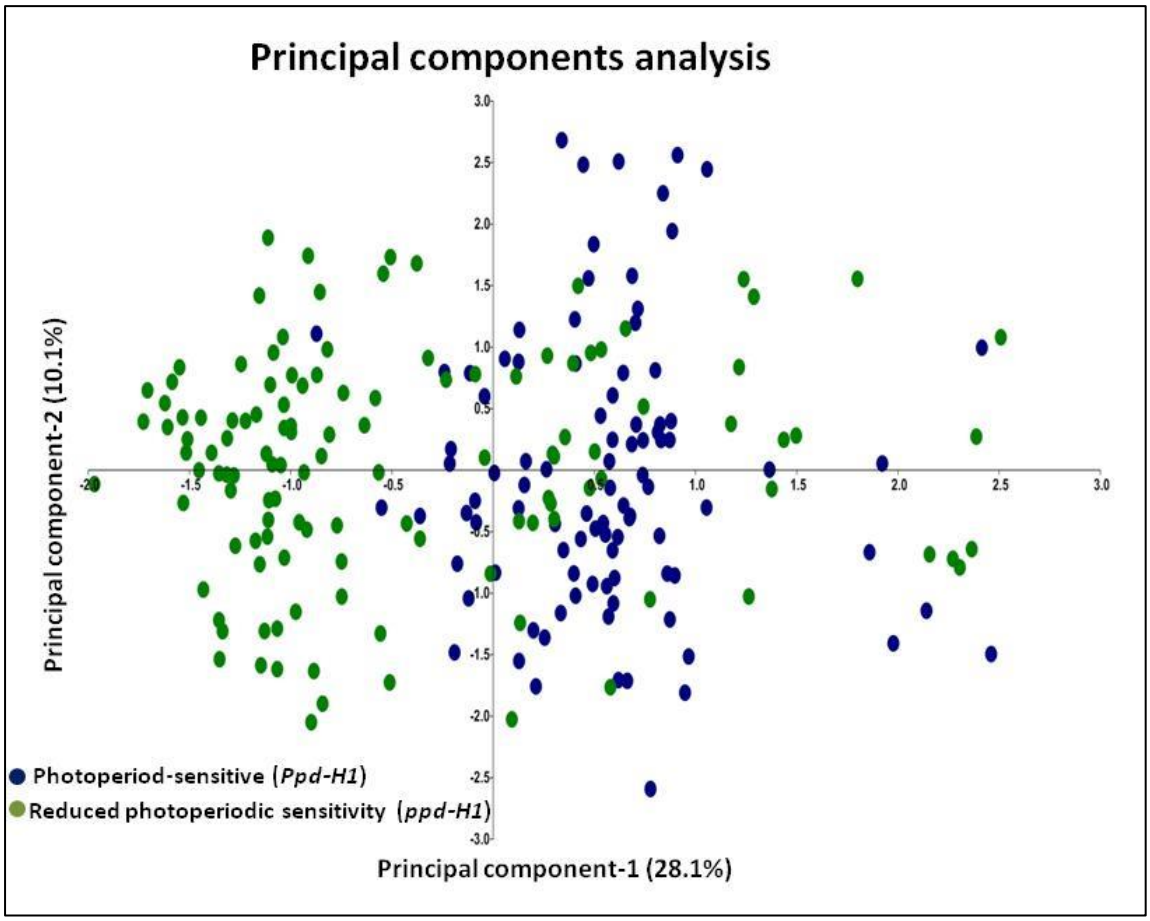
Supplementary Figure 3-1: Leaf traits per main culm at different stages for two- and six-rowed barley at different growth conditions.

A) Leaf number; B) leaf area; C) leaf dry weight; and D) leaf mass area. Small and capital letters differentiate two-rowed and six-rowed barley, respectively. Same letters for each row-type are not significantly different at $P \leq 0.05$ according to Least Significant Difference. Bars indicate standard deviation (n = 14 and 18 for two- and six-rowed barley in each growth condition, respectively). AP: awn primordium, Alqudah and Schnurbusch (2014); TIP: tipping, Z49; HD: heading, Z55; AE: anther extrusion, Z65, Zadoks et al. (1974). GH: greenhouse; 2R: two-rowed and 6R: six-rowed.



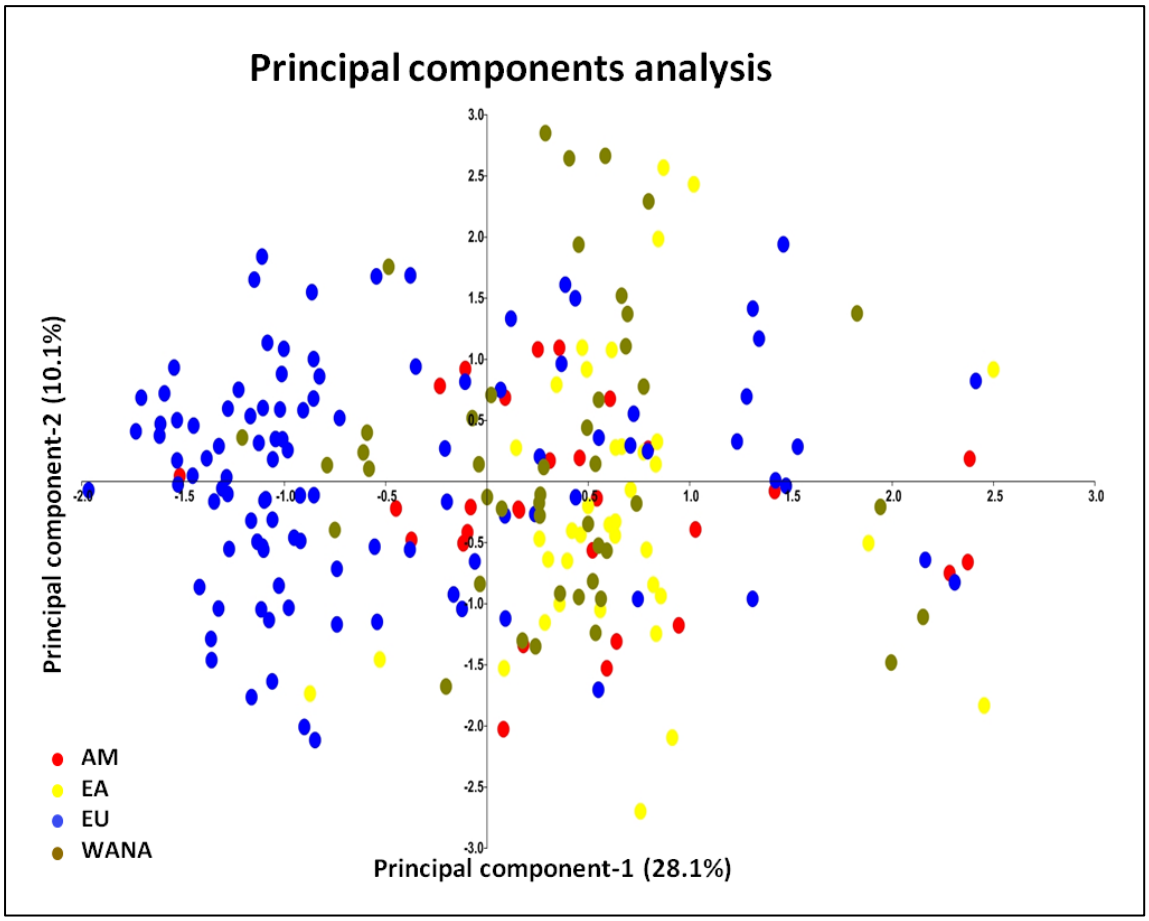
Supplementary Figure 2-2: Leaf growth rate per main culm at different stages for two- and six-rowed barley at different growth conditions.

A) Leaf fresh weight growth rate (LFWGR); B) leaf dry weight growth rate (LDWGR); C) leaf area growth rate (LAGR); and D) leaf dry matter content (LDMC). Small and capital letters differentiate two-rowed and six-rowed barley, respectively. Same letters for each row-type are not significantly different at $P \leq 0.05$ according to Least Significant Difference. Bars indicate standard deviation (n = 14 and 18 for two- and six-rowed barley in each growth condition, respectively). AP: awn primordium, Alqudah and Schnurbusch (2014); TIP: tipping, Z49; HD: heading, Z55; AE: anther extrusion, Z65, Zadoks et al. (1974). GH: greenhouse; 2R: two-rowed and 6R: six-rowed.



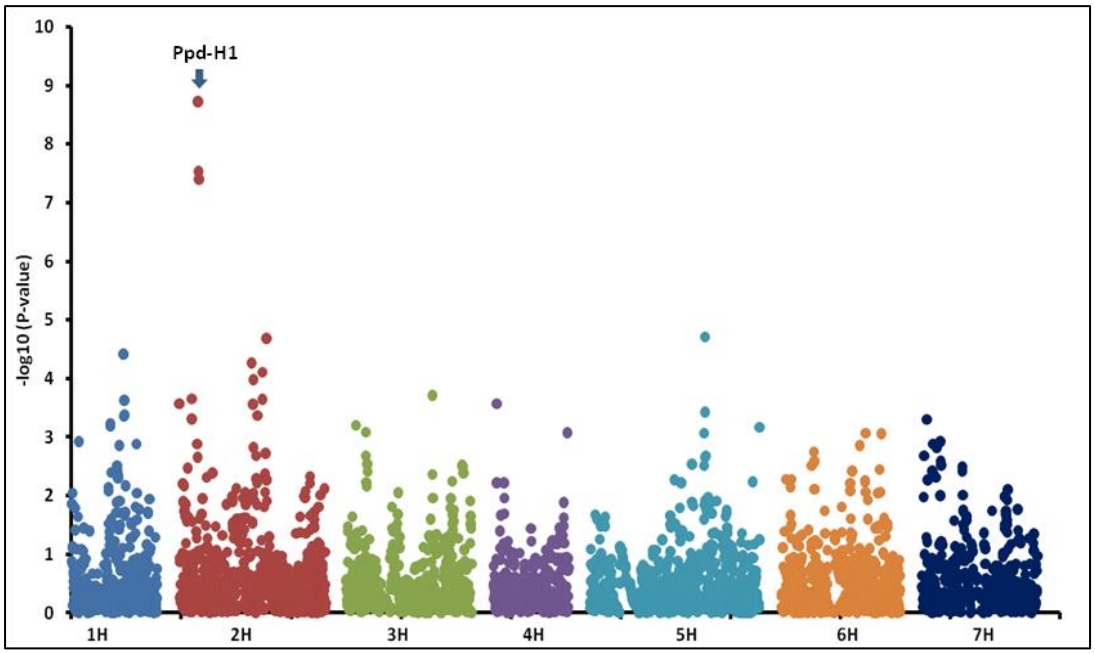
Supplementary Figure 3-1: Principal component analysis (PCA) of 218 spring barley accessions at heading stage using 6355 SNPs.

95 spring barley accessions with photoperiod-sensitive (*Ppd-H1*) and 123 accessions with reduced photoperiod sensitivity (*ppd-H1*).



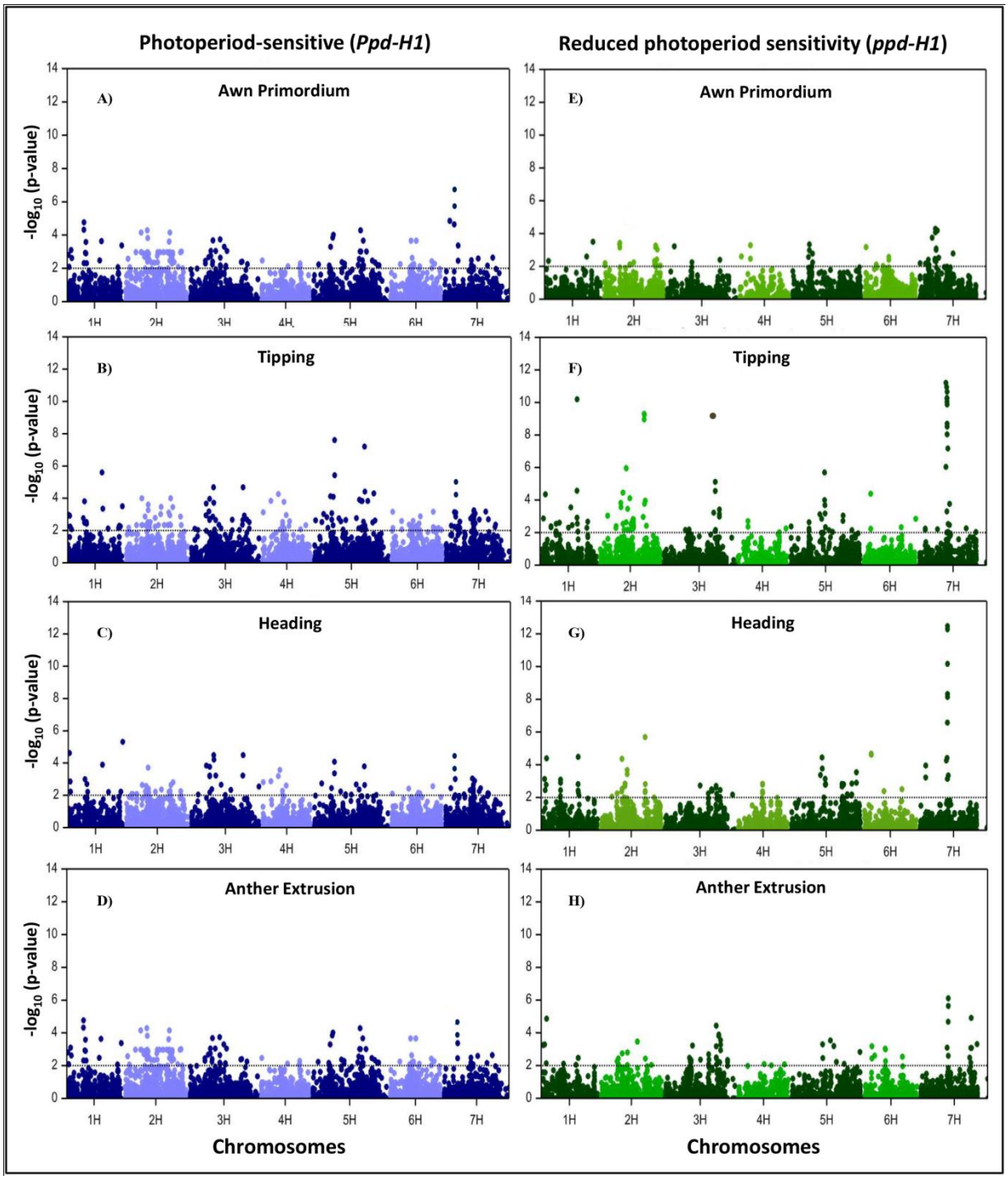
Supplementary Figure 4-2: Principal component analysis (PCA) of 218 spring barley accessions from different origins at heading stage using 6355 SNPs.

n = 45 for WANA, n = 108 for EU, n = 36 EA and n = 29 for AM.



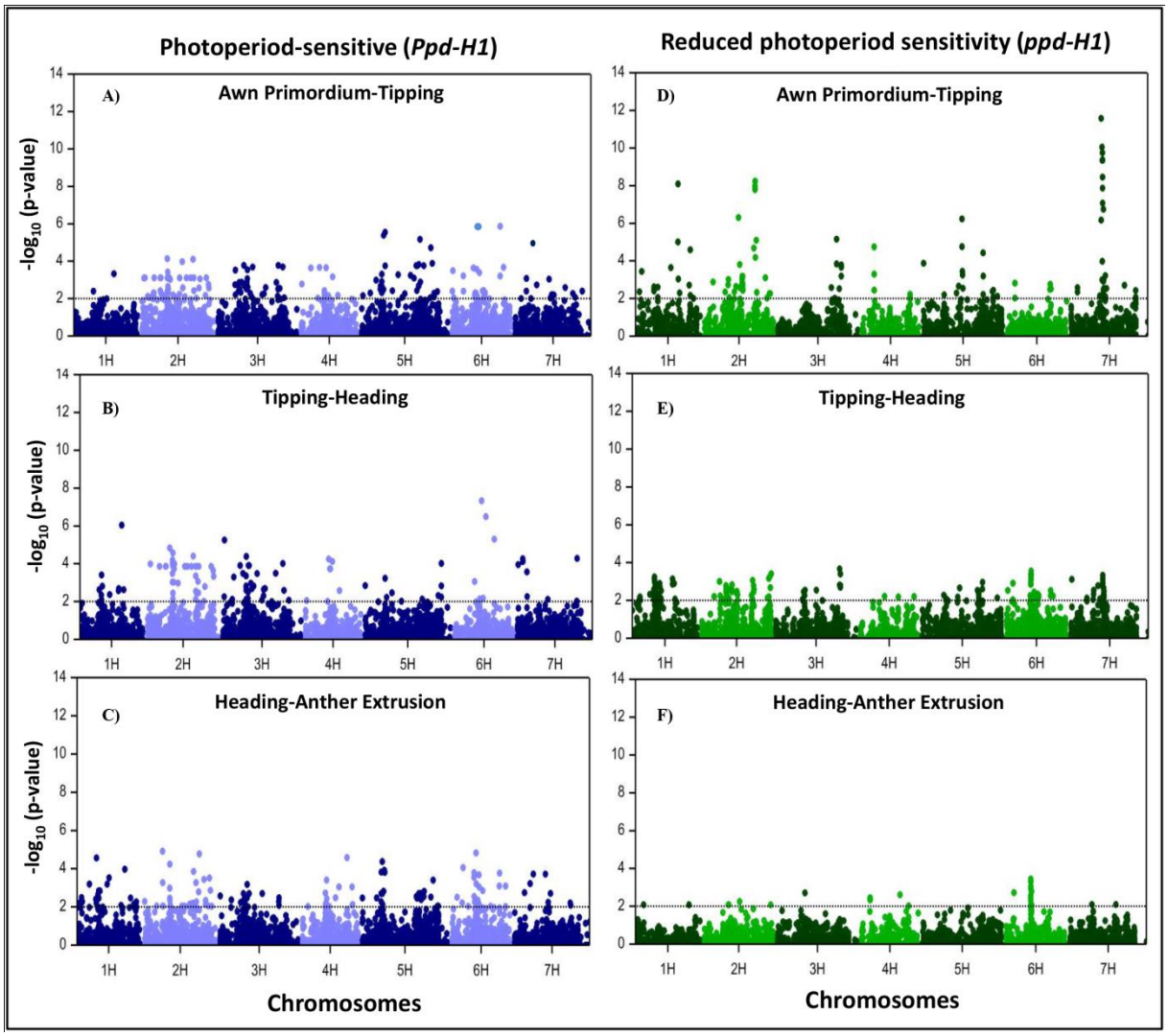
Supplementary Figure 4-3: Manhattan plots of association findings.

The figures summarize GWAS obtained for heading date known gene (*Ppd-H1*) in whole spring barley collection using the iSelect 9K SNP platform. n = 218 accessions.



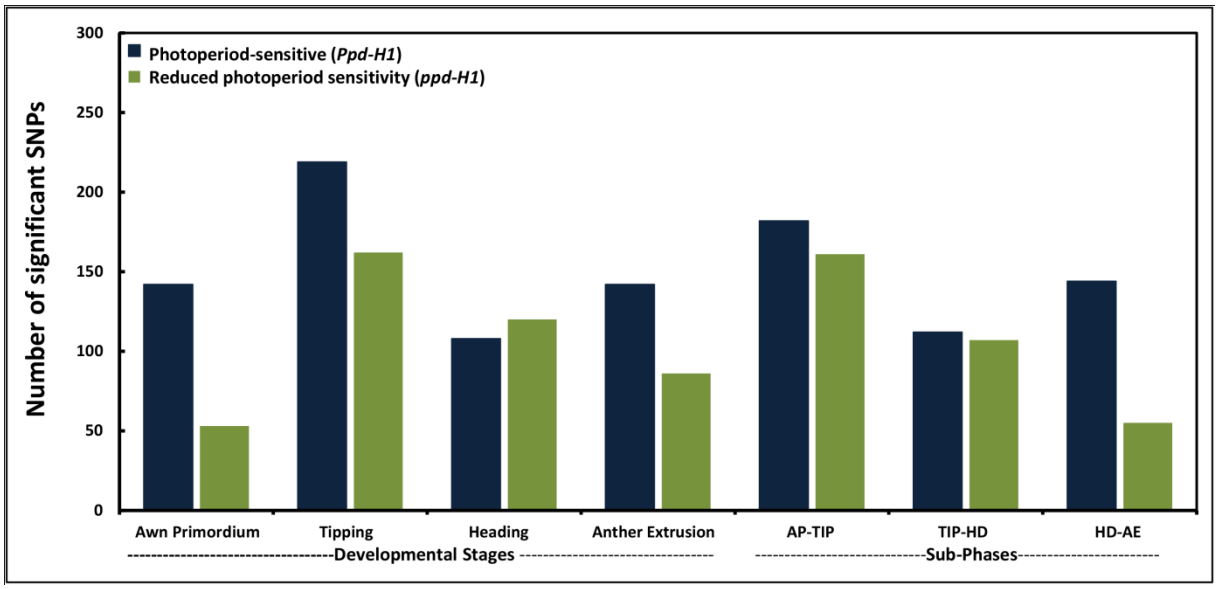
Supplementary Figure 4-4: Manhattan plots of association findings. The figures summarize GWAS obtained from dissecting heading time at different stages in photoperiod-sensitive (*Ppd-H1*) and reduced photoperiod sensitivity (*ppd-H1*) barley accessions using the iSelect 9K SNP platform.

Thermal time was taken from sowing to the beginning of awn primordium, tipping, heading and anther extrusion stages. The black dotted line marks the threshold significance levels ($-\log_{10}$ (*P-value* = 0.01)), and SNPs in loci exceeding this threshold are considered as significantly associated.



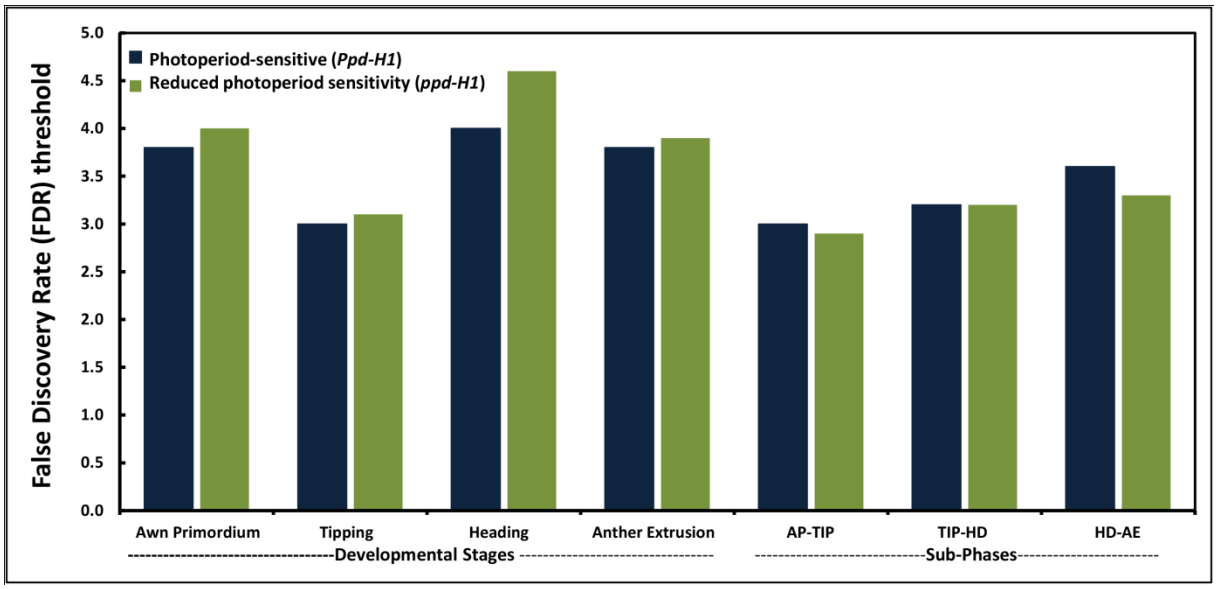
Supplementary Figure 4-5: Manhattan plots of association findings. The figures summarize GWAS obtained from dissecting heading time into sub-phases in photoperiod-sensitive (*Ppd-H1*) and reduced photoperiod sensitivity (*ppd-H1*) barley accessions using the iSelect 9K SNP platform.

Thermal time was taken for the duration between sub-phases. The black dotted line marks the threshold significance levels ($-\log_{10}$ (*P-value* = 0.01)), and SNPs in loci exceeding this threshold are considered as significantly associated.



Supplementary Figure 4-6: Number of significant SNPs for photoperiod-sensitive (*Ppd-H1*) and reduced photoperiod sensitivity (*ppd-H1*) at different developmental stages and sub-phases.

SNPs which have ($-\log_{10}>2$, *P-value* = 0.01) are considered as significant SNPs.



Supplementary Figure 4-7: False Discovery Rate (FDR) threshold (*P*=0.05) at each developmental stage and sub-phase in barley accessions with photoperiod-sensitive (*Ppd-H1*) and reduced photoperiod sensitivity (*ppd-H1*).

SNPs exceeding FDR threshold are considered as highly significant SNPs.

11.0 Supplementary Tables:

Supplementary Table 3-1: Coefficient of variation (CV %) for leaf traits at different developmental stages and sub-phases in two- and six-rowed barley.

Developmental stages/subphase*	Coefficients of Variation (CV %)					
	Two-rowed			Six-rowed		
	(GH)	(pots)	(soil)	(GH)	(pots)	(soil)
‡LA- AP	27	17	16	28	16	22
LA-TIP	73	24	31	32	24	28
LA-HD	80	14	28	41	24	31
LA-AE	74	18	35	27	21	23
LDW-AP	25	29	18	20	29	26
LDW-TIP	40	24	24	26	26	25
LDW-HD	46	20	27	33	27	23
LDW-AE	57	28	22	40	23	17
LMA- AP	73	68	55	73	70	60
LMA-TIP	65	49	39	61	53	50
LMA-HD	79	70	47	58	57	62
LMA-AE	84	78	33	76	57	72
LFWGR- AP	20	27	16	21	25	24
LFWGR-TIP	33	22	25	32	23	25
LFWGR-HD	27	22	26	37	28	24
LFWGR-AE	33	29	29	33	24	24
LFWGR (AP-TIP)	74	50	48	64	58	43
LFWGR (AP-HD)	76	67	46	56	49	53
LDWGR- AP	28	22	19	32	16	25
LDWGR-TIP	34	20	24	30	22	24
LDWGR-HD	32	27	19	37	26	31
LDWGR-AE	37	30	22	38	23	30
LDWGR (AP-TIP)	75	31	37	51	43	31
LDWGR (AP-HD)	53	42	30	50	33	40
LAGR- AP	30	16	14	22	16	19
LAGR-TIP	29	21	26	29	26	26
LAGR-HD	29	24	23	22	30	34
LAGR-AE	29	22	25	28	20	27
LAGR (AP-TIP)	83	44	51	53	61	43
LAGR (AP-HD)	67	44	41	39	61	48

*AP: awn primordium , Alqudah and Schnurbusch (2014); TIP: tipping, Z49; HD: heading, Z55; AE: anther extrusion, Z65, Zadoks et al. (1974); ‡LA: leaf area; LDW: Leaf dry weight; LMA: Leaf mass area; LFWGR: Leaf fresh weight growth rate; LDWGR: Leaf dry weight growth rate; LAGR: Leaf area growth rate; GH: greenhouse.

Supplementary Table 4-1: Thermal time for different developmental stages and sub-phases in (A) photoperiod-sensitive (*Ppd-H1*) and (B) reduced photoperiod sensitivity (*ppd-H1*) accessions.

(A) Photoperiod-sensitive (*Ppd-H1*) accessions:

Accession	Region of origin	Country of origin	Row-Type	Awn Primordium (GDD)	Tipping (GDD)	Heading (GDD)	Anther extrusion (GDD)	Awn Primordium-Tipping (GDD)	Tipping-Heading (GDD)
1	WANA	AFG	2	618	951	1014	.	333	63
2	WANA	IRQ	2	556	784	.	.	229	.
7	WANA	MAR	2	651	1190	1264	1450	539	74
8	WANA	MAR	2	612	1394	1617	1822	782	223
9	WANA	OMN	2	803	1264	1450	1786	461	186
10	WANA	PAK	2	784	1435	1478	1511	651	43
11	WANA	PAK	2	501	839	973	.	338	134
12	WANA	SYR	2	501	.	839	.	.	.
13	WANA	SYR	2	501	706	839	.	204	133
19	EA	CHN	2	549	868	1014	1095	319	146
29	EA	CHN	2	591	910	1014	1193	319	104
36	EA	IND	2	834	1276	1407	1737	443	131
37	EA	IND	2	670	1237	1295	1333	567	58
38	EA	IND	2	670	1102	1141	1517	432	39
39	EA	IND	2	834	1407	1445	2074	574	38
44	EA	KOR	2	586	814	1061	.	228	247
46	EA	KOR	2	.	875	967	.	.	93
91	EU	FRA	2	.	.	.	.	.	.
175	WANA	UZB	2	670	1005	1301	1394	335	296
182	EU	RUS	2	555	878	932	.	323	54
188	WANA	KAZ	2	555	1060	1134	1264	505	74
190	WANA	UZB	2	574	932	1134	1264	358	202
191	WANA	TJK	2	574	1005	1060	1190	431	55
214	WANA	TUR	2	525	940	1035	1095	416	95
221	EA	IND	2	503	967	1006	1517	464	39
222	WANA	SYR	2	525	742	884	1035	217	142
223	EU	ROM	2	628	1102	1141	1276	474	39
224	na	na	2	649	1407	1445	.	758	38
3	WANA	LBY	6	612	847	910	972	235	63
4	WANA	MAR	6	670	1141	1276	1407	471	135
5	WANA	MAR	6	628	1102	1141	1276	474	39
6	WANA	MAR	6	628	1065	1141	1333	437	76
14	WANA	SYR	6	611	1245	1446	1524	634	201
15	WANA	SYR	6	535	784	868	1014	250	83
16	WANA	TJK	6	593	973	1245	1446	380	272
17	WANA	TJK	6	722	1193	1287	1442	471	94
18	EA	CHN	6	640	1136	1287	1670	497	151
20	EA	CHN	6	465	.	.	.	.	.
22	EA	CHN	6	577	1014	1193	1442	437	179
24	EA	CHN	6	591	1095	1248	1557	504	153
25	EA	CHN	6	661	1055	1193	1410	395	138
30	EA	IND	6	661	1193	1557	1670	533	364
31	EA	IND	6	577	910	1014	1193	333	104
32	EA	IND	6	662	1065	1141	1276	403	76
33	EA	IND	6	722	910	1014	1055	188	104
34	EA	IND	6	690	1198	1333	1517	508	135
35	EA	IND	6	503	814	875	1141	311	61
40	EA	IND	6	593	1102	1141	1276	509	39
41	EA	JPN	6	794	1333	1445	1645	539	112
42	EA	JPN	6	670	1276	1407	1493	606	131
43	EA	KOR	6	794	.	.	.	.	.
48	EA	KOR	6	565	911	967	1160	346	56
50	EA	NPL	6	581	963	1020	1095	382	57
52	EA	NPL	6	520	790	828	904	269	39
53	EA	NPL	6	655	963	1095	1278	308	132
54	EA	NPL	6	618	924	963	1020	306	39
55	EA	NPL	6	581	924	963	1151	343	39
58	AM	MEX	6	581	673	732	.	93	59
59	AM	URY	6	520	673	732	.	153	59
60	AM	MEX	6	520	828	963	1352	308	135
61	AM	USA	6	581	828	885	1020	247	57
62	AM	USA	6	680	1278	1407	.	598	129
63	AM	USA	6	.	1223	1352	1518	.	129
64	AM	COL	6	656	1033	1071	1109	377	38
65	AM	USA	6	761	1095	1151	1278	334	56
66	AM	USA	6	565	875	948	1141	309	74
69	AM	URY	6	.	.	1518	1649	.	.
70	AM	URY	6	561	885	963	1020	324	78
76	AM	BOL	6	561	885	963	1020	324	78
77	AM	USA	6	600	963	1020	1151	363	57
81	AM	USA	6	642	1102	1276	1517	460	174

82	AM	USA	6	.	1223	1407	1518	.	184	111
83	AM	COL	6	587	1151	1352	1518	564	201	166
84	AM	PER	6	.	1095	1151	1352	.	56	201
86	WANA	TUR	6	752	1095	1151	1352	343	56	201
87	AM	USA	6	754	1102	1141	1250	349	39	109
88	EU	DEU	6	.	.	.	.	.	.	.
89	EU	NLD	6	.	1352	1407	1518	.	55	111
111	EU	NLD	6	692	1020	1095	1352	328	75	257
161	EU	RUS	6	565	967	1006	1160	402	39	154
163	EU	RUS	6	.	1339	1394	1450	.	55	56
171	WANA	UZB	6	651	1134	1190	1264	483	56	74
174	EU	UKR	6	670	932	1005	1134	262	73	129
189	WANA	KGZ	6	644	.	.	.	.	.	.
192	WANA	TKM	6	677	1264	.	.	587	.	.
194	EU	UKR	6	690	1060	1134	1264	370	74	130
198	EU	YUG	6	690	1060	1190	1264	370	130	74
200	EU	BGR	6	.	1229	1287	1428	.	58	141
201	EU	ALB	6	702	1095	1172	1229	394	77	57
204	WANA	AFG	6	702	1095	1229	1510	394	134	281
205	WANA	AFG	6	630	.	1580	1658	.	.	78
206	WANA	IRN	6	602	1035	1095	1172	433	60	77
210	WANA	TUR	6	623	1095	1172	1229	473	77	57
211	WANA	TUR	6	562	802	884	1035	240	81	152
212	WANA	TUR	6	618	1095	1193	1410	477	98	217

(B) Reduced photoperiod sensitivity (*ppd-H1*) accession:

Accession	Region of origin	Country of origin	Row-Type	Awn Primordium (GDD)	Tipping (GDD)	Heading (GDD)	Anther extrusion (GDD)	Awn Primordium-Tipping (GDD)	Tipping-Heading (GDD)	Heading-Anther extrusion (GDD)
21	EA	CHN	2	649	1407	1445	.	758	38	.
45	EA	KOR	2	.	1407	1518	.	.	111	.
57	AM	CAN	2	809	1095	1151	1352	286	56	201
72	AM	MEX	2	.	1006	1198	1389	.	192	191
78	AM	CHL	2	722	1557	2112	.	835	555	.
85	AM	CAN	2	803	1450	1580	1822	647	130	242
90	EU	FRA	2	728	1301	2029	.	573	728	.
92	EU	POL	2	617	1529	.	.	912	.	.
93	EU	GBR	2	617	1248	1529	1697	631	281	168
94	EU	NLD	2	690	1529	1880	2188	839	351	308
95	EU	DNK	2	727	.	.	.	.	.	.
96	EU	FRA	2	727	1324	1529	1642	597	205	113
97	EU	GBR	2	617	1567	1771	2006	950	204	235
98	EU	CZE	2	703	1529	1567	2006	826	38	439
99	EU	FRA	2	617	1379	1697	2059	762	318	362
100	EU	GBR	2	617	1436	2132	.	819	696	.
101	EU	GBR	2	727	.	.	.	.	.	.
102	EU	GBR	2	715	1379	1697	2006	664	318	309
103	EU	DEU	2	727	1880	.	.	1153	.	.
104	EU	POL	2	617	1174	1474	2006	557	300	532
105	EU	DEU	2	727	1529	.	.	802	.	.
106	EU	NLD	2	678	1324	1554	1697	646	230	143
107	EU	IRL	2	709	1435	1568	1642	726	133	74
108	EU	SWE	2	709	1655	2006	2188	946	351	182
109	EU	DEU	2	709	1655	.	.	946	.	.
110	EU	DNK	2	709	.	1880	2132	.	.	252
112	EU	NLD	2	635	1211	1268	1568	576	57	300
113	EU	SWE	2	635	1765	2059	.	1130	294	.
114	EU	HUN	2	674	1261	1542	1642	587	281	100
115	EU	HUN	2	709	1568	1642	1765	859	74	123
116	EU	SWE	2	635	1568	1642	1765	933	74	123
117	EU	FRA	2	709	1174	1529	1697	465	355	168
118	EU	DEU	2	746	1379	1568	1697	633	189	129
119	EU	SWE	2	746	1642	2059	.	896	417	.
120	EU	DEU	2	599	1045	1474	1529	446	429	55
122	EU	GBR	2	715	1499	1762	.	784	263	.
123	EU	AUT	2	746	1324	1529	1642	578	205	113
124	EU	GBR	2	774	1740	1873	1913	966	133	40
125	EU	AUT	2	702	1579	1740	1873	877	161	133
126	EU	SWE	2	666	1579	1873	1913	913	294	40
127	EU	DEU	2	630	1579	1740	1913	949	161	173
128	EU	SWE	2	666	1740	1873	1913	1074	133	40
129	EU	DEU	2	523	1343	1419	1740	821	76	321
130	EU	CZE	2	702	1740	1873	1913	1038	133	40
131	EU	GBR	2	666	1419	1740	2105	753	321	365
132	EU	SWE	2	774	1479	1740	1779	705	261	39
133	EU	DEU	2	702	1708	.	.	1007	.	.
134	EU	DNK	2	666	1017	1151	1740	351	134	589
135	EU	DEU	2	666	1479	1740	1779	813	261	39
136	EU	NLD	2	630	1479	1740	1779	849	261	39
137	EU	CZE	2	702	1151	1740	1779	449	589	39
138	EU	NLD	2	558	1343	1740	1779	785	397	39
139	EU	FRA	2	558	1209	1740	1779	651	531	39
140	EU	DEU	2	612	1209	1419	1740	597	210	321
141	EU	DEU	2	756	1740	1913	2008	984	173	95
142	EU	GBR	2	.	.	.	.	.	.	.
143	EU	FRA	2	756	1151	1286	1740	395	135	454
144	EU	AUT	2	.	1419	1779	1854	.	360	75

145	EU	CZE	2	720	1209	1343	1740	490	134	397
146	EU	DEU	2	648	1579	1740	1779	931	161	39
147	EU	CZE	2	732	1740	1854	1913	1008	114	59
148	EU	AUT	2	684	1286	1740	1854	602	454	114
149	EU	DEU	2	672	1286	1740	1779	614	454	39
150	EU	GBR	2	576	1017	1151	1740	441	134	589
151	EU	DEU	2	684	1579	1913	2105	895	334	192
152	EU	CZE	2	702	1287	1689	2056	586	402	367
153	EU	FRA	2	.	1343	1740	1779	.	397	39
154	EU	FRA	2	756	1343	1740	1779	587	397	39
160	EU	RUS	2	611	1870	2071	2211	1259	201	140
162	EU	RUS	2	681	1482	1726	1982	801	244	256
164	EU	RUS	2	611	1524	1580	1658	913	56	78
165	EU	RUS	2	611	1580	1793	1870	969	213	77
166	EU	UKR	2	724	1446	1580	1793	722	134	213
167	EU	UKR	2	538	1304	1446	1524	766	142	78
168	EU	BLR	2	611	.	.	.	.	.	.
169	WANA	KAZ	2	574	1304	1524	1735	730	220	211
170	WANA	KAZ	2	718	.	.	.	.	.	.
172	EU	ARM	2	661	1287	1442	1798	627	155	356
173	EU	LTU	2	611	1446	1580	1735	835	134	155
177	EU	RUS	2	593	1446	1580	1658	853	134	78
178	EU	RUS	2	724	1524	1580	1658	800	56	78
179	EU	RUS	2	593	1658	1870	2071	1065	212	201
180	EU	RUS	2	593	1446	1580	1735	853	134	155
183	EU	RUS	2	702	972	1442	1798	270	471	356
195	EU	UKR	2	555	973	1245	1446	417	272	201
196	EU	DEU	2	593	1446	1580	1989	853	134	409
202	EU	GRC	2	722	1136	1423	.	414	287	.
203	EU	ITA	2	722	1327	1726	.	605	399	.
207	WANA	IRN	2	602	1035	1095	1172	433	60	77
208	WANA	IRN	2	487	742	1035	1095	255	293	60
213	WANA	TUR	2	642	1442	1670	1982	800	228	312
215	WANA	TUR	2	525	940	1035	1172	416	95	137
216	WANA	ISR	2	562	1035	1095	1172	473	60	77
217	WANA	ISR	2	562	1172	1229	1287	610	57	58
218	WANA	ISR	2	623	1229	1366	1630	607	137	264
219	WANA	ISR	2	722	1384	1798	.	662	414	.
220	WANA	ISR	2	623	1229	1287	1366	607	58	79
23	EA	CHN	6	.	.	.	.	.	.	.
26	EA	CHN	6	581	1020	1151	1278	439	131	127
27	EA	CHN	6	618	1095	1151	1352	477	56	201
28	EA	CHN	6	649	1198	1276	1407	549	78	131
47	EA	KOR	6	752	1352	1407	1649	600	55	242
56	EA	NPL	6	636	963	1020	1095	327	57	75
67	AM	CAN	6	.	1264	1394	1525	.	130	131
68	AM	MEX	6	631	1134	1190	1450	503	56	260
71	AM	MEX	6	670	.	1598	.	.	.	.
73	AM	USA	6	670	1134	1264	1394	464	130	130
74	AM	CAN	6	.	1394	1543	.	.	149	.
75	AM	CAN	6	690	1264	1394	1525	574	130	131
79	AM	MEX	6	.	.	.	1264	.	.	.
155	EU	FIN	6	523	960	1017	1074	438	57	57
156	EU	FIN	6	574	.	.	.	.	.	.
157	EU	NLD	6	574	1245	1446	1524	671	201	78
158	EU	FIN	6	640	1265	1287	1442	626	22	155
159	EU	RUS	6	538	.	1580	1658	.	.	78
176	EU	RUS	6	618	910	972	1423	292	62	451
181	EU	RUS	6	520	1070	1446	1743	550	376	297
184	EU	RUS	6	724	.	1524	1580	.	.	56
185	EU	RUS	6	483	706	762	.	223	57	.
187	EU	UKR	6	520	.	1580	1735	.	.	155
193	EU	RUS	6	520	973	1446	1743	453	473	297
197	EU	AUT	6	630	.	.	.	.	.	.
209	WANA	IRN	6	681	1095	1442	1798	414	347	356

Supplementary Table 4-2: GenBank accession number for known heading time candidate genes with their POPSEQ genetic position and significantly associated markers (POPSEQ position in cM).

Chr.	Gene	Genebank accession number	Reference	cM (POP SEQ)	Contig identifier	Significantly associated SNP marker ≥FDR (cM POPSEQ)
1H	*HvCMF10*	JQ791225	(Cockram et al., 2012)	47.82	morex_contig_53826 CAJW010053826	12_30683 (46.6) SCRI_RS_149971 (48.08)
1H	*HvCO9/HvCMF11*	AY082965	(Griffiths et al., 2003;Comadran et al., 2012)	~60*	morex_contig_67944 CAJW010067944	
1H	*HvCMF5*	JQ791219	(Cockram et al., 2012)	81.72	morex_contig_79857 CAJW010079857	
1H	*Ppd-H2/HvFT3*	HM133570.1	(Casao et al., 2011a)	92.35	morex_contig_2551337 CAJW012551337	SCRI_RS_138010 (92.06) SCRI_RS_199689 (92.35)
1H	*HvAdh2*	AY184935.1	(Lin et al., 2002)	104.8	morex_contig_43476 CAJW010043476	SCRI_RS_238125 (103.82)
1H	*HvCMF6a*	JQ791220	(Cockram et al., 2012)	132.02	morex_contig_2548366 CAJW012548366	SCRI_RS_153896 (132.08)
1H	*Esp1L/HvELF3 /eam8*	JN180296.1	(Zakhrabekova et al., 2012)	132.36	morex_contig_80895 CAJW010080895	SCRI_RS_199945 (132.86)
1H	*HvCMF6b*	JQ791252	(Cockram et al., 2012)	132.57	morex_contig_174751 CAJW010174751	SCRI_RS_199945 (132.86)
2H	*PpdH1*	AY970701.1	(Comadran et al., 2012)	19.90	morex_contig_94710 CAJW010094710	BK_12 (19.90) BK_13 (19.90) BK_15 (19.90) BK_16 (19.90)
2H	*HvCO18*	JQ791251	(Cockram et al., 2012)	41.85	morex_contig_68173 CAJW010068173	12_10296 (40.08) SCRI_RS_154981 (41.20)
2H	*HvFT4*	DQ411320.1	(Faure et al., 2007)	50.04	morex_contig_6666 CAJW010006666	SCRI_RS_221843 (49.40)
2H	*eps2/HvCEN/eam6*	JX844786.1	(Comadran et al., 2012)	58.00	morex_contig_274284 CAJW010274284	12_30634 (58.07) 11_10358 (58.71) SCRI_RS_83731 (58.78)
2H	*HD6-2H*	DQ157464.1	http://kasetsartjournal.ku.ac.th/kuj_files/2008/A080402133514.pdf	59.41	morex_contig_1567582 CAJW011567582	
2H	*HvCO4*	AF490474	(Griffiths et al., 2003)	64.73	morex_contig_161048 CAJW010161048	SCRI_RS_59851 (64.58)
2H	*BFL/HvAPO2*	AB005620.1	(Kyozuka et al., 1998)	107.36	morex_contig_1567741 CAJW011567741	11_10989 (107.93)
2H	*HvAP2*	GQ403050.1	(Nair et al., 2010)	126.7	morex_contig_46950 CAJW010046950	
2H	*Ertr67 HvAP2 (Zeo)*	KC898651	(Houston et al., 2013)	127.05	morex_contig_43451 CAJW010043451	
3H	*HvGI*	AY740524.1	(Dunford et al., 2005a)	45.82	morex_contig_58270 CAJW010058270	12_31475 (45.82)

3H	*HvFT2*	DQ297407	(Faure et al., 2007)	52.03	morex_contig_1558556 CAJW011558556	11_20325 (52.02)
3H	*HvCMF1*	JQ791213	(Cockram et al., 2012)	98.22	morex_contig_43834 CAJW010043834	
3H	*HvLUX/eps3L/ eam10*	KC668273.1	(Gawronski et al., 2014)	149.00	morex_contig_2548416 CAJW012548416	
4H	*Vrn-H2/ HvZCCT-Ha/b/c*	AK365195.1	(Comadran et al., 2012)	114.94‡	morex_contig_2199658 CAJW012199658	SCRI_RS_151357 (114.94)
4H	*HvCO10*	JQ791236.1	(Cockram et al., 2012)	26.34	morex_contig_7813 CAJW010007813	SCRI_RS_207768 (26.3)
4H	*HvPhyA*	DQ201141.1	(Szucs et al., 2006)	34.56	morex_contig_9764 CAJW010009764	
4H	*HvCO16*	JQ791248.1	(Cockram et al., 2012)	51.13	morex_contig_44067 CAJW010044067	11_21071 (50.99) SCRI_RS_171142 (51.34)
4H	*HvPRR59*	JQ791228	(Cockram et al., 2012)	51.34	morex_contig_46739 CAJW010046739	11_21071 (50.99) SCRI_RS_171142 (51.34)
4H	*HvphyB*	DQ201142	(Szucs et al., 2006)	51.40	morex_contig_1557904 CAJW011557904	11_21071 (50.99) SCRI_RS_171142 (51.34)
4H	*HvPRR73*	JQ791230	(Cockram et al., 2012)	51.40	morex_contig_1563982 CAJW01156398	11_21071 (50.99) SCRI_RS_171142 (51.34)
4H	*HvCMF4*	JQ791217	(Cockram et al., 2012)	103.75	morex_contig_135706 CAJW010135706	SCRI_RS_179398 (103.75)
4H	*HvSOC1*	JN673265.1	(Papaefthimiou et al., 2012)	107.32	barke_contig_1803142 CAJV011657847	12_30385 (107.36)
5H	*HvCO3*	AF490473	(Griffiths et al., 2003)	43.76	morex_contig_67117 CAJW010067117	SCRI_RS_219574 (43.68)
5H	*HvTFL1*	DQ539338.1	http://www.ncbi.nlm.nih.gov/nuccore/DQ539338.1	44.09	morex_contig_2522905 CAJW012522905	i_11_20461 (43.95)
5H	*HvCMF13*	JQ791226.1	(Cockram et al., 2012)	46.45	morex_contig_1558212 CAJW011558212	11_10840 (47.79)
5H	*HvPRR95*	JQ791233	(Cockram et al., 2012)	97.29	morex_contig_41351 CAJW010041351	SCRI_RS_171047 (97.29)
5H	*HvPhyC*	AB827939.1	(Nishida et al., 2013)	125.76	morex_contig_106547 CAJW010106547	SCRI_RS_217212 (125.76)
5H	*Vrn-H1*	EF591648.1	(Cockram et al., 2007)	125.76	morex_contig_2552097 CAJW012552097	SCRI_RS_217212 (125.76)
5H	*HD6-5H*	DQ157464.1	http://kasetsartjournal.ku.ac.th/kuj_files/2008/A080402133514.pdf	128.19	morex_contig_40406 CAJW010040406	SCRI_RS_214130 (129.44)
6H	*HvCMF3*	JQ791216	(Cockram et al., 2012)	49.22	morex_contig_56141 CAJW010056141	
6H	*HvCO7*	AY082963	(Griffiths et al., 2003)	52.62	morex_contig_2550116 CAJW012550116	

6H	*HvCO5*	AY082958	(Griffiths et al., 2003)	55.02	morex_contig_243021 CAJW010243021	12_30857 (55.02)
6H	*HvCry1a*	DQ201149	(Szucs et al., 2006)	55.02	morex_contig_75574 CAJW010075574	12_30857 (55.02)
6H	*HvCry2*	DQ201155	(Szucs et al., 2006)	55.02	morex_contig_141897 CAJW010141897	12_30857 (55.02)
6H	*HvPRR1/HvTOC1*	JQ791234	(Cockram et al., 2012)	55.38	morex_contig_37494 CAJW010037494	SCRI_RS_237887 (55.10)
6H	*HvCry1b*	DQ201152	(Szucs et al., 2006)	59.06	morex_contig_48345 CAJW010048345	SCRI_RS_10810 (59.40)
6H	*HvCO14*	JQ791244	(Cockram et al., 2012)	67.91	morex_contig_367999 CAJW010367999	SCRI_RS_136724 (67.90)
6H	*HvCO2*	AF490469	(Griffiths et al., 2003)	68.20	morex_contig_6805 CAJW010006805	SCRI_RS_165594 (62.74)
6H	*HvCO11*	JQ791238	(Cockram et al., 2012)	69.26	morex_contig_1577721 CAJW011577721	11_20784 (69.05) 12_31250 (69.05)
7H	*Vrn-H3/HvFT1*	DQ100327	(Comadran et al., 2012)	34.43‡	morex_contig_54983 CAJW010054983	12_30894 (34.34) 12_30895 (34.34)
7H	*WAXY*	AF486515.1	(Patron et al., 2002)	13.88	morex_contig_49158 CAJW010049158	SCRI_RS_152931 (11.54)
7H	*HvCO8*	AY082964	(Griffiths et al., 2003)	41.99	morex_contig_368769 CAJW010368769	SCRI_RS_187827 (43.83)
7H	*HvSS1*	FN400939.1	http://www.ncbi.nlm.nih.gov/nuccore/239984689	54.39	morex_contig_1561797 CAJW011561797	
7H	*HvVRT-2*	AK355370	(Szucs et al., 2006)	61.75	morex_contig_37339 CAJW010037339	11_20975 (61.75)
7H	*HvCO12*	JQ791240	(Cockram et al., 2012)	67.77	morex_contig_140238 CAJW010140238	SCRI_RS_235641 (67.77)
7H	*HvCO13/HvM*	JQ791242	(Cockram et al., 2012)	67.77	morex_contig_45996 CAJW010045996	SCRI_RS_235641 (67.77)
7H	*HvCO1*	AF490468.1	(Griffiths et al., 2003)	67.91	morex_contig_138334 CAJW010138334	BK_03 (67.91) 12_31452 (67.91) 12_31452 (67.91) SCRI_RS_108830 12_31452 (67.91) SCRI_RS_124819 12_31452 (67.91) SCRI_RS_127040 12_31452 (67.91) SCRI_RS_132722 12_31452 (67.91) SCRI_RS_152696 12_31452 (67.91) SCRI_RS_171588 12_31452 (67.91) SCRI_RS_171786 12_31452 (67.91) SCRI_RS_192587 12_31452 (67.91) SCRI_RS_198541 12_31452 (67.91) SCRI_RS_207354 12_31452 (67.91)

						SCRI_RS_219081 12_31452 (67.91)
7H	*HvCO15*	JQ791246	(Cockram et al., 2012)	70.53	morex_contig_59119 CAJW010059119	11_10924 (70.53) 11_20828 (70.53) 12_30053 (70.67)
7H	*HvCCA1*	HQ850271.1	(Faure et al., 2012)	70.82	morex_contig_1567295 CAJW011567295	12_30053 (70.67) SCRI_RS_200107 (70.83)
7H	*HvLHY*	HQ222606.1	http://www.uniprot.org/uniprot/E9M5R6	70.82	morex_contig_1567295 CAJW011567295	12_30053 (70.67) SCRI_RS_200107 (70.83)
7H	*HvCMF7*	JQ791222	(Cockram et al., 2012)	91.78	morex_contig_104939 CAJW010104939	
7H	*HvCO6*	AY082960	(Griffiths et al., 2003)	120.82	morex_contig_7405 CAJW010007405	

* Based on genebank accession number we detected the contig identifier without POPSEQ position but Cockram *et al.* (Cockram et al., 2012) mapped it at around 60 cM on 1H.
‡ The position of these genes is based on genetic marker positions published in (Comadran et al., 2012).

Supplementary Table 4-3: Number of QTL (within confidence interval ±5 cM) between developmental stages at each chromosome in groups with A) photoperiod-sensitive (*Ppd-H1*) and B) reduced photoperiod sensitivity (*ppd-H1*).

QTL exceeding threshold significance level ($-\log_{10}>2$, *P*-value = 0.01) are considered as significantly associated.

A) photoperiod-sensitive (*Ppd-H1*)

	1H	2H	3H	4H	5H	6H	7H	Total
AP-TIP	4	10	5	3	6	6	6	**40**
AP-HD	3	6	4	1	5	4	5	**28**
AP-AE	3	5	7	2	8	5	6	**36**
TIP-HD	3	6	6	3	6	4	5	**33**
TIP-AE	4	5	5	4	8	5	5	**36**
HD-AE	3	5	4	2	6	3	5	**28**
Total	**20**	**37**	**31**	**15**	**39**	**27**	**32**	**201**

B) Reduced photoperiod sensitivity (*ppd-H1*)

	1H	2H	3H	4H	5H	6H	7H	Total
AP-TIP	5	5	2	1	3	0	2	**18**
AP-HD	3	2	1	0	2	1	1	**10**
AP-AE	3	4	2	0	2	1	0	**12**
TIP-HD	3	4	1	0	3	2	2	**15**
TIP-AE	3	4	2	1	3	2	3	**18**
HD-AE	2	3	1	1	4	2	2	**15**
Total	**19**	**22**	**9**	**3**	**17**	**8**	**10**	**88**

†AP: awn primordium, (Alqudah and Schnurbusch, 2014); TIP: tipping, Z49; HD: heading, Z55; AE: anther extrusion, Z65, (Zadoks et al., 1974).

12.0 Curriculum vitae

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Ph.D. work carried out at IPK Gatersleben under supervision of Dr. habil. Thorsten Schnurbusch, Plant Architecture Group, Genebank Department. Mentor: Prof. Dr. Andreas Graner.

M.Sc. 2007
Field Crops Production, Plant Production Department, Jordan University of Science and Technology (JUST), Irbid, Jordan.
Thesis title “Evaluation of Four Barely Cultivars under Terminal Drought Stress" Advisor: Prof. Nezar Samarah.

B.Sc. 2005
Plant Production, Plant Production Department, (JUST).

PEER-REVIEWED PUBLICATIONS BASED ON THE PRESENT DISSERTATION

Ahmad M. Alqudah; Thorsten Schnurbusch. (2014). Barley leaf area and leaf growth rates are maximized during the pre-anthesis phase. Agronomy. 5(2), 107-129. DOI: 10.3390/agronomy5020107

Ahmad M. Alqudah; Rajiv Sharma; Raj K Pasam; Andreas Graner; Benjamin Kilian; Thorsten Schnurbusch. Genetic dissection of photoperiod response based on GWAS of pre-anthesis phase duration in spring barley. PloS One. 9(11): e113120. DOI: 10.1371/journal.pone.0113120

Ahmad M. Alqudah; Thorsten Schnurbusch. (2014). Awn primordium to tipping is the most decisive developmental phase for spikelet survival in barley. Functional Plant Biology. 41(4): 424-436. DOI: http://dx.doi.org/10.1071/FP13248

ORAL OR POSTER PRESENTATIONS BASED ON THE PRESENT DISSERTATION

ORAL PRESENTATIONS

Ahmad M. Alqudah. Genetic Analysis of Pre-Anthesis Phase duration in Spring Barley (*Hordeum vulgare* L.). Triticeae workshop (ITMI and barley workshops). Plant and Animal Genome (PAG), CA, USA. January 10^{th}-14^{th}, 2015, San Diego, USA. **Invited speaker**

Ahmad M. Alqudah. Genetic Analysis of Pre-Anthesis Phase duration in Spring Barley (*Hordeum vulgare* L.). Upcoming BarleyGenomeNet (BGN), University of Viale Allegri, Reggio Emilia, Italy, Dec. 10^{th}-11^{th}, 2014

Ahmad M. Alqudah. Genetic Analysis of Pre-anthesis Development in Barley (*Hordeum vulgare* L.). Genetic Variation in Plant Breeding (GPZ, 2014). Plant Breeding Institute, Christian-Albrechts-University of Kiel, Kiel, Germany, 23^{th}-25^{th} Sep., 2014.

Ahmad M. Alqudah. Genetic Analysis of Pre-anthesis Development in Barley (*Hordeum vulgare* L.). Tenth Plant Science Student Conference (PSSC). Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany, 2^{nd}-5^{th} Jun, 2014.

Ahmad M. Alqudah. Genetics of Pre-anthesis Spike Development in Barley (*Hordeum vulgare* L.). Ninth Plant Science Student Conference (PSSC). Leibniz-Institute for Plant biochemistry, Halle (Saale), Germany, 26^{th}-31^{th} May, 2013.

POSTER PRESENTATIONS

Ahmad M. Alqudah; Raj Pasam; Benjamin Kilian; Thorsten Schnurbusch. Genetics of Pre-anthesis Spike Development in Barley (*Hordeum vulgare* L.). International Plant & Animal Genome XXI (PAG), San Diego, CA, USA, 12^{th}-16^{th} Jan., 2013.

Ahmad M. Alqudah; Benjamin Kilian; Thorsten Schnurbusch. Study of Pre-anthesis Development in Barley (*Hordeum vulgare* L.). International *Triticeae* Mapping Initiative (ITMI). Fargo, ND, USA, 25^{th}-29^{th} Jun., 2012.

Ahmad M. Alqudah; Benjamin Kilian; Thorsten Schnurbusch. The major differences between two- and six-rowed barley in growth, development and yield. Eighth Plant Science Student Conference (PSSC). Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany, 4^{th}-7^{th} Jun., 2012.

Ahmad M. Alqudah; Benjamin Kilian; Thorsten Schnurbusch. Study of Pre-anthesis Development in Barley (*Hordeum vulgare* L.). Eleventh International Barley Genetics Symposium (IBGS), Hangzhou, China, 15^{th}-20^{th} April, 2012.

Ahmad M. Alqudah; Thorsten Schnurbusch. Study of Pre-anthesis Development in Barley (*Hordeum vulgare* L.). Seventh Plant Science Student Conference (PSSC). Leibniz-Institute for Plant biochemistry, Halle (Saale), Germany, 14^{th}-17^{th} June, 2011.

PREVIOUS PEER-REVIEWED PUBLICATIONS AND BOOK CHAPTERS (PRIOR TO PHD DISSERTATION WORK)

Ahmad M. Alqudah; Nezar H. Samarah; Russell E. Mullen. (2011). Drought Stress Effect on Crop Pollination, Seed Set, Yield and Quality. In: Lichtfouse E (ed) Alternative Farming Systems, Biotechnology, Drought Stress and Ecological Fertilisation. Springer Netherlands, pp 193-213. DOI: 10.1007/978-94-007-0186-1_6

Nezar H. Samarah; **Ahmad M. Alqudah**. (2011). Effects of Late-Terminal Drought Stress on Seed Germination and Vigor of: Barley (*Hordeum vulgare* L.). Archives of Agronomy and Soil Science. 57 (1): 27–32. DOI: 10.1080/03650340903191663

Maher J. Tadros; Nezar H. Samarah; **Ahmad M. Alqudah**. (2011). Effect of Different Pre-Sowing Seed Treatment on the Germination of *Leucaena leucocephala* and *Acacia farnesiana* Species. New Forests. 42 (3): 397-407. DOI: 10.1007/s11056-011-9260-1

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Saba J. Goussous; Nezar H. Samarah; **Ahmad M. Alqudah**; Mohammad O. Othman. (2010). Enhancing Seed Germination of Four Crop Species Using Ultrasonic Technique. Experimental Agriculture. 46(2): 231-242. DOI: 10.1017/S0014479709991062.

Nezar Samarah; **Ahmad M. Alqudah**; Jumah Amayreh; Gina McAndrews. (2009). The Effect of Late-terminal Drought Stress on Yield Components of Barley in Semiarid Mediterranean Region. Journal of Agronomy and Crop Science. 195 (6): 427-441. DOI: 10.1111/j.1439-037X.2009.00387.x

Nezar H. Samarah; Russell E. Mullen; **Ahmad M. Alqudah**. (2009). An Index to Quantify Seed Moisture Loss Rate in Relationship with Seed Desiccation Tolerance in Common Vetch. Seed Science and Technology. 37: 413-422.

ORAL AND POSTER PRESENTATIONS (PRIOR TO PHD DISSERTATION WORK)

ORAL PRESENTATIONS

Nezar H. Samarah; Maha M. Al-Mahasneh; **Ahmad M. Alqudah**. Acquisitions of Seed Desiccation Tolerance and Vigor in Wheat (*Triticum durum*) Seeds during Maturation as Influenced by Drying Methods. The Fourth Conference on Scientific Research in Jordan, Amman, Jordan 7^{th}-8^{th} Nov., 2009.

Nezar H. Samarah; Russell. E. Mullen; **Ahmad M. Alqudah**. An Index to Quantify Seed Moisture Loss Rate in Relationship with Seed Desiccation Tolerance in Common Vetch. Annual Meeting of American of Official Seed Analyst (AOSA). Minnesota, USA, 5^{th}-15^{th} June, 2008.

POSTER PRESENTATIONS

Nezar H. Samarah; **Ahmad M. Alqudah**; Maha M. Al-Mahasneh. Effect of Developmental Stage of Durum Wheat on Seed Germination and Damage by *Rhyzopertha dominica* During Storage. The Twenty-ninth International Seed Testing Association (ISTA). Congress, Cologne, Germany, 16^{th}-22^{th} June, 2010.

Nezar H. Samarah; Maha M. Al-Mahasneh; **Ahmad M. Alqudah**. Acquisitions of Seed Desiccation Tolerance and Vigor in Wheat (*Triticum durum*) Seeds during Maturation as Influenced by Drying Methods. Annual Meeting of Crop Science Society of America (CSSA). Pittsburgh- Pennsylvania, USA, 1^{th}-5^{th} Nov., 2009.

Nezar H. Samarah; **Ahmad M. Alqudah**. Evaluation of Four Barley Cultivars under Terminal Drought Stress. Annual Meeting of Crop Science Society of America (CSSA). New Orleans-Louisiana, USA, 4^{th}-8^{th} Nov., 2007.

Herewith I declare that all of the aforementioned information is correct.

Ahmad M. Alqudah

13.0 Affirmation/ eidesstattliche Erklärung

Herr Ahmad M. Alqudah
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I hereby declare that the submitted thesis has been completed by me, the undersigned, and that I have not used any other than permitted reference sources or materials or engaged any plagiarism. All the references and the other sources used in the presented work have been appropriately acknowledged in the work. I further declare that the work has not been previously submitted for the purpose of academic examination, either in its original or similar form, anywhere else.

I hereby declare that I have no criminal record and that no preliminary investigations are pending against me.

Hiermit erkläre ich, dass ich diese eingereichte Dissertation selbständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Die den benutzten Hilfsmitteln wörtlich oder inhaltlich entnommenen Stellen habe ich unter Quellenangaben kenntlich gemacht. Die vorliegende Arbeit wurde in gleicher oder ähnlicher Form noch keiner anderen Institution oder Prüfungsbehörde vorgelegt.

Des Weiteren erkläre ich, dass ich weder vorbestraft bin noch dass gegen mich Ermittlungsverfahren anhängig sind.

Gatersleben, den…….

Ahmad M. Alqudah