Lehr- und Forschungszentrum für Landwirtschaft
Raumberg-Gumpenstein

60. Tagung der Vereinigung der Pflanzenzüchter und Saatgutkaufleute Österreichs 2009, 19 – 24
ISBN: 978-3-902559-37-1

Plant developmental patterns and environmental adaptation in barley

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Abstract

The effects of various environmental factors on flowering and on the activities and interactions of the photoperiod sensitivity (*PPD*) and vernalization response (*VRN*) loci were examined applying systematic phenotypic characterization in controlled growth chamber tests, functional QTL and association analyses based on gene-specific primers. The experimental materials were two bi-parental mapping populations (facultative × spring, facultative × winter) and a multi-varietal population consisting of genotypes of different geographic origins and with various growth habits. Small modifications in the controlled environment conditions led to dramatic changes in the flowering time phenotype. A genetical dissection of these changes via QTL and association analyses revealed novel effects and interactions of barley *VRN* and *PPD* genes. We hypothesize that the phenotypic reactions given to low light intensity, to sub-optimal temperature and to the synchronous application of photo and thermo cycles are connected with the mechanism and action of the circadian rhythm, which, in turn, alter the activity and role of *PPD-H1*, *VRN-H2* and *VRN-H1* in a manner distinct from that attributable to vernalization and photoperiod duration.

Keywords

Allele interactions, environmental cues, flowering, *Hordeum vulgare*, photoperiod, vernalisation

Introduction

The regulation of flowering has been dissected and evaluated to the greatest extent in *Arabidopsis* and this information was an indispensable platform for rapidly isolating the homologues from cereal crops, but still much less is known about the genetic determinants of flowering in cereals. Up till now the candidate gene sequences and functions have mostly been identified for the major genetic determinants, the *VRN* (vernalization response) and the *PPD* (photoperiod sensitivity) loci (COCKRAM et al. 2007, TREVASKIS et al. 2007, DISTELFELD et al. 2009, GREENUP et al. 2009). The advantages of barley as a model plant species include its diploid genome and a wide range of flowering time and geographical adaptation strategies.

Most of the experiments for identifying the genetic components of flowering in cereals, whether with the aims of QTL, association analyses or gene expression studies, were carried out under field or greenhouse conditions, where the various environmental factors could not be controlled completely and they acted in complex interactions. Controlled environment tests allow for trait dissection but usually a limited number of environmental cues are varied at a constrained number of levels due to the space and cost limitations. Thus the emphasis was laid mostly on the examination of the primary environmental cues, such as low temperature (vernalization response) and daylength (photoperiod response). Much less is known about the role of other environmental factors and about the signalling network through which they act. Controlled environmental tests makes it possible to dissect the complex environmental effects into individual factors (ambient temperature, light intensity, spectral composition of light, daily fluctuating factors) to study the effects of these individual factors on flowering and to identify the developmental genes, the activity of which are significantly influenced by the given environmental factor. The results of controlled environmental tests seem to prove that a small change in the parameters not affecting vernalization and photoperiod may result in dramatic variation in flowering time. However, these results underline the necessity of more careful set ups of experiments and cautious comparisons of the experimental data emerging from various environmental conditions, and they also represent challenge and possibility to identify and to better understand the regulation chains driven by various environmental cues and the interactions between major plant developmental genes. For this end it is of valuable contribution to carry out systematic characterisations of well-defined parental lines and progenies and sets of barley variety groups of different geographic origin and of various adaptation types under contrasting environments of field, greenhouse, controlled environments with constant conditions, and controlled environments with systematic introduction of varying conditions. These experimental designs make it possible to compare the effects of various treatments on the genetic determinants of flowering. This knowledge then may contribute to the manipulation of flowering without affecting major developmental requirements such as vernalization and photoperiodic response.

Thus, the aims of our research are to study the genetic determinants of flowering in barley through establishing the role of major flowering time loci, the effects of gene allele interactions and the role of various environmental factors as regulating cues. For this purpose a 'genetical phenomics' approach was applied, as we combined the functional mapping of the major genes (*VRN-H1*, *VRN-H2* and *VRN-H3* of vernalization response, and *PPD-H1* and *PPD-H2* of

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photoperiod sensitivity) of flowering, and the functional QTL analyses and association mapping with systematic phenotypic characterizations under various sets of environmental cues in controlled climatic chamber tests.

Materials and methods

Plant material

The cultivars Morex (M; spring), Dicktoo (D; facultative), and Kompolti korai (KK; winter) and the two DH mapping populations derived from the cross of D × M (DM) and D × KK (DK) used for these experiments have been well characterized at genotypic and phenotypic levels (PAN et al. 1994, KARSAI et al. 2005, 2006, 2007, 2008, von ZITZEWITZ et al. 2005, SZŰCS et al. 2006). The 169 barley varieties of the multi-varietal population originate from North and Central America (84), from Europe (75), from Asia (7) and Australia (3).

Phenotypic characterization

Controlled environmental experiments were carried out in the Phytotron facilities of the Agricultural Research Institute, Martonvásár, using Conviron PGV type growth chambers (Conviron Ltd., Winnipeg, Canada). The technical parameters of the growth chambers, including light sources and control systems for temperature and light intensity, are detailed in KARSAI et al. (2004). The individual effects of the following environmental factors were examined: two levels of light intensity at two photoperiod regimes, two levels of ambient temperature, and the effect of daily fluctuating vs. constant temperatures. The combinations of various environmental cues used for testing the developmental patterns of each bi-parental and multi-varietal population and for carrying out functional QTL analyses are listed in *Table 1*.

In the multi-varietal population, for comparing the plant developmental patterns of the varieties under long photoperiod and constant ambient temperatures, the vernalization requirements of the winter barleys were saturated. The following developmental phases were evaluated: beginning of tillering (DEV21 on Zadok's growth scale), first node appearance (DEV31), beginning of the extensive stem elongation (DEV30), flag leaf appearance (DEV37), heading (DEV49), end of the extensive stem elongation (SE_E), and reaching the final plant height (PH_final).

Genotypic characterization

The DM linkage map consists of 165 loci of various types (e.g. AFLP, RFLP, SSR, STS and ASGTs (allele-specific gene tags) with a total recombination length of 1040 cM and an average marker spacing of 6.3 cM (PAN et al. 1994, SZŰCS et al. 2006). The DK linkage map consists of 236 loci of various types, with a total recombination length of 1107 cM and an average marker distance of 4.5 cM (KARSAI et al. 2005, 2007, SZŰCS et al. 2006). The *VRN-H1*, *VRN-H2*, *VRN-H3* and *PPD-H1*, *PPD-H2* loci were mapped, when possible with allele-specific primers in the DM and DK populations (KARSAI et al. 2005, TURNER et al. 2005, von ZITZEWITZ et al. 2005). Linkage maps were constructed using JoinMap 4.0 (VAN OOIJEN 2006). QTL analyses were performed using composite interval mapping (CIM) Model 6, with forward regression and backward elimination as implemented in WinQTL Cartographer v. 2.5 (WANG et al. 2007). Threshold levels were set using 500 permutations. For the multi-varietal population, the same allele-specific primers were used.

Results

Effect of VRN-H1 on plant development in the absence of VRN-H2

In the Dicktoo × Morex population there is functional segregation in the *PPD-H1* locus, in addition to the *VRN-H1* functional polymorphism. These two loci were the major determinants of flowering in the various temperature treatments applied under long photoperiod (*Table 2*). Under all conditions the Dicktoo type winter allele in the *VRN-H1* locus, and the Morex type insensitive allele in the *PPD-H1* locus significantly delayed the plant development. Of the

Table1: **Lists of environmental factors studied in the bi-parental and multi-varietal populations**

Photoperiod (hrs)	Temperature (°C)	Light intensity (μmol m^{-2} s^{-1})	DM	DK	Multi varieties
12	18 constant	340		+	
12	18 constant	170		+	
16	18 constant	340		+	
16	18 constant	220	+	+	
16	18 constant	170		+	+
16	18/16 thermo cycle	220	+	+	
16	10 constant	220	+		

Table 2: **Effects of the *VRN-H1* and *PPD-H1* genes on flowering time in the Dicktoo × Morex mapping population under various temperature treatments**

Temperature treatment		*VRN-H1*			*PPD-H1*		
		LOD	R^2	Add. eff.	LOD	R^2	Add. eff.
18°C	Constant	22.7	30.3	9	31.2	55.0	-12
18°C	Thermo cycle	22.3	41.6	24	12.0	17.2	-13
10°C	Constant	8.7	17.1	7	18.6	47.6	-12

Table 3: **Flowering time values of the four *VRN-H1/PPD-H1* allele classes of the Dicktoo × Morex mapping population and the two parents at the various temperature treatments**

VRN-H1/PPD-H1 allele combination	Flowering time at 18°C constant temperature	Flowering time at 18°C thermo cycle	Flowering time at 10°C constant temperature	% of changes from 18°C constant temperature to 18°C thermo cycle	% of changes from 18°C constant temperature to 10°C constant
Dicktoo	36	128	61	356	169
DD/DD	38	84	60	221	159
DD/MM	75	121	89	161	119
MM/DD	30	44	53	148	176
MM/MM	45	66	71	145	157
Morex	41	54	70	132	171

two genes, *VRN-H1* determined a greater proportion of the phenotypic variance at 18°C thermo cycle and *PPD-H1* at 18°C constant and at 10°C constant temperature treatments. Compared to the 18°C constant temperature, the application of thermo cycle increased the phenotypic effects of *VRN-H1*, while significantly decreased that of *PPD-H1* to a ratio of one-third. The low constant temperature of 10°C did not influence the effect of *PPD-H1* but significantly decreased the effect of the *VRN-H1* locus.

The combined effects of *VRN-H1* and *PPD-H1* explained most of the phenotypic variation in the experiments (two-locus R^2 values were 83.5%, 74.0%, and 83.1% in the 18°C constant, 18°C thermo cycle, and 10°C constant treatments, respectively). As a result, the mean flowering times of lines with the parental allele combinations at the two loci were statistically the same as the respective parents under the two constant temperature treatments (18°C and 10°C) (*Table 3*). At 18°C thermo cycle, however, the DD (*VRN-H1/PPD-H1*) lines headed significantly earlier than Dicktoo (84 vs. 128 days, respectively), while the MM lines were significantly later than Morex (66 vs. 54 days, respectively). The non-parental allele combinations were responsible for the significant phenotypic transgressive segregation, which were apparent at each temperature treatment.

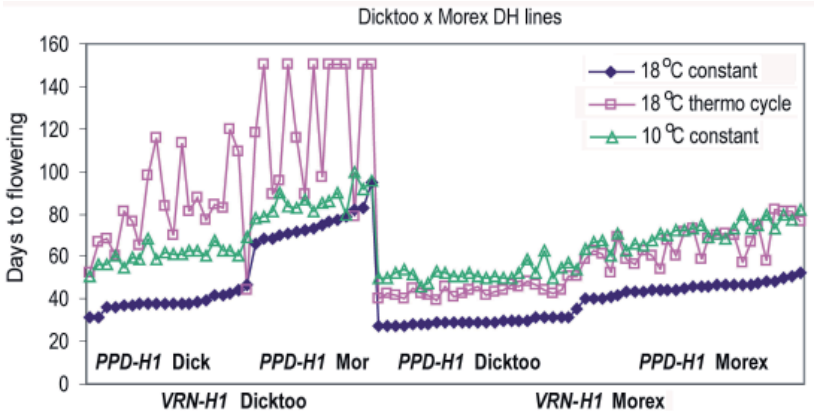

Figure 1: **Effect of sub-optimal temperature and synchronous photo and thermo cycles on major developmental genes in the Dicktoo × Morex mapping population**

Lines with MD alleles at *VRN-H1/PPD-H1* headed significantly earlier, while lines with DM alleles at *VRN-H1/PPD-H1* headed significantly later than the parents and parental allele combinations. In addition, the reactions of the non-parental combinations to a sub optimal temperature or thermo cycle were significantly different from those of the parents or parental allele combinations. The MD (*VRN-H1/PPD-H1*) was the only subclass with a relatively uniform reaction to all the treatments including the thermo cycle, but its flowering was delayed to the largest extent by the sub-optimal temperature. Conversely, the sub-optimal temperature had the smallest delaying effect on the flowering of the DM (*VRN-H1/PPD-H1*) subclass, but the largest scattering was observed in this subclass when the thermo cycle was applied (*Figure 1*).

Effect of VRN-H1 on plant development in the presence of VRN-H2

In the Dicktoo × Kompolti korai population there is only functional polymorphism in the *VRN-H2* gene, all the lines carry the sensitive allele in the *PPD-H1* locus based on the SNP22 haplotype (TURNER et al. 2005). There was allelic variation at region of *VRN-H1* not known to affect phenotype and this variation allowed us to monitor allelic segregation at this locus. When the role of the two *VRN* loci in flowering time was examined, it was found that photoperiod, light intensity, and the application of thermo cycle influenced their activity (*Table 4*). When active, the presence of the *VRN-H2* gene and the Dicktoo type winter allele at *VRN-H1* delayed plant development, irrespective to the environmental composition.

Under a long photoperiod (16 h) *VRN-H2* explained the largest portion of the phenotypic variance irrespective of the light intensity. The *VRN-H1* gene alone was only a significant though minor source of variance under high light intensity. The two genes together contributed more than 90% of the variance at both light intensities (R^2 high = 96.9%; R^2 low = 91.9%). Light intensity had the strongest effect on the *VRN-H* genes under the 12 hr photoperiod regime, which represents the borderline between long and short photoperiod regimes. While the effect of *VRN-H2* was highly significant under high light intensity, the activity of this gene could not be detected when low light intensity was applied. The effect of *VRN-H1*, on the other hand, was tripled at low light intensity. Thus under a 12 hr photoperiod more than 50% of the phenotypic variance in the flowering time was explained by *VRN-H2* under high light intensity and by *VRN-H1* under low light intensity. The bi-locus effect was highly significant at both light intensities (R^2 high = 78.8%; R^2 low = 52.8%).

In comparing the effects of constant temperature and daily thermo cycle, *VRN-H2* had a very large effect on flowering

Table 4: **Effects of the *VRN-H1* and *VRN-H2* genes on flowering time in the Dicktoo × Kompolti korai mapping population under various environmental conditions**

Treatments		*VRN-H1* LOD	*VRN-H1* R^2	*VRN-H1* Add. eff.	*VRN-H2* LOD	*VRN-H2* R^2	*VRN-H2* Add. eff.
Photoperiod and light intensity treatments							
16 hrs	340 μmol m^{-2} s^{-1}	5.1	2.0	3	32.8	89.6	-16
16 hrs	170 μmol m^{-2} s^{-1}		ns		32.3	70.3	-14
12 hrs	340 μmol m^{-2} s^{-1}	5.8	16.6	8	12.8	48.2	-14
12 hrs	170 μmol m^{-2} s^{-1}	13.5	51.6	10		ns	
Temperature treatments							
18°C	Constant	3.7	3.2	3	34.5	63.8	-12
18°C	Thermo cycle	24.6	49.5	12	11.0	15.5	-7

time at 18°C constant temperature accounting for 64% of the phenotypic variance (*Table 4*). The *VRN-H1* locus had a significant effect under this condition, but it explained a very low portion of the phenotypic variance. The application of both light and thermo cycles resulted in a shift in the significance of the effects of these two loci: at 16T *VRN-H1* explained close to 60% of the phenotypic variance and *VRN-H2* only 16%.

As the bi-locus effects of the two *VRN-H* genes contributed the highest proportion of the phenotypic variance under 16 and 12 hr photoperiod regimes, the flowering characteristics of the four possible allele combinations were compared (*Figure 2*). Under a long photoperiod the light intensity did not influence the type or degree of interaction between the allele phases of the two *VRN-H* genes. The Kompolti allele in *VRN-H2* (presence of the gene) resulted in later flowering irrespective of the light intensity level applied and this effect was not modified by the allele composition of the *VRN-H*1 gene. The lack of the *VRN-H2* gene caused earlier flowering and made the effect of the allele composition of *VRN-H1* significant under both light intensities. Under a 12 hr photoperiod, however, the light intensity exerted a strong modifying effect on the interaction between the two *VRN-H* genes. At high light intensity the interaction between *VRN-H2* and *VRN-H1* was similar to that observed for the 16 hr photoperiod, except that the importance of the *VRN-H1* allele composition increased. At low light intensity level, the quantitative effect of the *VRN-H2* gene in repressing flowering diminished significantly. The presence or absence of the *VRN-H2* gene only influenced flowering when the Kompolti korai allele was present in the *VRN-H1* gene. In the case of the Dicktoo *VRN-H1* allele, the *VRN-H2* gene had no apparent effect on flowering. When the effects of the two light intensities on flowering were compared under the 12 hr photoperiod regime, it became apparent that low light intensity only resulted in earlier flowering when the *VRN-H2* gene was present. In this case, however, its effect was mostly independent of the allele composition of *VRN-H1*.

Similar environment dependent interaction between the *VRN-H2* and *VRN-H1* genes was apparent in the comparisons of constant vs. daily fluctuating temperatures. *VRN-H2* and *VRN-H1* jointly accounted for most of the phenotypic variation, irrespective of growth condition: the two-locus R^2 values were 0.83 for 18°C constant temperature and 0.69 for 18/16°C thermo cycle. The average flowering times of lines with parental allele combinations at these two loci were statistically the same as respective parent under all the three conditions, with one exception. At 18°C constant temperature the average flowering of the DD lines was again significantly earlier than that of Dicktoo (86 vs. 109 days).

As shown in *Figure 3*, there is a pattern of growth condition-dependent epistasis between these two loci. Two features are noteworthy. First, the K allele at *VRN-H1* always resulted in

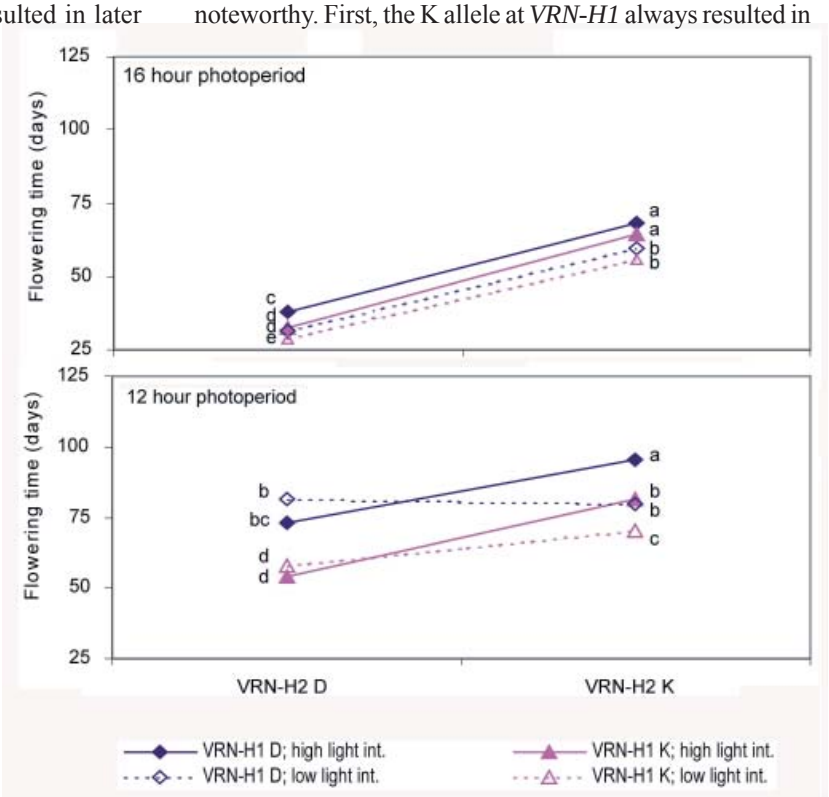

Figure 2: **Effects of photoperiod (12, 16 hr) and light intensity (high: 340 μmol $m^{-2}s^{-1}$, low: 170 μmol $m^{-2}s^{-1}$) on the association between the allele phases of the *VRN-H2/VRN-H1* genes in the Dicktoo (D) × Kompolti korai (K) mapping population, measured in terms of flowering time. (Within each photoperiod, data points labelled with the same letter were not significantly different from each other at the P=0.05 level)**

significantly earlier flowering than the D allele, regardless of growth condition or allele phase of *VRN-H2*. Second, the winter allele (K) at *VRN-H2* delayed flowering, with one exception: under 18/16°C thermo cycle, the D allele at *VRN-H1* locus resulted in extremely delayed flowering irrespective of the allele phase at *VRN-H2*.

Developmental patterns in the multi-varietal population

In the principal component analysis of the allele compositions of the 169 barleys the first factor showed a strong correlation with *VRN-H1* and *VRN-H2* in one direction and with *PPD-H1* and *PPD-H2* in the other direction underlining the higher probability of the parallel occurrences of some alleles. The putative spring and winter alleles at the *VRN-H3* locus showed a pattern more independent both from the sensitivity and growth habit groups. Taking into account the basic allele versions of the *VRN-H1*, *VRN-H2* and the two *PPD* loci, there are 16 possible allele classes. In this group of barley varieties, members belonging to 15 of these classes were identified, but the majority of them proved to represent rare combinations. There were only two classes, with frequencies higher than 10%. Of these two, the class containing 78% of the spring growth habit varieties was characterised with the dominant allele at *VRN-H1*, the recessive allele at *VRN-H2* and the insensitivity alleles at both *PPD-H* loci. The other frequent class containing 56.7% of the winter growth habit barleys could be characterized with the opposing allele combination as carried the recessive *VRN-H1*, and the dominant *VRN-H2* alleles and the sensitivity alleles at both *PPD-H* loci.

In this group of barley varieties, all the developmental phases were significantly determined by the *VRN-H* and *PPD-H* loci, together explaining more than 50% of the phenotypic variance. The only exception was the beginning of tillering. Of the loci, the allele phase in *VRN-H1* was the most significant determinant of the developmental patterns (its individual effect were between 29 and 53%), followed by *VRN-H2* (with individual effects between 22 and 38%) and the *PPD-H* loci (with individual effects between 11 and 32%), while the effect of *VRN-H3* was small, or not significant.

Comparing the developmental patterns of the *VRN-H1*, *VRN-H2* and *PPD-H1* classes, at the stage of first node appearance only two classes were significantly later, than the others, those which carried the winter alleles at both *VRN-H* loci. The time elapsed between first node appearance and the beginning of intensive stem elongation was the shortest in classes with the sensitive allele in *PPD-H1*, irrespective to the allele combination in the *VRN-H* loci. This difference between the insensitive and sensitive alleles remained throughout the further plant development. In addition, two other phenomena became evident. In the presence of the dominant *VRN-H2* allele, heading followed the flag leaf appearance significantly earlier in the classes with the sensitive *PPD-H1* allele, irrespective to the allele composition in *VRN-H1*, while in the absence of the dominant *VRN-H2* allele, the extensive stem elongation phase reached its end sooner followed up the heading in classes with the sensitive *PPD-H1* allele, irrespective to the allele composition in *VRN-H1*. These two phenomena accentuated further the plant developmental fastening effects of the *PPD-H1* sensitive allele.

Discussion

Gene expression, QTL and segregating population studies proved that the presence or absence of the vernalization critical region in the intron 1 of the *VRN-H1* gene basically determines the growth habit (FU et al. 2005, KARSAI et al. 2005, von ZITZEWITZ et al. 2005, KÓTI et al. 2006, SZŰCS et al. 2007). The spring allele (deletion of the vernalization critical region) shows complete dominance over the winter allele (YAN et al. 2004, DUBCOVSKY et al. 2006, KÓTI et al. 2006, SZŰCS et al. 2007). Our results emphasise, that in addition to determining the growth habit, the *VRN-H1* gene also quantitatively influences the flowering time, and that the *VRN-H1* gene is also subject to regulation by environmental stimuli other than low temperature vernalization. The site(s) of this additional regulation is partly different from that of the vernalization regulation site (KARSAI et al. 2005, KÓTI et al. 2006, SZŰCS et al. 2007). Photoperiod, low light intensity, the ambient temperature and the various combinations of daily fluctuating factors all practiced modifying effects on the *VRN-H1* gene in an allele specific way (von ZITZEWITZ et al. 2005, KARSAI 2008). The dominant spring allele showed greater sensitivity to the sub optimal temperature, while the synchronous photo and thermo cycles had the strongest effect on the recessive winter allele. In addition, significant differences were identified between the reaction types of two recessive winter alleles from the facultative

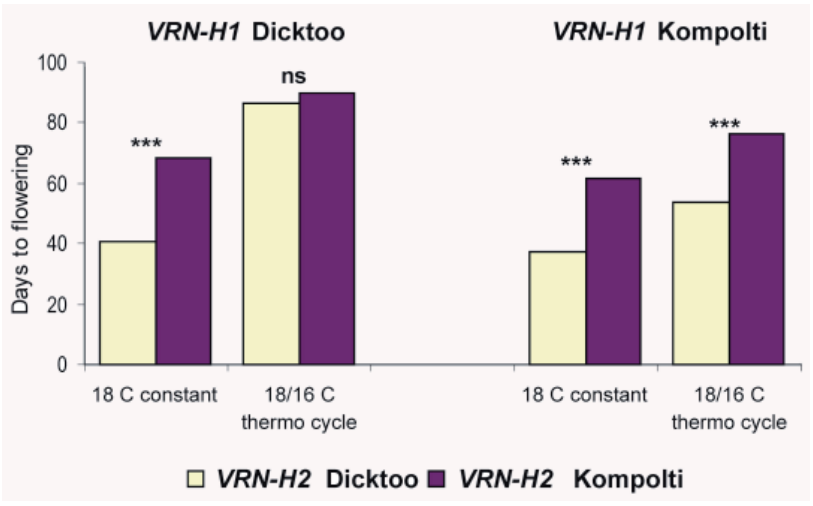

Figure 3: **Effects of daily fluctuating environmental factors on the association between the allele phases of *VRN-H2* and *VRN-H1* genes in the Dicktoo × Kompolti korai mapping population**

Dicktoo and from the winter Kompolti korai, which were completely the same in the vernalization critical region (von ZITZEWITZ et al. 2005). This may be due to as yet uncharacterised functional polymorphisms in other regions of the 17 kb gene. The Dicktoo type *VRN-H1* allele was more sensitive to the application of synchronous photo and thermo cycle than the Kompolti type allele. In addition, low light intensity differentially influenced the activating effect of the two parental recessive alleles on flowering under an intermediate photoperiod regime.

The various environmental factors influenced not only the activity of the *VRN-H1* gene, but also its specific interactions with the allele types of the *PPD-H1* and *VRN-H2*. The non-parental allele combinations in the *PPD-H1* and *VRN-H1* were responsible for the significant phenotypic transgressive segregation resulting in the early and late flowering genotypes (PAN et al. 1994, KARSAI et al. 1997). These combinations also showed specific reactions to the various environmental cues. The environmental dependent allele interactions were the most characteristic under the sub optimal temperature, and under the synchronous application of the photo and thermo cycle. There was also a pattern of growth condition-dependent epistasis between the *VRN-H2* and *VRN-H1* loci, which became evident under two growth conditions: applying low light intensity under an intermediate photoperiod regime, and the synchronous application of photo and thermo cycles.

In summary, the systematic phenotypic characterizations combined with functional QTL and association analyses proved to be efficient in identifying environmental factor dependent gene functions and allele interactions. Thus this approach produces valuable additional information to gene expression studies. The genetic dissection of the phenotypic changes via QTL and association analyses revealed novel effects and interactions of the barley *VRN* and *PPD* genes, different from that characteristic to them in the vernalization and photoperiod driven regulation pathways.

Acknowledgements

This project was funded by the OTKA NK72913, OM-00047/2005 research grants and by the Bolyai János research fellowship grant of the Hungarian Academy of Sciences.

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