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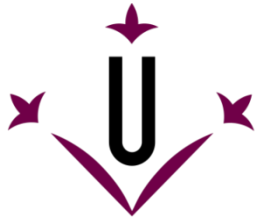
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Effects of ambient temperature in association with photoperiod on phenology and on the expressions of major plant developmental genes in wheat (*Triticum aestivum* L.)

Running head: Ambient temperature on plant development in wheat

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Abstract

In addition to its role in vernalization, temperature is an important environmental stimulus in determining plant growth and development. We used factorial combinations of two photoperiods (16H, 12H) and three temperature levels (11°C, 18°C, and 25°C) to study the temperature responses of 19 wheat cultivars with established genetic relationships. Temperature produced more significant effects on plant development than photoperiod, with

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strong genotypic components. Wheat genotypes with *PPD-D1* photoperiod sensitive allele were sensitive to temperature; their development was delayed by higher temperature, which intensified under non-inductive conditions. The effect of temperature on plant development was not proportional; it influenced the stem elongation to the largest extent, warmer temperature lengthened the lag phase between the detection of first node and the beginning of intensive stem elongation. The gene expression patterns of *VRN1*, *VRN2* and *PPD1* were also significantly modified by temperature, while *VRN3* was more chronologically regulated. The associations between *VRN1* and *VRN3* gene expression with early apex development were significant in all treatments, but was only significant for later plant developmental phases under optimal conditions (16H and 18°C). Under 16H, the magnitude of the transient peak expression of *VRN2* observed at 18°C and 25°C associated with the later developmental phases.

Key words: wheat, temperature, photoperiod, apex and plant development, *VRN1*, *VRN2*, *VRN3* and *PPD1* gene expressions

Highlights: Following vernalization ambient temperature has a significant effect in determining plant development in cereals via influencing the start and the speed of intensive stem elongation. Ambient temperature significantly influences the expression levels of *VRN1*, *VRN2* vernalization response and *PPD1* photoperiod sensitivity genes in gene specific manners indicating that these genes may participate in the regulation network of ambient temperature sensing.

Introduction

In temperate cereals the basic and best described environmental cues determining plant development are low temperatures and photoperiod, based on which, distinct categories of genotypes with or without vernalization requirements (winter vs. spring growth habits), and of photoperiod sensitivity or insensitivity can be clearly distinguished. The genetic basis of these developmental mechanisms have already been studied in detail, as a result of which the structure, allele types and functions of the major developmental genes contributing to the regulation of vernalization response and photoperiod sensitivity have mostly been revealed (Campoli & von Korff 2014; Fjellheim *et al.* 2014; Cockram *et al.* 2015; Deng *et al.* 2015).

In cereals, the vernalization requirement is determined by the epistatic interactions among three major vernalization response genes, *VRN1*, *VRN2*, and *VRN3*; *VRN1* and *VRN3* encode dominant flowering activators, while *VRN2* encodes a dominant repressor of flowering (Trevaskis *et al.* 2003; Trevaskis *et al.* 2007; Yan *et al.* 2003; Yan *et al.* 2004; Fu *et al.* 2005; Karsai *et al.* 2005; von Zitzewitz *et al.* 2005; Faure *et al.* 2007; Diaz *et al.* 2012). In a general model, flowering of a winter type cereal is repressed by high levels of *VRN2* transcript during autumn which suppress the expression of *VRN3*. During the winter months, the low temperature activated *VRN1* transcription down-regulates *VRN2* expression in parallel with the saturation of the vernalization requirement enabling the plant to flower. When the photoperiod is longer than 12 hours, *VRN1* up-regulates the expression of *VRN3*, which further enhances flowering (Distelfeld *et al.* 2009; Campoli & von Korff 2014; Deng *et al.* 2015). *PPD1* is the major gene of photoperiod sensitivity (Turner *et al.* 2005; Beales *et al.* 2007) and acts both with the photoperiod regulating pathway and the circadian clock, *PPD1* further activates the expression of *VRN3* under long photoperiods (Campoli & von Korff 2014). Thus the vernalization and photoperiodic pathways converge on *VRN3*, which

integrates the various environmental signals (Campoli & von Korff 2014). The functional polymorphisms in these plant developmental genes have been studied in detail. In the case of *VRN1*, various sequence differences were detected in the promoter, exon1 and intron1 regions of *VRN-A1*, but fewer polymorphisms have been found in *VRN-B1* and *VRN-D1* (Yan *et al.* 2004; Fu *et al.* 2005; Milec *et al.* 2013). Notably, in all three *VRN1* genes, the basis of the dominant spring growth habit alleles is a large deletion which occurred in intron1. In the case of *VRN2*, the deletion of the entire locus or point mutations in the CCT domain result in spring varieties (Yan *et al.*, 2004; Karsai *et al.*, 2005; von Zitzewitz *et al.*, 2005; Distelfeld *et al.*, 2009). In wheat, the photoperiod insensitivity alleles in the *PPD1* genes result in rapid heading irrespective of the photoperiod. While the role of *PPD-A1* is not significant in *T. aestivum*, several functional polymorphisms were identified in *PPD-B1* and *PPD-D1* leading to significant variations in heading date (Beales *et al.* 2007; Diaz *et al.* 2012; Kiss *et al.* 2014). The basis of insensitivity is the increased gene copy number in the *PPD-B1* locus, while in the case of *PPD-D1* gene it is a large deletion of 2089 bp in the promoter region leading to over-expression (Beales *et al.* 2007; Díaz *et al.* 2012; Nishida *et al.* 2013).

In addition to and building on the basic developmental mechanisms of vernalization and photoperiod sensitivity, there are several other environmental stimuli which influence the rate of plant growth and development, temperature being one of the most influential among them. Temperature as an important seasonal cue is more complex than photoperiod in relation to both its characteristics and its effects. Photoperiod follows a predictable pattern from year to year, whereas the temperature profile of a given area can show tremendous and unpredictable variation among seasons, years and even within 24 hours. On the plant level, increases in temperature accelerates crop development more than growth, thus the period available for the production of biomass shortens to the extent that plants at the time of harvest are usually smaller and with fewer tillers (Atkinson & Porter 1996). Thus the physiologically optimal

temperature can be defined as the range in which the plant growth and development are proceeding at maximal rate but there are no strong negative effects on plant biomass production. Temperatures extending beyond (heat stress) or below (cold stress) of this range may severely affect plant fitness and survival (Bahuguna & Jagadish 2014). The majority of research has focused on understanding plant responses to these extreme temperatures, with limited attention directed to the dissection of the molecular mechanisms underlying plant responses to changes in the temperature range between the two extremes.

All crops and all phases of development are sensitive to temperature; (Slafer *et al.* 2015), but there is genotypic variation in the magnitude of response (Rawson & Richards 1993; Slafer & Rawson 1995). The exact nature of the involvement of temperature in the genetic regulatory network for flowering is not completely understood yet. In *Arabidopsis thaliana*, the existence of a thermosensory pathway has already been established, which acts via complex crosstalk both with the low-temperature vernalization and photoperiod pathways (Wellmer & Riechmann 2010). This interplay of temperature, light and endogenous factors in temperature signalling confer adaptive plasticity to plants under fluctuating seasonal conditions. In *A. thaliana*, the existence of natural variation in responses to temperature has also been revealed, though it was found to be smaller in magnitude than that of responses to vernalization and photoperiod (Lempe *et al.* 2005).

The experimental data suggest that the control of the floral transition in response to temperature is regulated differently in different plant species (Hemming *et al.* 2012; Capovilla *et al.* 2015). Compared to *A. thaliana*, much less is known about the genetic components of temperature sensing in cereals, where most of the research has been conducted to evaluate the effects of temperature on plant development and on the duration of the different plant phenophases. It has been proven that there is a wide range of responses to temperature among cereal genotypes (Slafer & Rawson 1995; Slafer & Rawson 1996;

Atkinson & Porter 1996; Karsai *et al.* 2013). In addition, the sensitivity of cereal genotypes to temperature was proven to show marked changes in association with the various developmental phases (Slafer & Rawson 1996; Borrás-Gelonch *et al.* 2012; Karsai *et al.* 2013). With very few exceptions, however there is limited information either on the genetic components of temperature sensing or on how temperature influences the activities of the major plant developmental genes controlling vernalization requirement and photoperiod sensitivity (Bullrich *et al.* 2002; Appendino & Slafer, 2003; Lewis *et al.* 2008; Borrás-Gelonch *et al.* 2010; Hemming *et al.* 2012). In a series of field experiments differing in photoperiod and temperature levels Borrás-Gelonch *et al.* (2012), suggested that the magnitude of the *PPD-H1* photoperiod sensitivity gene affecting plant development was influenced not only by the daylength but also by the average temperature of the field. However, the experimental set up and the population type used does not make it possible to determine exactly the type of associations between temperature and plant development. Hemming *et al.* (2012) examined the changes in gene expressions of plant developmental genes in certain barley cultivars under long and short photoperiods applying two temperature levels, and they did not identify any significant changes in the expressions of the vernalization response (*VRN-H1*, *VRN-H2*, *VRN-H3*) and the photoperiod sensitivity (*PPD-H1*) genes across the temperature levels. In wheat there is even less information on the genetic background of temperature response, though the interaction between temperature and photoperiod has been suggested to be a significant component in eliciting different developmental responses of wheat genotypes (Slafer & Rawson 1996; Borrás-Gelonch *et al.* 2012).

In the present work our first objective was to study the effect of temperature via its interaction with photoperiod on plant development of wheat by evaluating both apex development and series of consecutive developmental sub-phases from seedling stage to

heading. In addition, we examined how the expression patterns of the three major vernalization response genes (*VRN1*, *VRN2* and *VRN3*), and the major photoperiod sensitivity gene (*PPD1*) are influenced by temperature under different photoperiods and finally we aimed at establishing causal relationships between the gene expression levels and the various plant developmental phases. For this purpose we conducted a series of experiments using factorial combinations of two photoperiod and three temperature levels under controlled environments to exclude the possible confounding effects of other environmental factors. Under these conditions the plant developmental patterns of 19 wheat cultivars of various geographic origins were thoroughly evaluated. In addition, the time course changes (both thermal and chronological) in the expression of the major vernalization response and photoperiod sensitivity genes were also evaluated in a subset of 11 wheat cultivars.

Materials and methods

Plant materials

In order to examine the effect of temperature in association with photoperiod in wheat, 19 wheat cultivars of various geographic origins were included into a series of controlled environmental tests. The basic information on the cultivars is listed in Table 1. In parallel to the controlled experiments discussed in this article, we also determined the vernalization response, and photoperiod sensitivity of these cultivars, and measured their heading date under field conditions and included these data in Table 1 as additional information.

Phenotypic characterization

The controlled tests consisted of the factorial combinations of 2 photoperiods (16 hour light period in a 24-hour day, referred to as LD; and 12 hour light period in a 24-hour day referred to as SD) and three constant ambient temperature levels (11, 18 and 25°C) in the phytotron

facilities of the Centre of Agricultural Research of the Hungarian Academy of Sciences (MTA ATK), Martonvásár, Hungary, with all the other environmental factors kept uniform across the treatments. The temperature levels we used in the experiments were between the two extremes of cold or heat stress. As Porter & Gawith (1999) identified the optimum ambient temperature for wheat being in the range of 17–23°C over the course of the entire growing season, the three temperatures we applied represented sub-optimal (11°C), optimal (18°C) and supra-optimal but no-heat-stress (25°C) temperature levels. To refer to this range we use the term of ambient temperature only for simplifying the presentation of our results. The same three Conviron PGR-15 growth chambers (Controlled Environments Limited, Winnipeg, Canada) were used for these purposes, each representing one temperature level; first the three temperatures at LD were tested in parallel, which was followed by the three temperature treatments at SD. The light source was metal halide lamps and the light intensity was set to 240 $\mu\text{molm}^{-2}\text{s}^{-1}$ photosynthetic photon flux density (PPFD), at plant level. For all the experiments, the plants were germinated in Jiffy pots at room temperature for one week, which was followed by the vernalization treatment at 3°C for 45 days under short photoperiod (9 hour light period in a 24-hour day) and low PPFD (20 $\mu\text{molm}^{-2}\text{s}^{-1}$). At the end of the vernalization the plants were at 1-2 leaf stages. They were then transplanted into pots 12 cm in diameter and 18 cm in height, with a soil capacity of 1.5 kg filled with a 4:1 mixture of garden soil and sand, and they were placed into the growth chambers. Four plants of each genotype were planted in two pots as replications giving an average plant density of approximately 60 plants/m² in the chamber space.

The plants were measured in 5-day intervals for number of leaves and plant height from soil to last leaf sheath on the main stem. In comparing the different treatments, thermal time (TT) was calculated as the sums of daily average temperatures (i.e. assuming a base temperature of 0°C and an optimum temperature higher than 25°C for all G × E conditions analysed). The

connection between thermal and chronological times under the various ambient temperature levels is listed in Supplementary Table 1. The associations between thermal time and time course data for plant height, and number of leaves were calculated as described by Kiss *et al.* (2011). Regular monitoring of the plant developmental traits made it possible to identify a series of consecutive phenophases that spanned the life cycle from seedling stage to attainment of maximum plant height. Thus, the phenophases were defined based on the Zadoks' scale (Zadoks *et al.* 1974) as follows: appearance of the first main stem node (ZD31), onset of intensive stem elongation (ZD30), appearance and full expansion of the flag leaf (ZD37, ZD39), booting stage (ZD49), heading (ZD59) and the end of intensive stem elongation (ZDSE). The experiments were terminated after 140 days at 25°C (equivalent to 3500 °Cd on thermal time), 150 days at 18°C (2700 °Cd) and 180 days at 11°C (2000 °Cd). For genotypes not reaching a given developmental stage till then the respective thermal time values were given for statistical purposes.

In each treatment, sets of extra plants for each genotype were also grown for regular sampling of three plants as replications (at 100, 200, 300, 500, 1000, and 1500 °Cd, if necessary) in order to examine the apex structure by measuring the length of the apex and evaluating the apex developmental phase using the Waddington scale (Waddington & Cartwright 1983). In addition, the thermal time to double ridge (DR) and terminal spikelet (TS) formation in the apex was determined for all the genotypes in all treatments.

The statistical analyses were carried out using Statistica 6, GenStat 18.0 and SPSS 16.0 program packages. The variance components were estimated using a restricted maximum likelihood method (REML). Broad-sense heritability (h^2) was calculated as genetic variance divided by total variance. The phenotypic diversity of the 19 wheat cultivars based on the trait matrix (19 genotypes \times 6 environments \times 16 phenology traits) was established using the UPGMA grouping module in Statistica 6.

Genotypic characterization

The major allele types of the 19 wheat cultivars in the *VRN* and the *PPD* genes (Table 1) were determined using gene allele specific primers as listed in Kiss *et al.* (2014). In addition, they were subjected to the 15K Illumina SNP platform (TraitGenetics Ltd, Gatersleben, Germany), which resulted in 4971 polymorphic markers after leaving out those with any missing values, being monomorphic, or rare alleles (characteristic to only one genotype) or showing complete linkage within the same genetic distance in the consensus marker map provided by the company. The genetic diversity of the 19 wheat cultivars was established by calculating the Kinship matrix in the TASSEL 5.0 program.

Studying the gene-expression levels

For studying the gene expressions of the major plant developmental genes, leaf tissues were collected at 0, 100, 200, and 300 thermal times (°Cd) after the end of the vernalization treatment. The last fully expanded leaf was collected one hour after the beginning of the light cycle in each particular treatment. The time-point for sample collection was chosen based on the results of a previous experiment (Allard *et al.*, personal communication), where no strong diurnal cycle was observed for the studied genes. The only exception was *PPD1*, the expression level of which allowed discriminating photoperiod sensitive and insensitive genotypes only at the start of the day. Leaves of three plants were pooled for one biological replicate, with a total of three biological replicates. Total RNA was isolated using the Qiagen RNeasy plant mini kit after Trizol extraction, with an extra step of DNase treatment programmed in the QIAcube equipment (Qiagen Ltd). The cDNA transcription was performed from 1.0 µg of total RNA using the RevertAid First Strand cDNA synthesis kit (Thermo Scientific Ltd) with the standard protocol provided by the company.

For the expression studies 11 wheat cultivars (listed with bold and italic letters in Table 1) were selected to represent the three phenotypic subgroups which were identified based on the plant developmental patterns (as shown in Results). Changes in the expression levels were analysed for the vernalization response genes, *VRN1*, *VRN2* and *VRN3*, and for the photoperiod sensitivity gene *PPD1* using the generic gene specific primers listed in Supplementary Table 2. The primer pairs amplify all homoeoalleles (A, B and D copy) of each studied gene. The quantitative real-time PCR was carried out in three biological and two technical replicates in a Rotor-Gene Q equipment (Qiagen Ltd) applying the syber-green technology of the company. Expression was normalized to Actin using the Rotor-Gene software, which also takes amplification efficiency into account.

In order to establish causal relationships between the gene expression levels and the various plant developmental phases Principal Component Analysis (PCoA) was carried out on the data matrix of the 11 wheat cultivars containing the gene expression data and the apex developmental data measured at 0, 100, 200, and 300 °Cd as well as plant phenology data. In order to simplify the complex associations we distinguished three, partially technical categories of plant development; (1) early apex development (EA) stands for the Waddington stage and apex length measured at 0, 100, 200, and 300 °Cd, (2) the late apex development (LA) includes the thermal times for reaching double ridge (DR) and terminal spikelet (TS) formation and the interval between these two phases in thermal time (1 and 2 show opposing signs in correlations, making it necessary to separate them), while (3) later plant developmental stages (post TS) cover the thermal times between TS and first node appearance (ZD31TS), and those of first node appearance (ZD31) and booting (ZD49).

Results

Phenology in the context of temperature × photoperiod

When development was evaluated in thermal time requirements of each phases, the effects of temperature were stronger than those of photoperiod on plant development, especially on the two phases of DR and ZD30. At these developmental phases the temperature effect amounted to one third of the variance, and was also dominant for all the other phases with around a quarter of the variance explained (Table 2). The effect of photoperiod was limited during the early developmental phases, but continuously increased starting from the onset of the intensive stem elongation, reaching its maximum at ZD49, ZD59 and ZDSE phases when the photoperiod contributed to about 22% of the phenotypic variance. Genotype as a main variance component was significant at all the developmental phases, covering a larger portion of the variance at the earlier development, especially at the TS and ZD31 phases (30-40%). Following these phases the effect of genotype gradually decreased to its lowest value of 19.8% at the ZDSE phase. Genotype × temperature interaction covered a significant proportion of the variance at all the phases, being the highest around the terminal spikelet formation and onset of the intensive stem elongation. The genotype × photoperiod and the three way interactions were also significant variance components, but only explained less than 10% of the variance in most of the cases. The interaction between photoperiod and temperature was not significant throughout the development. Due to the high genotypic ratio, the broad-sense heritability/reproducibility (h^2) values of the developmental phases were significant and high, between the values of 0.649 (for ZDSE) and 0.80 (Z31).

The 19 wheat genotypes represented a wide genetic pool with two separate groups organised by the geographic origin (Fig 1). One group contained all the West-European wheat cultivars,

while the Hungarian cultivars together with the two American genotypes were placed into the other group. The population structure was moderately associated with the *PPD-D1* allele type. The insensitive allele was more frequent in the Hungarian and American wheats, but there was one insensitive genotype among the West-European wheats, as well. When the phenotypic responses under the six environments were taken into account, the *PPD-D1* allele type became the major separating factor; the reaction patterns of the six wheat genotypes carrying the insensitive allele (PPDins) were distinctly different from those with photoperiod sensitivity (PPDsens). Three cultivars of the sensitive group however (PPDsens, VRN++), with the largest vernalization reactions (Suppl Fig 1.) were the most distinct of all. Due to the strong group patterns detected in the phenotypic values, the results will be presented as averages over the genotypes belonging to each of the three groups in the following sections of the article.

The developmental patterns and the responses to photoperiod and growing temperature were significantly different between the three phenotypic groups (Fig 2). The ***PPDins group*** exhibited the fastest plant development under all environmental combinations. Temperature and photoperiod effects were small even if the LD 18°C, LD 11°C and SD 18°C treatment exhibited a small but significantly faster developmental time than LD 25°C, SD 25°C and SD 11°C. In this group, the duration of early phases until terminal spikelet formation (TS) within the whole plant development decreased parallel to the lower temperature levels, irrespective to photoperiod (Fig 3a). The effect of temperature on the interval length between the first node appearance (ZD31) and the beginning of intensive stem elongation (ZD30) however was strongly influenced by the photoperiod, there was a significant increase in Z3031 due to 11°C only under SD. Other developmental phases were relatively proportional at all the six environments.

Under LD, the response patterns of the *PPDsens group* and the relative duration of the phases were very similar to those of the PPDins (Fig 2 and Fig 3b). Under SD, however the effect of temperature became very pronounced on the development of the PPDsens group. High temperature significantly delayed plant development which was the most evident in the interval between first node appearance (ZD31) and the start of intensive stem elongation (ZD30) (Fig 3b). At SD 25°C, the interval of Z3031 took 28% of the whole developmental span mostly at the expense of the interval of the start of intensive stem elongation (ZD30) to flag leaf appearance (ZD37); the flag leaves had already expanded before the start of intensive stem elongation.

The reaction patterns of the *PPDsens, VRN++ group* were influenced by the temperature to the largest extent (Fig 2). The plant development of this group was extremely delayed both by the 25°C temperature and by SD. Plant development completed normally only at LD 18°C, LD 11°C and SD 18°C, under all the other conditions their development stopped at various developmental stages (Fig 3c). At 25°C they reached the ZD31 stage, but the intensive stem elongation did not start at all (LD) or was extremely delayed (SD) as it can be seen by the increased ratio of Z3031 interval in Fig3c. Under SD 11°C again the process of intensive stem elongation was disrupted, but in a different way; the intensive stem elongation started right after the first node appearance and proceeded till the flag leaf extended. Afterwards however the stem growth stopped between booting (ZD49) and heading (ZD59), as a result of which the heads remained in the flag leaf sheaths in most of the cases.

Expression patterns of the major developmental genes in the context of temperature × photoperiod

In the case of 11 wheat cultivars, the expression patterns of *VRN1*, *VRN2*, *VRN3* and *PPD1* genes were also evaluated at early stages of the plant development at four thermal time points

of 0, 100, 200, and 300 °Cd for all treatments. In addition, at the same thermal time points the apex developmental stages were also determined.

In analysing the gene expressions both the thermal time and the calendar time course data were compared in order to be able to determine the true effects of the environmental factors on the individual gene (Fig 4 and Suppl. Fig 2). This parallel evaluation revealed that with the exception of *VRN3*, temperature significantly influenced the gene expression levels of the major plant developmental genes, *VRN1*, *VRN2* and *PPD1*, the magnitude of which was dependent on the photoperiod.

The *VRN1* transcript was already present at a low level by the end of the vernalization treatment (Fig 4a). This transcript level remained the same at 25°C, irrespective to the photoperiod, showed only a slight increase under 18°C by 300 °Cd, while the largest increase (6-7 fold) occurred at 11°C. *VRN1* expression in the three phenotypic groups was compared at 300 °Cd, when its average level was the highest. Under LD, the difference between the PPDins and PPDsens increased with the lower temperature and became significant at 11°C, while under SD, PPDins had higher *VRN1* level not only at 11°C but also at 25°C (Fig 5a). The *VRN1* level of the PPDsens, VRN++ group was significantly the lowest at 25°C and 18°C, but at 11°C this group had the same gene expression level as the PPDsens group.

VRN3 was not yet expressed at the end of the vernalization treatment; its expression increased with thermal time (Fig 4c). The increase in gene transcript however proved to be proportional to the chronological time elapsed till the same thermal time equivalents of the three temperatures, especially under LD (Suppl Fig 3c). At its maximum transcript level, the *VRN3* expression of the PPDins group was significantly the highest at 18°C and 11°C under LD, while only at 11°C under SD (Fig 5c). The *VRN3* level of the PPDsens, VRN++ group was significantly the lowest in all treatments.

Temperature had a very strong effect on the expression pattern of *VRN2* (Fig 4b). The *VRN2* transcript level was very low at the end of the vernalization treatment. *VRN2* expression slightly increased after the transfer to 11°C; at higher temperatures, however *VRN2* showed a significant increase to a peak level which was then followed by a decrease as development proceeded. At 18°C this peak was smaller and occurred at 100 °Cd (around 10 – 12 days in chronological time), which was followed by a gradual decline. At 25°C, the peak occurred at 200 °Cd (around 7 – 8 days in chronological time) and was significantly larger, followed by a sharper decline. The effect of the photoperiod increased with the higher temperature levels. At 11°C and 18°C there was no difference between the two photoperiods, while at 25°C, photoperiod had a very strong modifying effect. A temperature of 25°C together with LD resulted in the highest transient increase of the *VRN2* expression. Under LD the transient expression peak was the highest in the case of the PPDsens, *VRN++* group, irrespective to the temperature level, while the PPDins and PPDsens groups had similar activities (Fig 5b). This tendency was also characteristic of the SD 18°C and SD 11°C treatments. Under SD 25°C, however the PPDins cultivars had the highest transient expression of *VRN2*.

The appearance of transient expression peaks at the higher temperature levels (18°C and 25°C) was also characteristic of the *PPDI* gene (Fig 4d). The magnitudes of the transient peaks were similar, the differences were only apparent in the timing of the peak and in the significance of photoperiod as a modifying factor. At 18°C the transient expression occurred only under SD with a peak at 100-200 °Cd (10 - 12 days) followed by a gradual decline, while at 25°C the pattern of this transient expression was independent of the photoperiod, with a peak at 200 °Cd (5 – 6 days), followed by a sharper decline. *PPDI* activity was lowest at 11°C and showed a gradual but continuous decrease in expression over both time courses. Under LD the *PPDI* activity in the PPDins group was 2 – 4 times higher compared to the two PPDsens groups, irrespective to the ambient temperature (Fig 5d). Under SD this difference

between the insensitive and sensitive groups mostly diminished and only at 25°C was the activity of the insensitive group somewhat higher.

The associations between the gene expression levels, the apex and later plant developmental phases (as defined in the MM section) across the 11 wheat genotypes were also evaluated for all the six environments applying principal component analyses. Under the standard environment of LD 18°C, the first 5 factors had Eigenvalues higher than 1, and together they explained 91.6% of the variation. The *VRN1*, *VRN3* gene expression appeared to be closely correlated with each other and also with the early developmental phases of the apex as characterised by the Waddington scale (WD) and the apex length (APL) (Fig 6); the higher expression levels were significantly associated with the more developed and longer apices. Opposing to this group was *VRN2* expression together with both the early apex development (formation of double ridges (DR) and terminal spikelet (TS)) and the later plant developmental phases (interval between terminal spikelet and first node appearance (Z31TS), first node appearance (ZD31), and booting stage (ZD49), underlining the strong negative correlations between these two groups. The *PPD1* activity was independent from all the other variables.

The changes in the association patterns between gene expressions and development were evaluated by correlating the significant background PCoA factors of the traits under the various environments (Suppl. table 3 and 4), the results of which are summarised in Table 3. Under LD, the *VRN1*, *VRN2*, and *VRN3* expression levels strongly correlated with the early apex development, irrespective to the temperature. Higher *VRN1* and *VRN3* levels were paralleled with more developed apices, while in the case of *VRN2* the correlation was opposite. The association of the expression levels with the later apex and plant developmental phases were temperature dependent for all the *VRN* genes. The correlation was similar for *VRN1* and *VRN3*. At 11°C there was connection only with the early apex development, at

18°C the correlation was significant at all stages, while at 25°C their expressions correlated with apex development until the later stages, but not with the plant development. In contrast the *VRN2* expression significantly correlated with early apex, later apex and later plant development both at 18°C and 25°C. The *PPD1* gene expression showed significant association with apex and plant development only at 11°C, and this was strongest with the later plant developmental phases.

Under SD, the associations between gene expressions and developmental phases were less pronounced and more variable across the temperatures. The early apex development correlated with the *VRN3* expression to the greatest extent at all three temperature levels, followed by the negative association with *VRN2*. Though the correlation between *VRN1* expression and apex development was weak or not significant, the activity level of this gene strongly correlated with the later plant developmental phases at 18°C and 11°C.

Discussion

Temperature and photoperiod are two of the most tractable environmental stimuli which influence the rate and development of plants. Here we have presented a series of factorial experiments which start to dissect the combined roles of temperature and photoperiod. In particular we further investigate the previous observations which show that the effects of temperature signalling are concentrated on certain developmental phases (Slafer & Rawson 1996; Borrás-Gelónch *et al.* 2012; Karsai *et al.* 2013).

Based on the response patterns to ambient temperature the wheat cultivars presented formed three distinct categories: fully vernalized (or with spring habit) photoperiod insensitive, fully vernalized photoperiod sensitive, and partially vernalized photoperiod sensitive genotypes, which are similar to categories observed in barley (Karsai *et al.* 2013). This classification

highlights that photoperiod sensitivity and vernalization requirement largely determines the temperature response. Our results indicate that in temperate cereals ambient temperature as an environmental cue acts on plant development under inductive photoperiods for that genotype, reinforcing its effect on the sensitive genotypes during and after vernalization. This is of particular significance when considering the effects of warm periods during winter or early spring which would allow periods of active growth.

Our results identify that temperature most significantly influenced the process of intensive stem elongation. This was most apparent in the photoperiod sensitive genotypes, particularly under the 12 hour photoperiod. As in barley, where the beginning of intensive stem elongation was found to be determined by higher temperatures (Borras-Gelonch *et al.* 2012; Karsai *et al.* 2013) we found compartmentalization in how temperature acted on wheat stem elongation. A warmer temperature delayed the start of stem elongation irrespective of vernalization whilst lower temperatures only influenced genotypes with partially saturated vernalization requirement, which often caused the premature abortion of stem elongation around the booting stage.

To start to understand the molecular basis of how temperature and photoperiod interact we measured the expression of the major vernalization response (*VRN1*, *VRN2*, and *VRN3*) and photoperiod sensitivity (*PPD1*) genes. Our findings identify that temperature exerted significant effects on the expression of most of these genes in a gene-specific manner, which was modulated by photoperiod to a small extent. *VRN3* was the exception, which under long photoperiod showed its expression to be chronologically regulated and not influenced by temperature, which is in complete agreement with results in barley (Hemming *et al.* 2012). Ambient temperature had the strongest and opposing effect on the expression of *VRN1* and *VRN2*. Higher temperatures down regulated the expression of *VRN1* while the expression of *VRN2* increased, although this increase in expression was transient. This trend of increased

VRN2 expression under warmer temperatures was clearest in the partially vernalized genotypes but remained true, with a lower amplitude, in the fully vernalized genotypes. Our results suggest that *VRN2* has an important role in determining plant development even after vernalization, which further expands its role from that proposed by Yan *et al.* (2004) and Fjellheim *et al.* (2014). The expression of *PPD1* also showed a transient increase under both long day and short day photoperiods, the pattern of which varied depending on temperature. The mechanism of this regulation may relate to its sequence and possible functional similarity to its *A. thaliana* ortholog *AtPRR7* which is part of the circadian clock network and involved in temperature sensing (Farre *et al.* 2005). Evidence shows that the cereal *PPD1* is under circadian regulation as well as participating in a photoperiod regulating pathway which has evolved specifically in the grasses (Campoli *et al.* 2012).

The results also show that the expression levels of *VRN1* and *VRN2* strongly correlated with the apex development and later plant development phases. Notably the association between *VRN1* gene expression and the different developmental stages, especially the later ones, was only significant under fully inductive conditions. This indicates that partially different regulation mechanisms exist for the generative apex development and the later plant developmental phases. Particularly interesting is the observation that there is a photoperiod dependent reverse turn in the correlation between *VRN2* and later plant developmental phases. Under long photoperiod with increased temperature higher *VRN2* expression was associated with delayed development however under the short photoperiod the transient high *VRN2* expression peak was most pronounced in the photoperiod insensitive genotypes, which showed normal initiation and procession of stem elongation.

One of the significant differences between the genetic control of *A. thaliana* and temperate cereals is that in *A. thaliana* the vegetative – generative transition and the generative apex development take place synchronously with the process of bolting, thus the factors

determining bolting practices inseparable effects on flower development (Holt *et al.* 2014). In cereals, however these two processes are partially separated from each other; the generative development of the apex with the formation of terminal spikelet has already been accomplished by the time of the beginning of intensive stem elongation. Our results show that the regulation of vegetative to generative apex and the subsequent stem elongation respond to distinct environmental signals, with this partially independent regulation being more evident under non optimal conditions for a particular genotype. In barley, candidate genes from early maturing (*eam*) loci were identified to belong to the circadian clock and when mutated not only resulted in early heading but also in faster plant and stem growth rates (Faure *et al.* 2012; Campoli *et al.* 2013; Boden *et al.* 2014). These, combined with our own findings suggest that intensive stem elongation phase could be under the control of the circadian clock, possibly in a more direct way than the apex to generative phase which are controlled by the gene cascades of vernalization and photoperiod (Dubcovsky *et al.* 2006; Fjellheim *et al.* 2014). If so, then the environmental factors such as ambient temperature and light quality which influence the entrainment of the clock should also strongly influence stem elongation. Here we demonstrate that ambient temperature exerts such an important impact on stem elongation in wheat. This intricate regulating system which integrates temperature signals in the later developmental phases builds on the primary control mechanisms of vernalization and photoperiod, and leads to the environmentally driven plasticity of plant development, as such it is an important element in ecological adaptation in cereals.

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Author's contributions

I.K. and S.G. designed the research, T.K, A.S, J.B., M.M. and K.B. performed the experiment, T.K, A.S., L.D. and K.B. processed the data, I.K, T.K, V.A. S.G, G.S. O.V., and G.G. analysed and interpreted the data, T.K, L.D. and I.K wrote the manuscript, S.G, L.D., V.A., G.S., G.G, and O.V. edited the manuscript and all authors approved the final version.

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Table 1 List of the wheat genotypes examined under the factorial combinations of two photoperiod and three temperature levels in controlled experiments

Abbr	Name	Origin	Spring allele ¹	Insensitive allele ²	Vern resp ³ (days)	Photoperiod sens ⁴ (days)	HD in field ⁵
AT1	<i>Mv Toborzó*</i>	H	-	PPD-D1	44	2	133
AT2	<i>Mv Verbunkos</i>	H	-	-	50	23	146
AT3	<i>Tommi</i>	D	-	-	66	44	146
AT4	<i>Ellvis</i>	D	-	-	68	67	146
AT5	Plainsmann V	USA	-	PPD-D1	49	-2	131
AT6	Mv Magma	H	VRN-B1	PPD-D1	44	1	144
AT7	Soissons	F	-	PPD-D1	52	-4	138
AT8	Fleischman 481	H	-	-	30	29	139
AT9	Mv Hombár	H	-	-	43	29	142
AT10	<i>Ukrainka</i>	UKR	-	PPD-D1	51	0	137
AT11	<i>Avalon</i>	GB	-	-	56	37	147
AT12	<i>Cadenza</i>	GB	VRN-A1	-	4	28	145
AT13	<i>Spark</i>	GB	-	-	18	42	151
AT14	Rialto	GB	-	-	18	51	149
AT15	<i>Paragon</i>	GB	VRN-A1, VRN-B1	-	5	53	147
AT17	Weebil	Mex	VRN-D1	PPD-D1	9	5	141
AT18	Savannah	GB	-	-	43	33	149
AT19	<i>Buster</i>	GB	-	-	43	36	146
AT20	<i>Charger</i>	GB	-	-	67	61	145

1 Presence of spring allele(s) in the vernalization response loci of VRN1

2 Presence of insensitive allele(s) in the photoperiod response loci of PPD1

3 Difference in heading date (ZD59) after 0 and 45 days vernalization

4 Difference in heading date (ZD59) at 12 and 16 hours photoperiods (after 45 days vernalization)

5 Days to heading from Jan 1 (ZD59) in autumn sown experiment, Martonvásár, 2014/15; LSD_{0.05} = 1.8 days

* Genotypes with bold and italic fonts are included to the gene expression studies

Table 2 Effects of genotype, photoperiod and temperature on the consecutive developmental phases evaluated in thermal times as characterised with their respective percentages of the total variance in wheat by using the MLM module of GenStat 18.0

Variance comp (σ^2 (%))	df	DR	TS	ZD31	ZD30	ZD37	ZD39	ZD49	ZD59	ZDSE
Residuals	1	0.3	0.6	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Genotype (G)	18	28.1*	32.3*	39.4*	21.1*	26.8*	25.9*	23.3*	24.1*	19.8*
Photoperiod (P)	1	0.9	4.9	2.9	15.1	14.6	18.3	21.9	21.3	22.8
Temperature (T)	2	34.8*	26.0	26.1	29.6*	26.5	25.1	22.4	21.5	22.0
G x P	18	12.9*	9.0*	4.5	6.8*	7.6*	8.4*	10.0*	9.5*	8.3*
G x T	36	12.1**	20.4***	18.4**	15.0**	17.8**	16.5***	12.9**	13.0**	13.9**
P x T	2	1.3	0.0	0.2	1.9	0.0	0.0	1.1	1.7	1.7
G x P x T	36	10.4***	6.8***	8.3***	10.3***	6.6***	5.6***	8.3***	8.9***	11.4***
h^2		0.696	0.722	0.801	0.686	0.690	0.695	0.686	0.695	0.649

DR: double ridge; TS: terminal spikelet; ZD31: first node appearance; ZD30: onset of the intensive stem elongation; ZD37: flag leaf appearance; ZD39: flag leaf fully expanded; ZD49: booting stage; ZD59: heading; ZDSE: end of the intensive stem elongation

Table 3 Associations between the gene expressions and the apex and plant developmental patterns under the six environments of two photoperiods × three temperatures evaluated on the data matrices of 11 wheat cultivars based on the r values between the significant background PCoA factors of the various traits.

(the largest r values over the three sampling times and over the different early and late developmental phases subtracted from Suppl Tables 3 and 4 are presented at each cell)

Envir	<i>VRN1</i>			<i>VRN2</i>			<i>VRN3</i>			<i>PPD1</i>		
	Early apex ¹	Late apex ²	Plant stages ³	Early apex	Late apex	Plant stages	Early apex	Late apex	Plant stages	Early apex	Late apex	Plant stages
LD_25C	0.97	-0.79	ns	-0.95	0.93	0.96	0.93	-0.84	ns	ns	ns	ns
LD_18C	0.99	-0.85	-0.93	-0.97	ns	0.99	1.00	-0.90	-0.84	ns	ns	ns
LD_11C	0.95	ns	ns	-0.79	ns	ns	0.89	ns	ns	ns	0.71	0.82
SD_25C	0.88	ns	ns	ns	ns	ns	0.90	-0.78	ns	-0.83	ns	ns
SD_18C	0.74	-0.93	-0.91	-0.88	0.91	0.88	0.95	ns	ns	-0.70	ns	ns
SD_11C	ns	ns	-0.92	-0.83	ns	ns	0.84	ns	-0.92	ns	ns	ns

¹ early apex development as measured by apex length and Waddington scale at the thermal times of 0, 100, 200, and 300 °Cd

² Thermal times of two apex developmental phases of double ridge (DR) and terminal spikelet formation (TS) and the thermal time interval between them (TSDR)

³ thermal time between TS and first node appearance (ZD31TS), first node appearance (ZD31) and booting (ZD49)

Figure legends

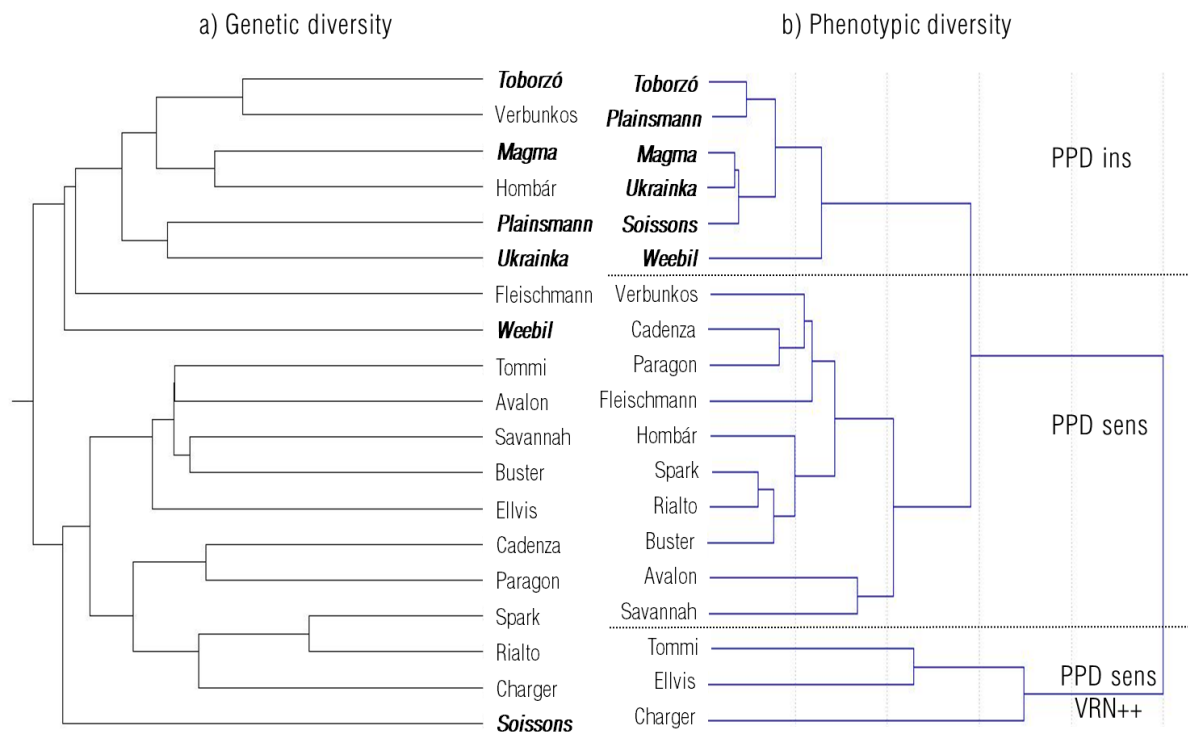


Figure 1 Genetic and phenotypic diversities between the 19 wheat cultivars, based on (a) 4971 SNP markers and (b) 16 phenology traits measured at 6 various environments combining 2 photoperiod \times 3 temperature regimes (Wheat genotypes with bold italics carry the *PPD-D1* insensitive allele)

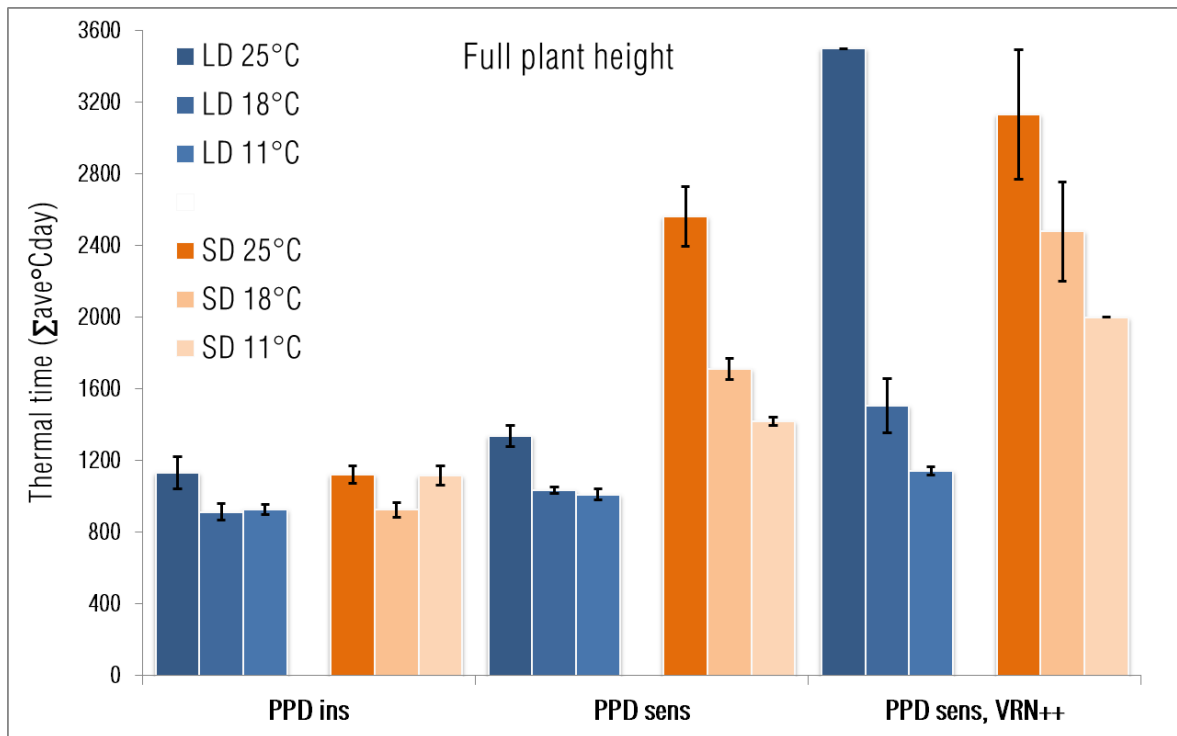


Figure 2 Thermal times collected till attaining the final plant height in the three phenotypic groups of 19 wheat cultivars at 6 various environments combining 2 photoperiod \times 3 temperature regimes (Error bars are based on standard deviations among wheat genotypes belonging to a subgroup (n= 6 for PPD ins; n= 10 for PPD sens; n=3 for PPD sens, VRN++))

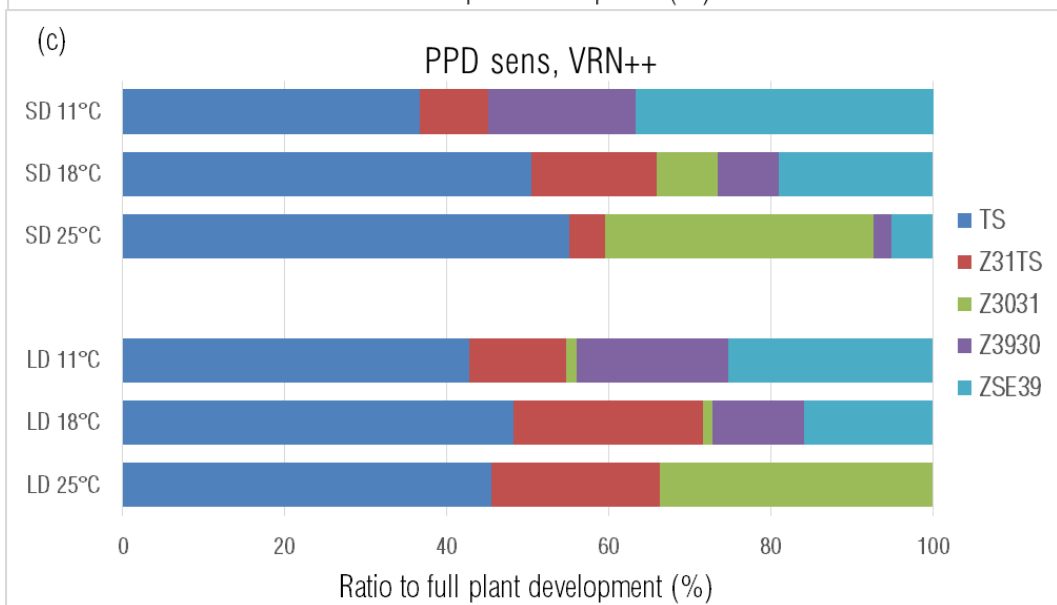
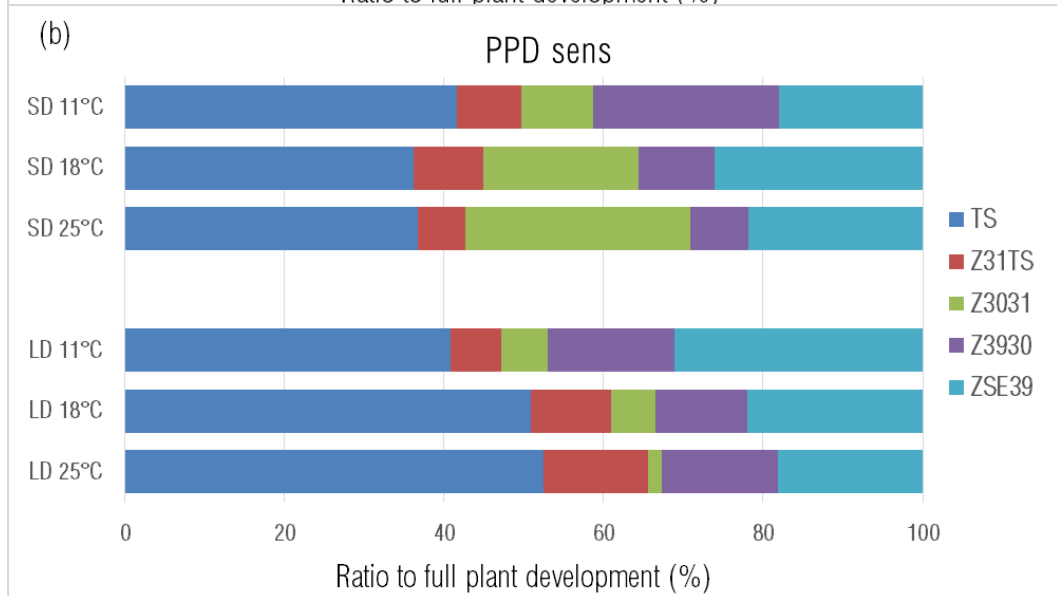
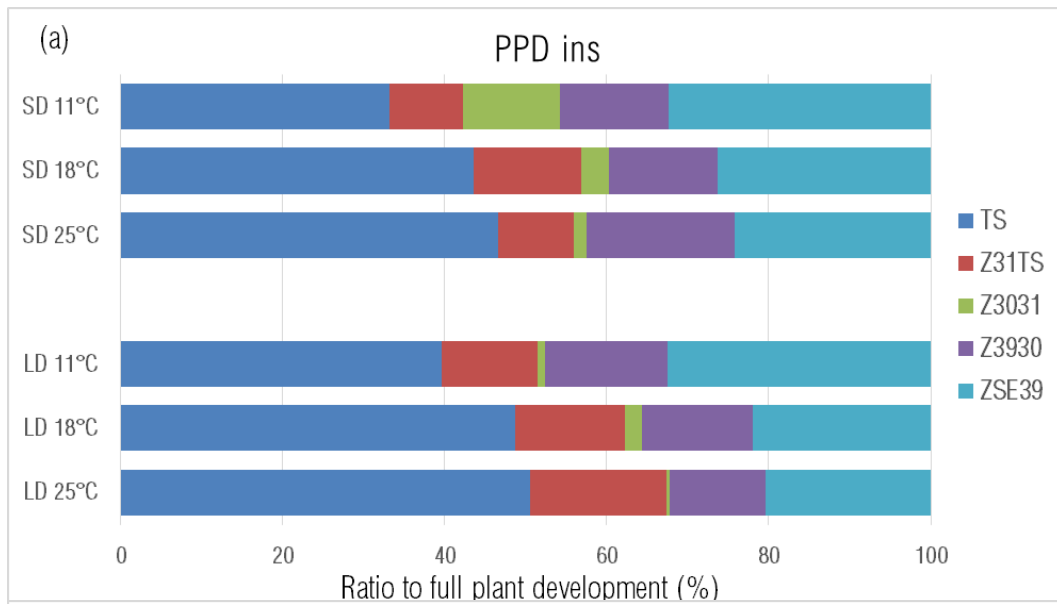
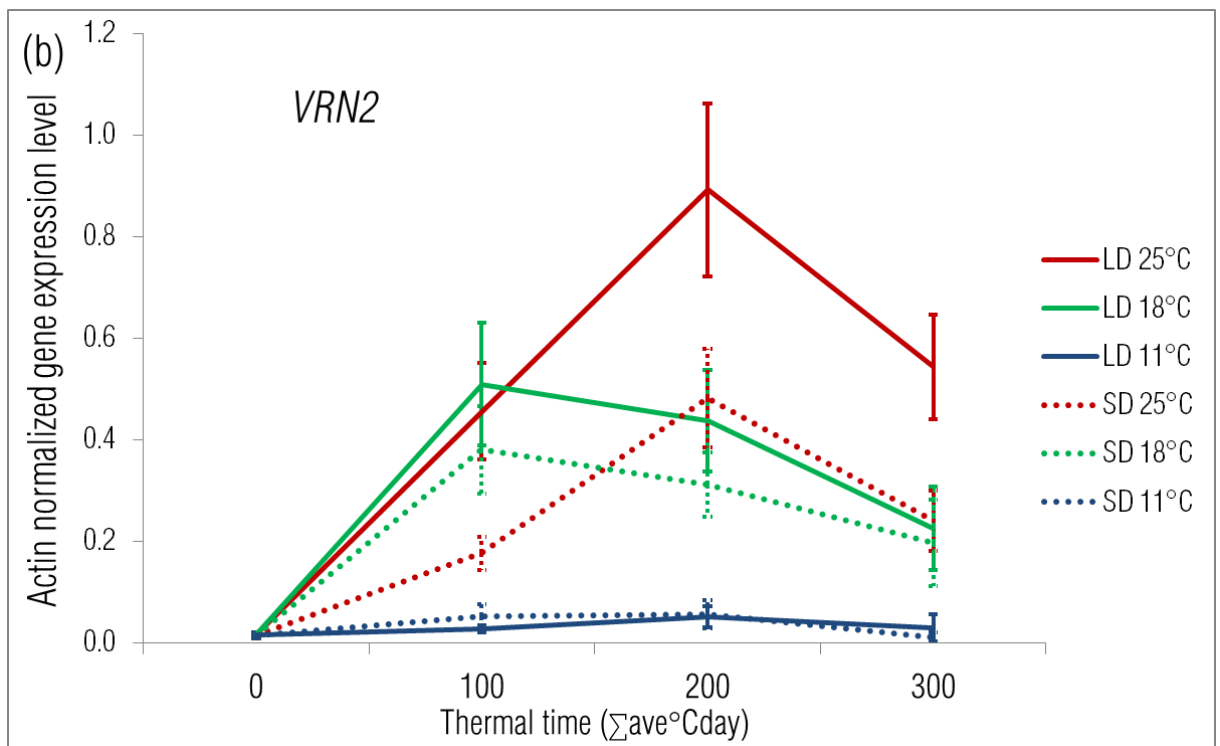
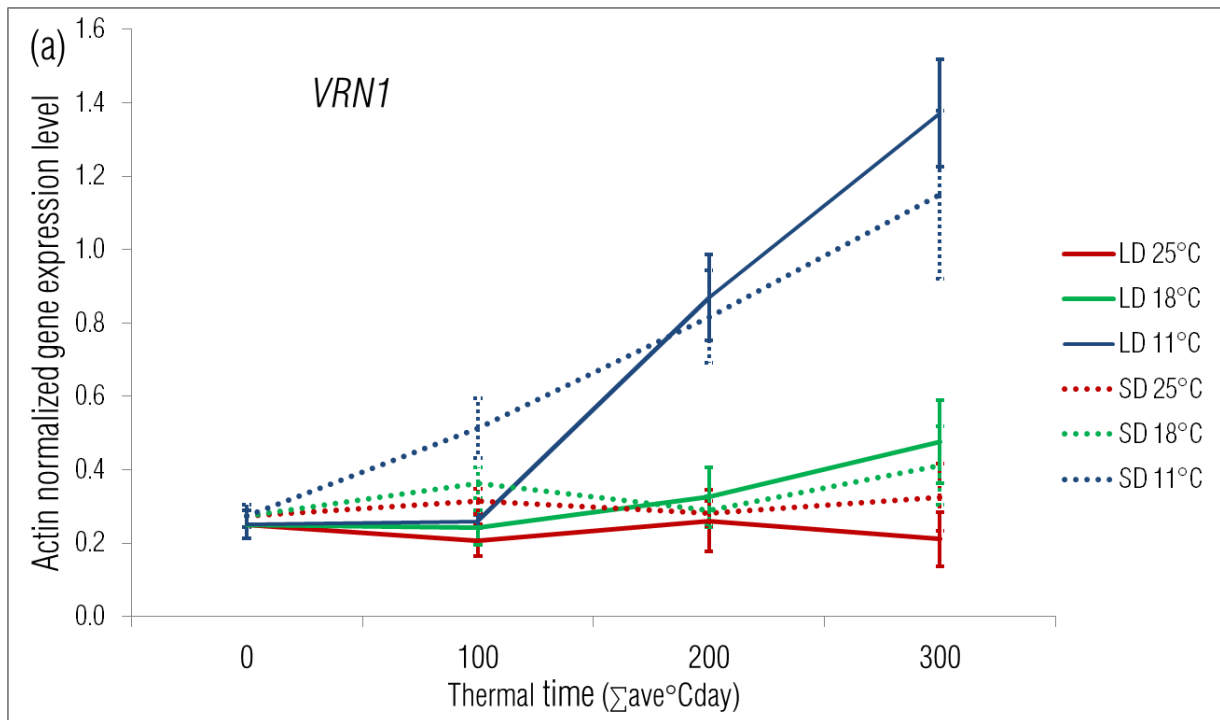


Figure 3 Proportions of the consecutive developmental phases until attaining the full plant height in the three phenotypic groups of 19 wheat cultivars at 6 various environments combining 2 photoperiod \times 3 temperature regimes; (a) PPD insensitive (PPD ins), (b) PPD sensitive (PPD sens) and (c) PPD sensitive group with strong vernalization requirement (PPD sens, VRN++) (Length of developmental intervals: TS – terminal spikelet formation, Z31TS – between first node appearance and terminal spikelet, Z3031 – between the start of intensive stem elongation and first node appearance, Z3930 – between fully emerged flag leaf and the start of intensive stem elongation, ZSE39 – between fully emerged flag and the end of intensive stem elongation)



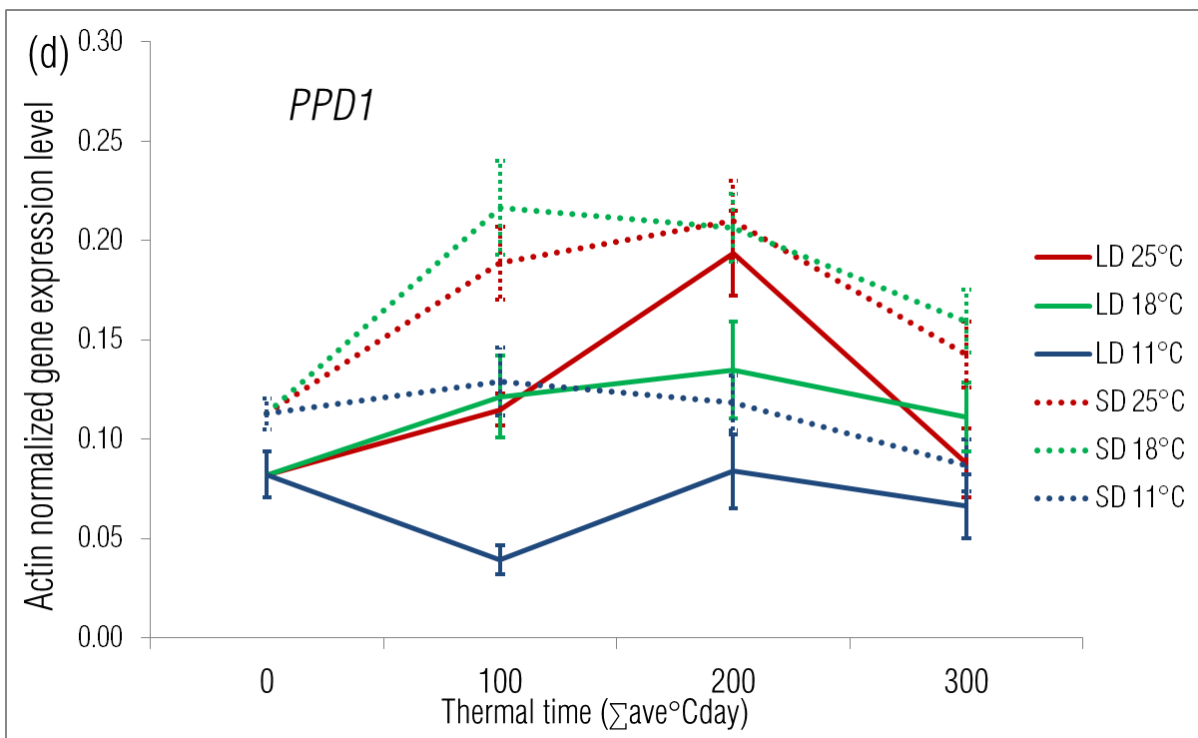
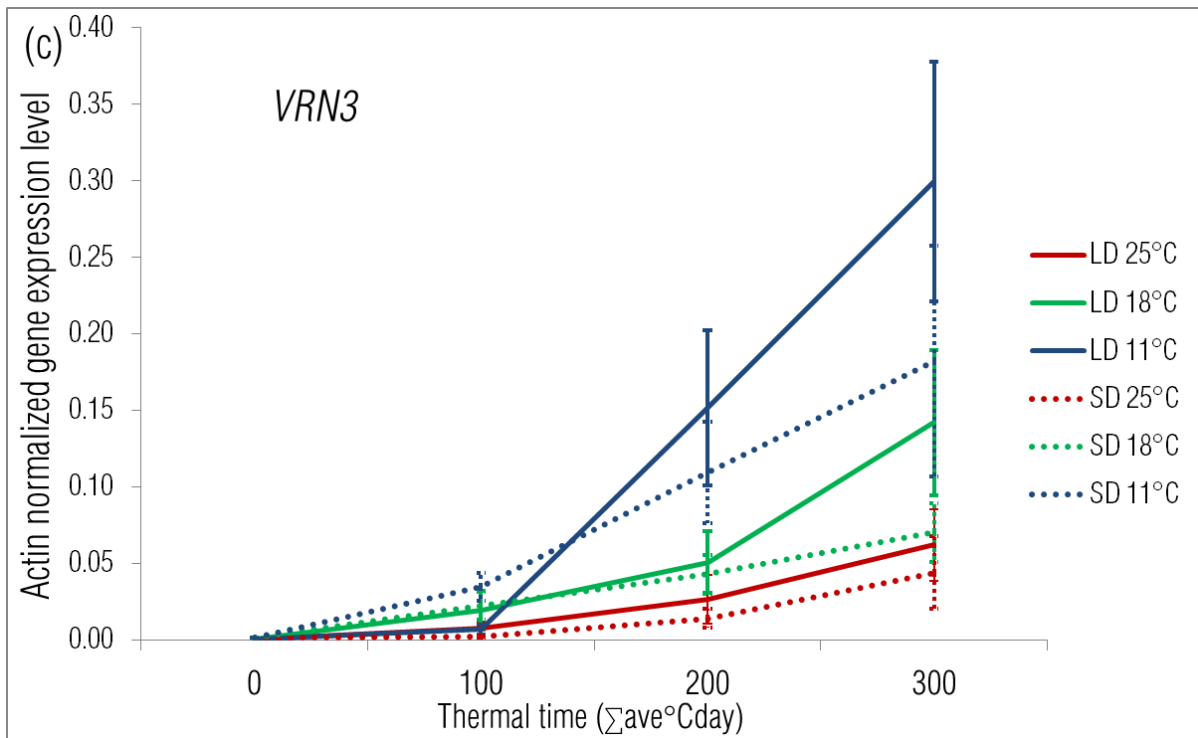
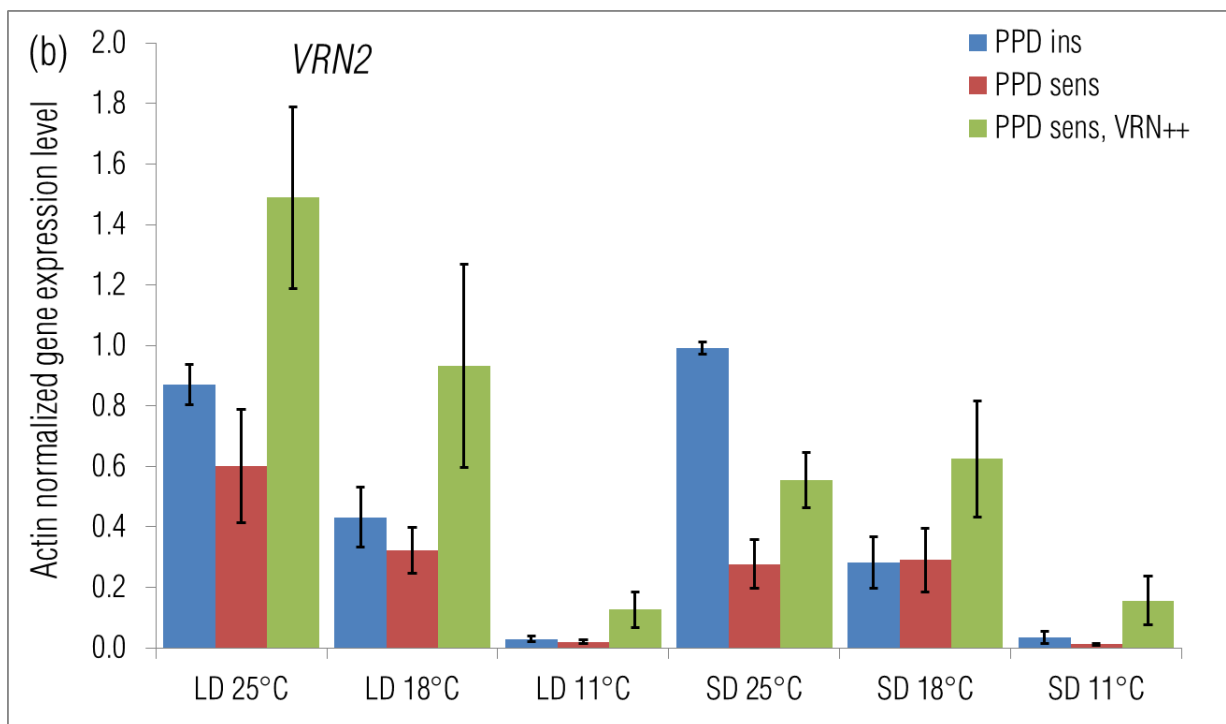
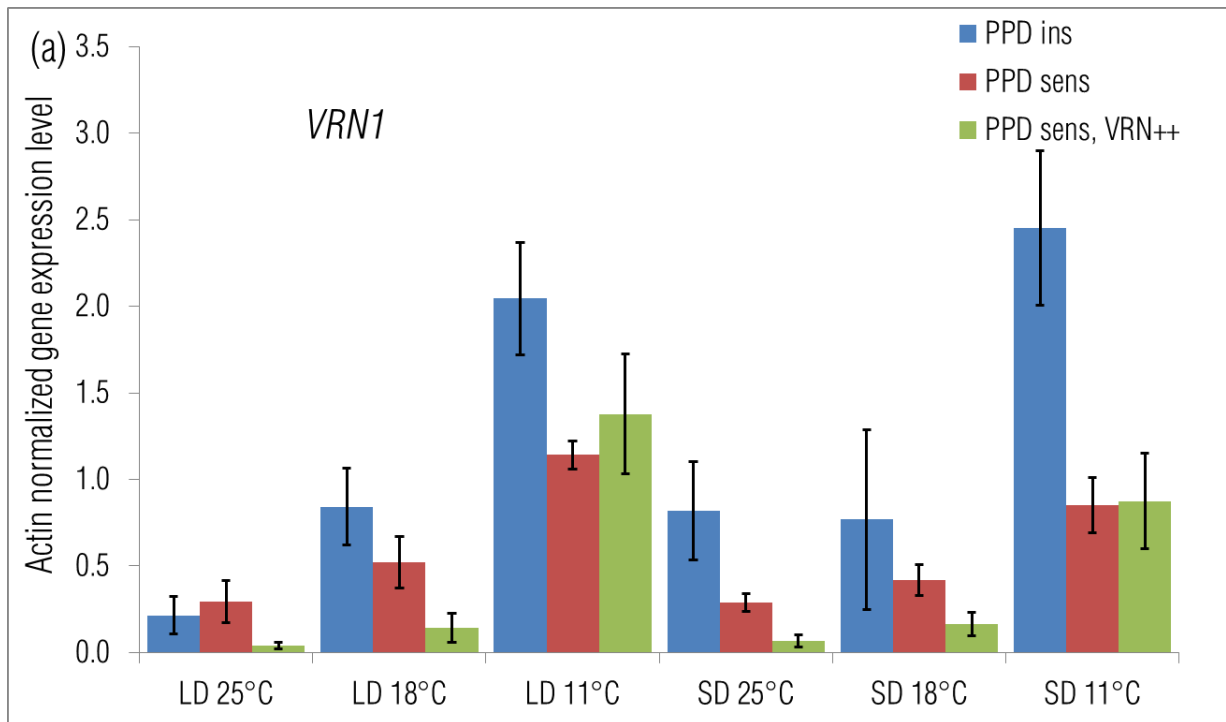


Figure 4 Dynamics of expression levels of *VRN1* (a), *VRN2* (b), *VRN3* (c) and *PPD1* (d) genes in association with the thermal times averaged over 11 wheat cultivars across the factorial combinations of 2 photoperiods and 3 temperature levels (Error bars are based on standard deviations among the 11 wheat genotypes)



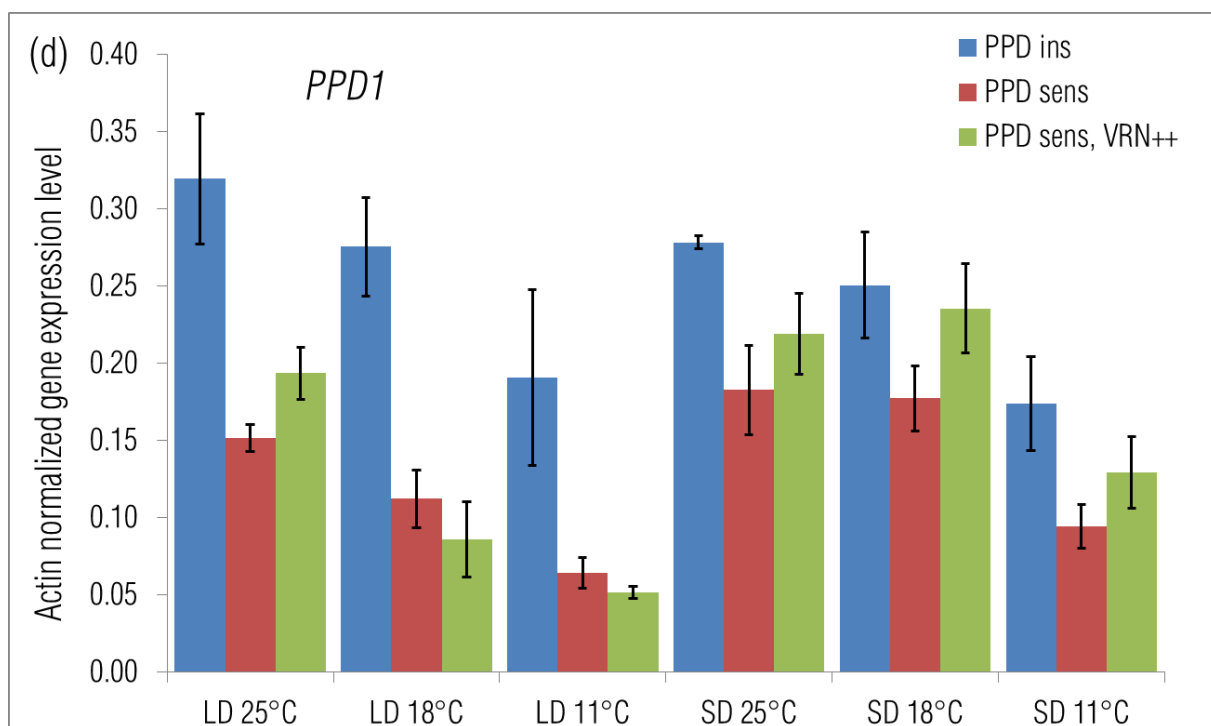
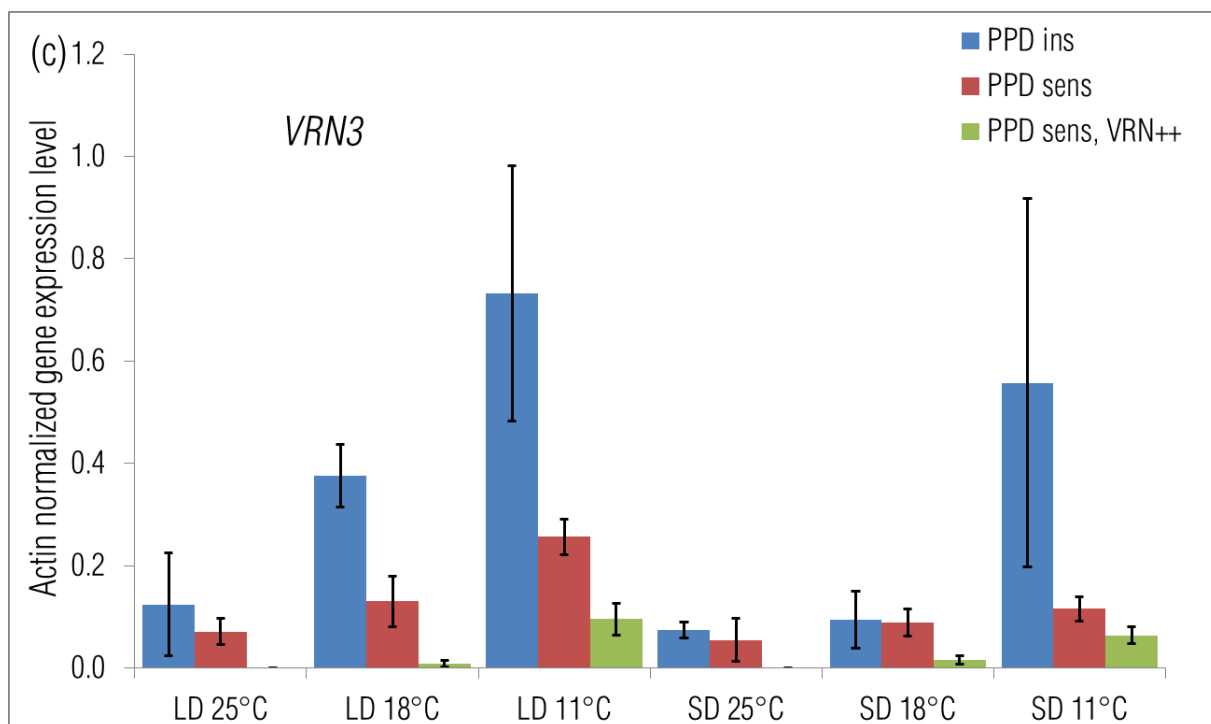


Figure 5 Mean values of the three phenotypic groups of 11 wheat cultivars at the respective maximum activities of *VRN1* (a), *VRN2* (b), *VRN3* (c), and *PPD1* (d) genes (Error bars are

based on standard deviations among wheat genotypes belonging to a subgroup (n= 2 for PPD ins; n= 6 for PPD sens; n=3 for PPD sens, VRN++)

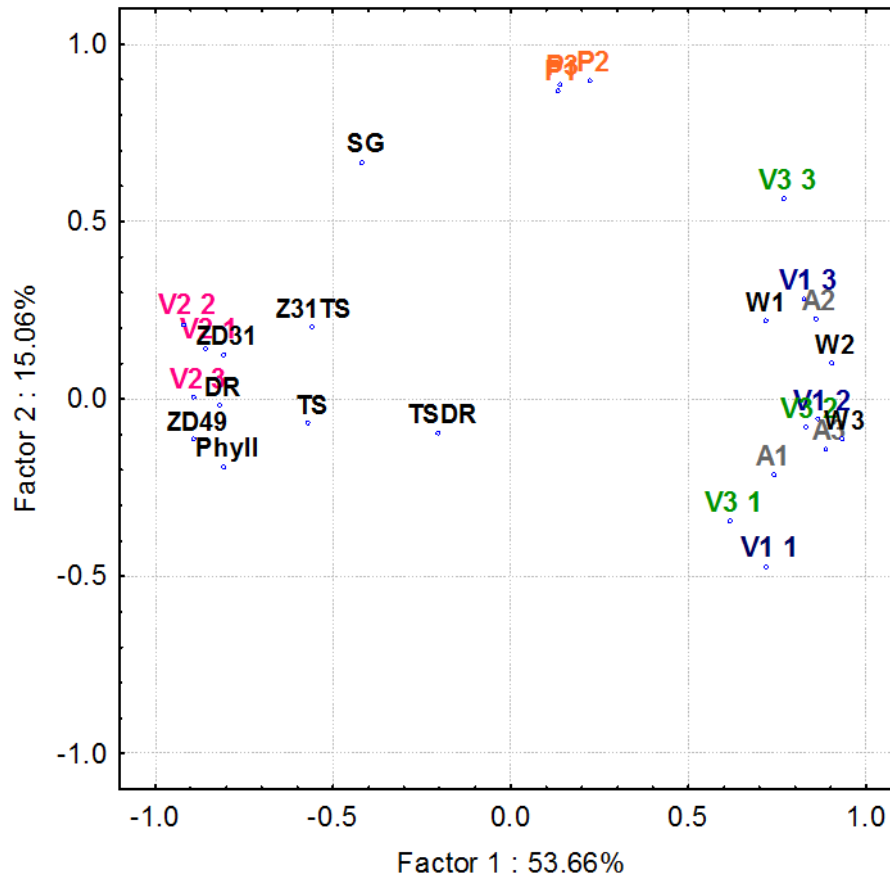


Figure 6 Principal component analysis (PCoA) carried out on the data matrix of gene expression levels and plant developmental parameters of 11 wheat cultivars under the environment of 16 hour photoperiod and 18°C temperature.

Abbreviations:

V1 – *VRN1*; V2 – *VRN2*; V3 – *VRN3*; P – *PPD1*; A – Apex length, W – Waddington stage; DR – double ridge; TS – terminal spikelet; TSDR – interval length between DR and TS; ZD31 – first node detectable; Z31TS – interval length between TS and ZD31; ZD49 – booting stage; SG – rate of stem growth, Phyll – phyllochron.

1 – at 100 °Cd; 2 – at 200 °Cd, 3 – at 300 °Cd