https://doi.org/10.17221/76/2025-CJGPB

Seasonal and microclimate-responsive expression of *VRN-A1* and *VRN-B1* in wheat under field conditions

Nicole Frantová[1], Ilja Tom Prášil[2], Ludmila Holková[1]*

[1]*Department of Crop Science, Breeding and Plant Protection, Faculty of AgriSciences, Mendel University in Brno, Brno, Czech Republic*
[2]*Plant Stress Biology and Biotechnology, Czech Agrifood Research Center, Praha, Czech Republic*

**Corresponding author: xfrantov@mendelu.cz*

Citation: Frantová N., Prášil I.T., Holková L. (2026): Seasonal and microclimate-responsive expression of *VRN-A1* and *VRN-B1* in wheat under field conditions. Czech J. Genet. Plant Breed., 62: 53–63.

Abstract: The need of vernalisation, controlled by the gene *VRN-1*, impacts wheat adaptation and yield stability, yet field evidence on the plasticity of *VRN-1* homoeologs expression is limited. We quantified *VRN-1* homoeolog dynamics across two sites and two seasons in seven cultivars, by sampling their apex and leaf. *VRN-A1* varied with genotype ($P < 0.001$***), tissue (apex > leaf; $P < 0.001$***), apex development ($P < 0.001$***), day length ($P < 0.001$***), and to a lesser extent, on short-term freezing exposure, quantified as a 5-day freezing-degree sum (FDS; $P = 0.019$*). Photoperiod class (*Ppd-D1a* vs *Ppd-D1b*) added an additional effect ($P = 0.001$***). *VRN-B1* showed strong genotype effects ($P < 0.001$***), a modest effect of site on its expression ($P = 0.025$*), and pronounced associations with microclimate variables (day length, thermal sums, freezing exposure; all $P < 0.001$***). Directionally, *Ppd-D1a* backgrounds tended to advance the development while showing earlier apex *VRN-A1* peaks. Overall, *VRN-A1* expression mainly reflected developmental stage and seasonal forcing, whereas *VRN-B1* might be more microclimate-responsive, indicating complementary roles for timing and stress-response plasticity. To isolate causal effects and to further explain these dynamics, targeted sequencing and tests in near-isogenic lines will be needed in future work.

Keywords: field-based gene expression; flowering time adaptation; photoperiod sensitivity; *Triticum aestivum*; *VRN-1* homoeologs

Control of flowering time is a key determinant of wheat adaptation and yield stability, as the transitions from stem elongation to heading and maturity determine how efficiently plants convert resources into grain (Chen et al. 2010; Langer et al. 2014). These developmental phase changes are regulated by genetic pathways that sense temperature (vernalisation) and day length (photoperiod) and coordinate developmental rate within the cropping season (Snape et al. 2001; Shcherban et al. 2015). Vernalisation requirements vary among genotypes and are most efficiently fulfilled by prolonged exposure to 0–10 °C, with strongly reduced effects above 11 °C (Brooking 1996; Curtis et al. 2002). In European winter cultivars, this corresponds to 5–13 weeks of cold exposure (Košner & Pánková 2002; Petr & Hnilička, 2002). Allelic composition of the *VRN-1* homoeologs largely determines this requirement, with dominant alleles shortening and recessive haplotypes extending it (Yan et al. 2004; Kamran et al. 2014).

Supported by the National Agency for Agricultural Research (NAZV), Ministry of Agriculture of the Czech Republic (Project QL24010142), and by institutional funding from the Ministry of Agriculture of the Czech Republic (Project MZE-RO0425).

https://doi.org/10.17221/76/2025-CJGPB

The central role in the vernalisation pathway has *VRN-1*, a MADS-box transcription factor with three homoeologous copies in hexaploid wheat: *VRN-A1*, *VRN-B1* and *VRN-D1* on chromosome 5 (Galiba et al. 1995; Kiss et al. 2014). Winter habit typically carries recessive *vrn-A1/vrn-B1/vrn-D1* (vernalisation required), whereas the presence of ≥1 dominant *Vrn-1* allele confers a spring habit (no vernalisation required). To avoid ambiguity, we define 'facultative' as a phenotypic subset of spring lines that do not require vernalisation yet overwinter reliably when autumn-sown. The homoeologs differ in their effect size on earliness (A1 > B1 > D1), corresponding to heading-time contrasts observed in the field (Stelmakh 1992; Zhang et al. 2008, 2022).

VRN-1 activity is embedded in a wider regulatory network. *VRN2* encodes CCT-domain zinc-finger transcriptional repressors of flowering that are down-regulated by prolonged cold (vernalisation) (Yan et al. 2004; Distelfeld et al. 2009). Conversely, *VRN-3/FT1* integrates vernalisation and photoperiod signals from leaves to the shoot apex and promotes *VRN-1* transcription, assisted by 14-3-3 and bZIP proteins (Yan et al. 2006; Li & Dubcovsky 2008; Li et al. 2015; Sehgal et al. 2023). In turn, *VRN-1* down-regulates *VRN-2* in leaves, completing a regulatory feedback loop (Hemming et al. 2008; Chen & Dubcovsky 2012). Epigenetic modifications, particularly within intron 1, provide a "memory" of cold exposure, referring to vernalisation-associated chromatin remodelling at *VRN-1*, such as DNA-methylation changes, while a *VRN-1*-derived long non-coding RNA enhances promoter activation (Khan et al. 2013; Oliver et al. 2009; Xu & Chong 2018; Xu et al. 2021).

This architecture functions within broader transcription-factor families: MADS-box proteins (*VRN-1*) provide meristem identity, zinc-finger/CCT proteins (*VRN-2*) enforce seasonal repression, and *VRN-3* transmits environmental cues to transcriptional change (Takatsuji 1998; Smaczniak et al. 2012).

We hypothesised that microclimate affects *VRN-1* homoeolog expression in the field, with tissue specificity and *Ppd-D1* alleles further shifting expression level and timing.

MATERIAL AND METHODS

Plant material. Seven bread wheat cultivars were studied: six winter types (Balitus, Bohemia, IS Conditor, RGT Sacramento, Tobak, Tonnage) and one facultative type (Tybalt). Basic characteristics (origin, earliness, vernalisation requirement, and freezing tolerance, LT50) are summarised in Table 1. (Frantová 2024). Seeds for genetic analyses were provided by the Gene Bank, Czech Agrifood Research Center (Prague-Ruzyně); seeds for field trials (commercial cultivars) were supplied by a seed company. For each cultivar we also list the *VRN-1* homeolog combination (*VRN-A1/VRN-B1/VRN-D1*) and the *Ppd-D1* allele (Frantová 2024). *Ppd-D1* has the strongest effect on photoperiod in hexaploid wheat (Beales et al. 2007).

Table 1. Specifics about the chosen varieties

Variety	Origin	Earliness	Vernalisation requirements (weeks)	Freezing tolerance (LT50) (°C)	*VRN-1/Ppd-D1* allelic profile	Photoperiod sensitivity
Balitus	Austria	very early	long ≥ 7	–16.0	*vrn-A1/vrn-B1/ vrn-D1/Ppd-D1a*	insensitive
Bohemia	Czech Republic	very early	long ≥ 7	–18.0	*vrn-A1/vrn-B1/ vrn-D1/Ppd-D1a*	insensitive
IS Conditor	Slovak Republic	moderately early	medium 4–6	–13.5	*vrn-A1/vrn-B1/ vrn-D1/ppd-D1b*	sensitive
RGT Sacramento	France	moderately early	medium 4–6	–13.5	*vrn-A1/vrn-B1/ vrn-D1/Ppd-D1a*	insensitive
Tobak	Germany	semi-late	short 3–4	–14.5	*vrn-A1/vrn-B1/ vrn-D1/ppd-D1b*	sensitive
Tonnage	Denmark	semi-late	short 3–4	–15.0	*vrn-A1/vrn-B1/ vrn-D1/ppd-D1b*	sensitive
Tybalt	Netherlands	semi-late	none	–13.5	*vrn-A1/vrn-B1/ Vrn-D1/ppd-D1b*	sensitive

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Figure 1. Monthly precipitation totals and mean air temperature for the study area in (A) 2020 and (B) 2021

We use the standard *VRN-1* nomenclature in which *Vrn-A1* denotes a dominant spring-like allele linked to intron-1/promoter deletions (Fu et al. 2005).

Field sites, climatic conditions, and crop management. The sites differed markedly in edaphic conditions. Site A (N 49°1'22.246", E 16°37'2.896", Nosislav, South Moravia, Czech Republic) had a finer texture with a higher clay fraction and a shallow water table (mean depths 1.35 m in 2020, 1.05 m in 2021), whereas Site B (N 49°0'45.941", E16°33'47.083", Žabčice, South Moravia, Czech Republic) was sand-rich/coarser with a deep water table (16–18 m). Monthly precipitation and 2-m air temperature were obtained from the on-site station (Figure 1). In 2020, an early warm anomaly in February was followed by colder conditions in March and then a gradual temperature increase, whereas the 2021 season remained generally colder, with February also below the long-term normal and no comparable early warming.

The plots were arranged as randomised complete blocks (three replicates at site A, four at site B); the net plot area was 10.3 m^2 (7.3 × 1.37 m). Sowing occurred in early October each season (site A: 15. 10. 2019 and 8. 10. 2020; site B: 8. 10. 2019 and 7. 10. 2020). Fertilisation regimes followed standard station practice. Further information can be found in Frantová (2024).

Phenology scaling and environmental variables. Development of the main-stem shoot apex was recorded and measured under a stereomicroscope with the Waddington scale (WS; 0–10 with decimal subdivisions, Waddington et al. 1983). Sampling frequency increased from winter to spring as development accelerated (Table 2).

From the available environmental cues, we selected four for analysis: daylength; growing degree days accumulated from the first November observation to each subsequent sampling (25 Nov 2019; 12 Nov 2020); the low-temperature sum over the same period; and a short-term freezing-degree sum computed from the 5-day 07:00–07:00 window preceding sampling. All temperature metrics were derived from above-ground, near-surface air temperature.

Daylength (DL) was computed as the interval between apparent sunrise and apparent sunset for the study coordinates on each sampling date, using the NOAA Global Monitoring Laboratory Solar Calculator (NOAA 2025).

Above-ground temperature was recorded by the sensors at each site (TepUni temperature sensor, AMET Velké Bílovice). Growing degree days (GDD) expressed in hours quantify thermal time above 5 °C (baseline), computed from the first November sampling as:

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$$GDD^{h}_{5} = \Sigma_{hours}\max(0, T_{hours} - 5\ °C)\ (°C{\cdot}h) \quad (1)$$

Cold exposure (low temperature sum, LTS) was computed from daily mean temperatures using a ≤ 5 °C inclusion rule: values > 5 °C contribute 0; values ≤ 5 °C contribute their magnitude, negatives values turned into positive. To quantify recent sub-zero exposure (freezing-degree sum, FDS), we computed a 5-day freezing-degree sum from 15-min air-temperature series using a 0 °C base. For each sampling date t_s, we integrated from 07:00 on the sampling day back 5 days. This window was chosen because tissue was sampled at 08:00–09:00, ensuring only pre-sampling exposure contributes.

Transcriptome analysis. For expression analyses, two shoot apexes with subtending stems and the 1st–3rd youngest and healthy-looking leaves of the main stem were sampled with two biological replicates. Each biological replicate was a pooled sample (two leaves pooled = one leaf sample; two apices pooled = one apex sample). Field sampling on each date was conducted between 08:00 and 09:00 local time (CET/CEST) to minimise diurnal variation in transcript levels. Material was snap-frozen in liquid N_2 in the field and stored at −80 °C until RNA extraction. Total RNA was extracted using the RNeasy Plant Mini Kit (QIAGEN; spin-column protocol; 80 µl RNase-free water elution). Residual genomic DNA was removed with TURBO DNA-free™ (Invitrogen), the input was 3 000 ng of RNA per 20 µL of each sample. First-strand cDNA was synthesised using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific).

Real-time PCR analysis. Quantitative PCR was performed with SYBR Green chemistry using LightCycler® 480 SYBR Green I Master (Roche) on a Bio-Rad iQ™5 (96-well plates; 20 µL reactions, 1 µL cDNA; 40 cycles, kit-recommended cycling). Primer sequences and optimised annealing conditions were as follows: *ACTIN* (Aslam et al. 2017; annealing 60 °C for 15 s), *VRN-A1* (Loukoianov et al. 2005; annealing 64 °C for 15 s) and *VRN-B1* (Frantová 2024; annealing 64 °C for 15 s). *ACTIN* served as the reference gene, samples were diluted 10×, and normalised relative expression was calculated according to Pfaffl (2001). Melt-curve analysis confirmed single specific amplicons, and amplification efficiencies ranged from 88% to 100% (E = 1.88–2.00).

Statistical analysis. Preliminary linear-model/ANOVA fits to the qPCR expression data were used to check assumptions. For *VRN-A1*, residuals showed clear deviations from normality and strong heteroscedasticity, and expression was analysed using a generalised linear model (GLM) with a Gamma distribution and log link. In contrast, *VRN-B1* expression approximately satisfied normality and homoscedasticity, and was therefore analysed using a Gaussian GLM with an identity link. Fixed factors were genotype, tissue and site; covariates were apex developmental stage (Waddington scale) and microclimate variables (FDS, LTS, GDD, DL), including their interactions. Significance of effects was assessed with Type III Wald χ^2 tests ($\alpha = 0.05$); we report generalised partial η^2 as an effect-size measure and marginal R^2 as an indicator of model fit. Because of CPU/memory limits, the effect of photoperiod-sensitivity class (*Ppd-D1*) was tested in a separate reduced model for each gene. GLM analyses were performed in jamovi Ver. 2.7 (the jamovi project, Australia) based on the R environment. Bivariate associations between expression, phenology and environmental variables were evaluated using Spearman's rank correlation

Table 2. Sampling dates with daylength and 5-day freezing-degree sum

Growing season 2019–2020	Day length (h) both sites	FDS (h of < 5 °C)		Growing season 2020/2021	Day length (h) both sites	FDS (h of < 5 °C)	
		site A	site B			site A	site B
8.1.2020	8.5	251.8	184.0	4. 12. 2020	8.6	104.5	84.0
12.2.2020	10.0	76.3	39.3	28. 1. 2021	9.2	72.8	51.0
10.3.2020*	11.6	35.0	25.8	25. 2. 2021	10.8	17.5	6.3
18.3.2020	12.1	93.5	50.0	18. 3. 2021	12.1	62.0	13.3
1.4.2020	12.9	141.0	133.5	30. 3. 2021*	12.8	13.8	0
15.4.2020	13.7	46.8	15.8	12. 4. 2021	13.6	85.0	39.0
28.4.2020	14.5	11.5	0	23. 4. 2021	14.2	12.5	0.3
				28. 4. 2021	14.5	10.5	0

FDS – freezing-degree sum; *denotes the Waddington W2.0 (double ridge) apex stage, the onset of spikelet initiation

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(two-tailed, $P < 0.05$) in XLSTAT (Excel add-in; Lumivero, USA), with Excel used for data handling and heat-map graphics.

RESULTS AND DISCUSSION

***VRN-A1* transcripts seem lower in a facultative cultivar and earlier in *Ppd-D1a* genotypes.** Across seasons and tissues, the winter cultivars showed cold-responsive *VRN-A1* induction under field conditions (Figure 2), with transcript levels generally higher in the apex than in leaves. In the relatively mild 2020 season, characterised by a warm February followed by colder spells in March, *Ppd-D1a* carriers (Balitus, Bohemia) tended to show earlier and stronger apex induction, whereas *ppd-D1b* carriers more often displayed slower, delayed peaks. In the colder 2021 season, with February temperatures also below the long-term normal, *VRN-A1* peaks were generally shifted later and were weaker, especially in photoperiod-sensitive (*ppd-D1b*) genotypes. This pattern is consistent with the view that "insensitive" *Ppd-D1a* alleles retain some photoperiod responsiveness but favour earlier or stronger *VRN-A1* activation when vernalisation is partially satisfied (Slafer et al. 2023). The facultative cultivar Tybalt maintained low *VRN-A1* expression (often < 2.0) in both years, in line with reports that facultative wheats require little or no vernalisation and therefore accumulate lower *VRN-1* under winter conditions (Prášil et al.

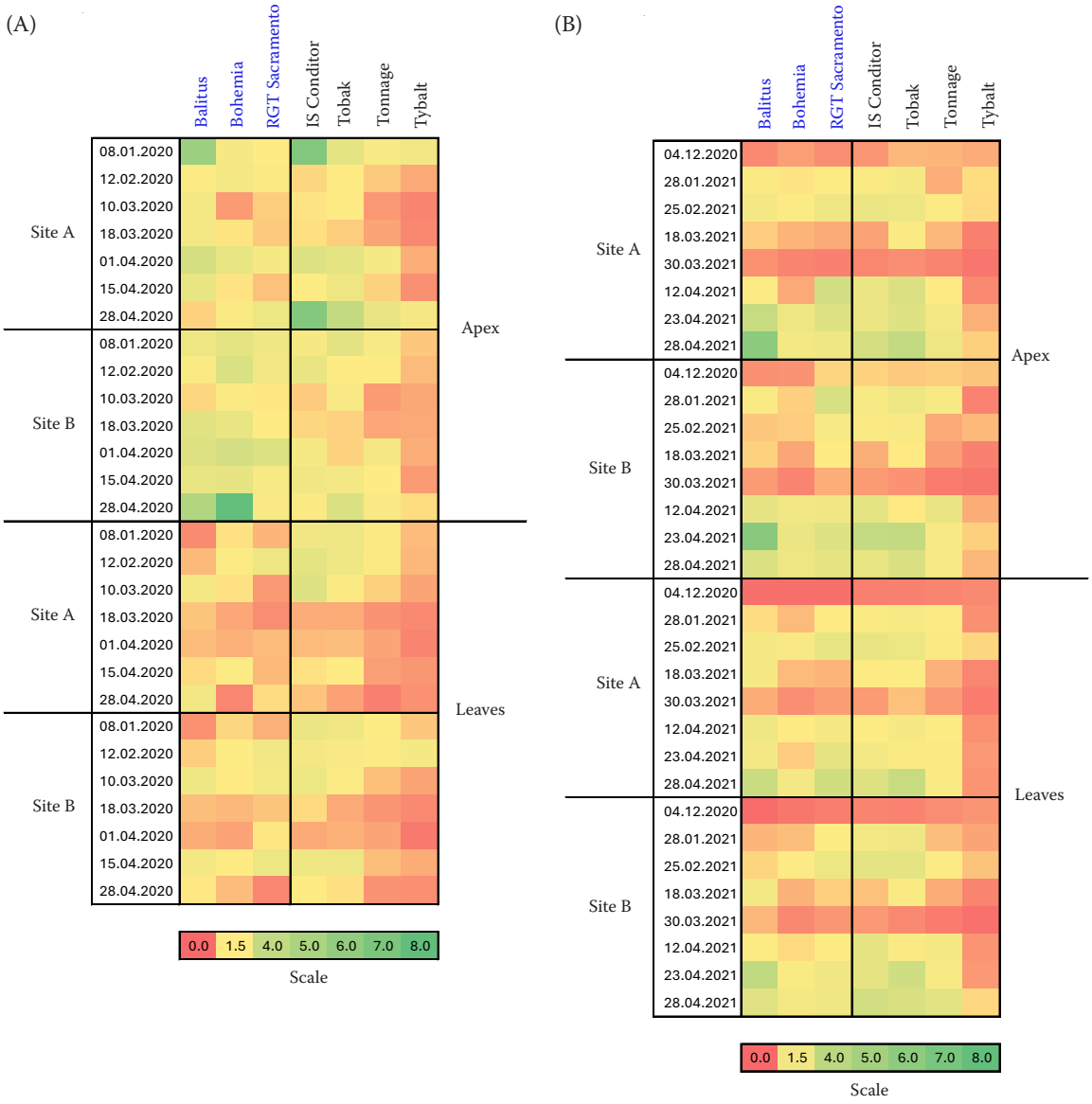

Figure 2 continues on the next page

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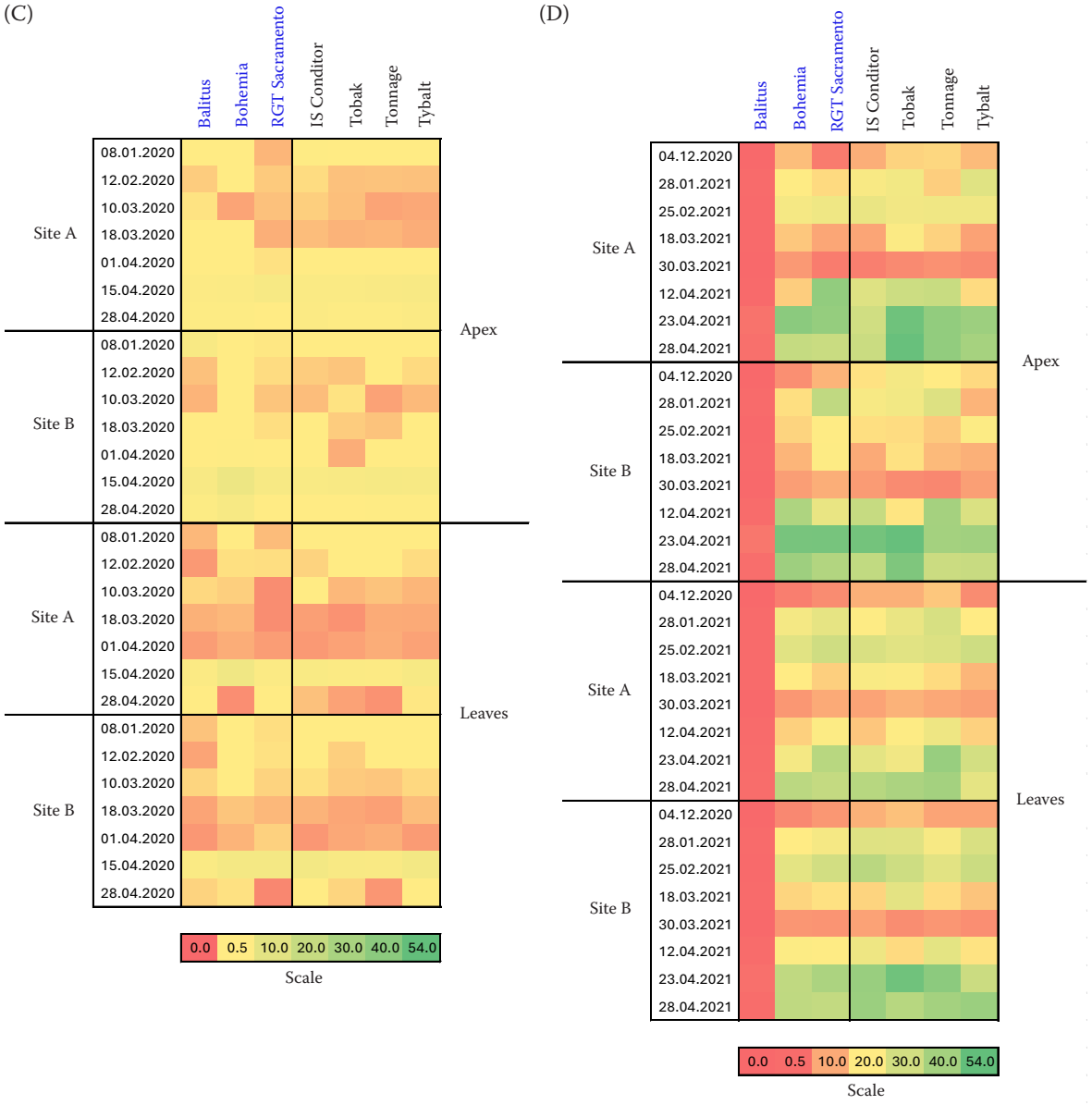

Figure 2. Heat maps of normalised relative expression (NRE): *VRN-A1* (A–B), *VRN-B1* (C–D) (two seasons × two sites × two tissues); blue labels indicate genotypes carrying the photoperiod-insensitive allele *Ppd-D1a*

2025). From an applied perspective, combining information on *VRN-A1* apex dynamics with *Ppd-D1* background and typical seasonal temperature patterns may help prioritise cultivars for late-autumn sowing (Wenda-Piesik et al. 2016).

***VRN-B1* appears more environmentally responsive than *VRN-A1*.** Compared with *VRN-A1*, *VRN-B1* expression was more variable and strongly season-dependent. In the milder winter of 2020, *VRN-B1* transcripts remained low across genotypes, whereas in the colder 2021 season they increased markedly, with pronounced April peaks in Tobak and Tonnage while Balitus stayed low. Two non-exclusive mechanisms may underlie this pattern. First, allelic differences at *VRN-B1*. This gene shows extensive sequence diversity (promoter changes, intron-1 indels and several non-synonymous SNPs) and allele-dependent expression under controlled vernalisation, implying genotype-specific baselines and responsiveness (Strejčková et al. 2023).

Second, environmental dose and timing of cold. *VRN1* transcription in leaves is promoted by prolonged low temperatures and contributes to repression of *VRN-2*, so winter-to-winter differences in cold

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accumulation and fluctuation can shift expression amplitude or timing (Chen & Dubcovsky 2012). In 2021, intermittent warm spells followed by renewed cold likely reinforced vernalisation signalling and favoured stronger *VRN-B1* induction, while the milder 2020 season remained below this threshold. Tybalt, which showed weak *VRN-A1* but strong *VRN-B1* induction in 2021, further suggests partial functional compensation between homeologs, with *VRN-A1* more strongly promoted by genotype/development and *VRN-B1* acting as the more environmentally responsive copy in our field conditions.

Photoperiod group and tissue shape *VRN-A1*. Generalised linear models (Table 3) showed that *VRN-A1* expression varied strongly with genotype ($P < 0.001$; $R^2 = 0.319$) and tissue, with consistently higher levels in the apex than in leaves ($P < 0.001$). Apex developmental stage ($P < 0.001$), day length (DL; $P < 0.001$) and short-term freezing (FDS; $P = 0.019$) were also significant predictors, whereas site and all tested interactions were not. The significant DL effect should not be read as a simple linear day-length response; it most likely reflects mediation by the photoperiod pathway, where *FT1* produced in leaves under long days (*Ppd-D1* dependent) forms an FT–14-3-3–FD complex that promotes *VRN-1* transcription in the apex. In a reduced model, photoperiod class (*Ppd-D1a* vs *ppd-D1b*) further modified *VRN-A1*

Table 3. Generalised linear models (GLMs) results for *VRN-A1* (Gamma–log) and *VRN-B1* (Gaussian–identity)

Gene		χ^2	*df*	*P*-value	η^2	R^2
VRN-A1	genotype	195.95	6	< 0.001***	3.30×10^{-1}	0.319 (*P* < 0.001***)
	tissue	30.44	1	< 0.001***	5.13×10^{-2}	
	site	1.58	1	0.209	2.66×10^{-3}	
	apex development	31.54	1	< 0.001***	5.31×10^{-2}	
	FDS	5.51	1	0.019*	9.27×10^{-3}	
	LTS	1.01	1	0.316	1.70×10^{-3}	
	GDD	0.27	1	0.607	4.46×10^{-4}	
	DL	14.12	1	< 0.001***	2.38×10^{-2}	
	genotype × tissue	7.73	6	0.258	1.30×10^{-2}	
	genotype × site	4.54	6	0.605	7.64×10^{-3}	
	tissue × site	0.08	1	0.780	1.31×10^{-4}	
	genotype × tissue × site	4.94	6	0.552	8.32×10^{-3}	
	photoperiod sensitivity	10.40	1	0.001***	1.75×10^{-2}	0.012 (*P* 0.001***)
VRN-B1	genotype	81.65	6	< 0.001***	$5^{-}96 \times 10^{-5}$	0.258 (*P* < 0.001***)
	tissue	0.74	1	0.390	5.39×10^{-6}	
	site	5.05	1	0.025*	3.68×10^{-5}	
	apex development	1.16	1	0.282	8.43×10^{-6}	
	FDS	26.97	1	< 0.001***	1.97×10^{-4}	
	LTS	23.00	1	< 0.001***	1.68×10^{-4}	
	GDD	37.49	1	< 0.001***	2.74×10^{-4}	
	DL	49.63	1	< 0.001***	3.62×10^{-4}	
	genotype × tissue	1.23	6	0.975	9.01×10^{-6}	
	genotype × site	1.58	6	0.954	1.15×10^{-5}	
	tissue × site	0.41	1	0.524	2.97×10^{-6}	
	genotype × tissue × site	1.24	6	0.975	9.04×10^{-6}	
	photoperiod sensitivity	17.60	1	< 0.001***	1.28×10^{-4}	0.021 (*P* < 0.001***)

FDS – freezing-degree sum; LTS – low temperature sum; GDD – growing degree days; DL – daylength; *df* – degree of freedom; η^2 – effect size (proportion of variability in the response explained by the factor)

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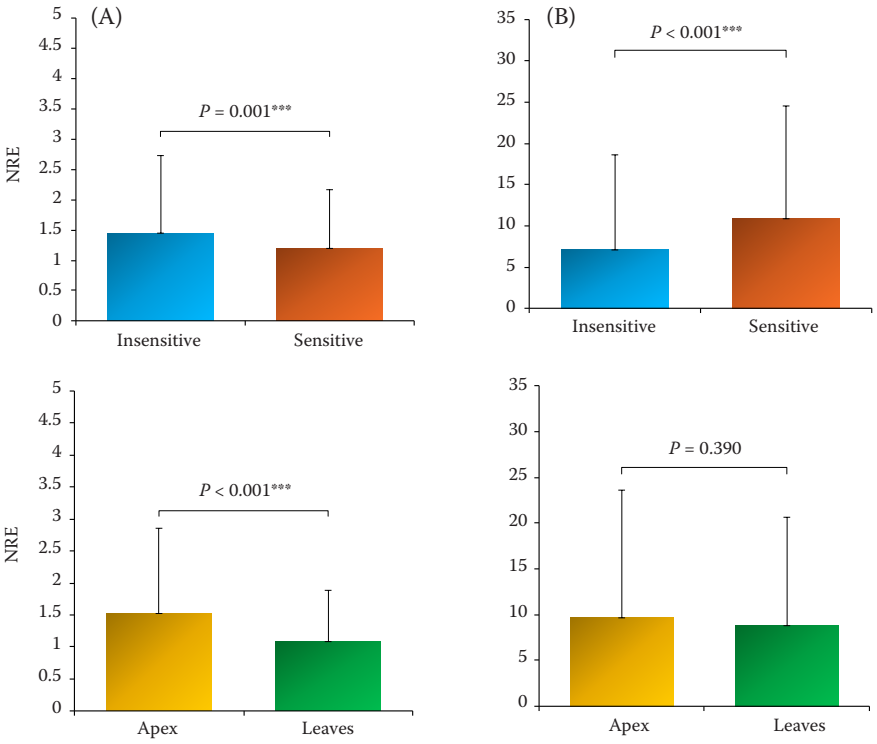

Figure 3. Normalised relative expression (NRE) by photoperiod type and tissue: *VRN-A1* (A), *VRN-B1* (B)
Error bars represent standard deviation

expression ($P = 0.001$), with photoperiod-insensitive genotypes tending to reach higher apex transcript levels earlier in development. Together with the more environment-sensitive behaviour of *VRN-B1*, these results support *VRN-A1* as the primary, apex-localised homeolog whose expression is strongly promoted by genotype background and photoperiod signalling, while *VRN-B1* provides additional adjustment to year-to-year environmental variation.

VRN-B1* appears more environmentally responsive than *VRN-A1*.** For *VRN-B1*, genotype again contributed strongly ($P < 0.001$; $R^2 = 0.258$), whereas tissue was not significant ($P = 0.390$), indicating a weaker apex–leaf contrast than for *VRN-A1*, although leaf expression may still aid cold acclimation (Dhillon et al. 2010). Site showed a small but significant effect ($P = 0.025$*), and microclimate covariates (DL, GDD, TLS and FDS) were all highly significant ($P < 0.001$***), while interactions were not. In the reduced model, photoperiod class again affected *VRN-B1* ($P < 0.001$***; $R^2 = 0.021$), with photoperiod-sensitive *ppd-D1b* genotypes showing

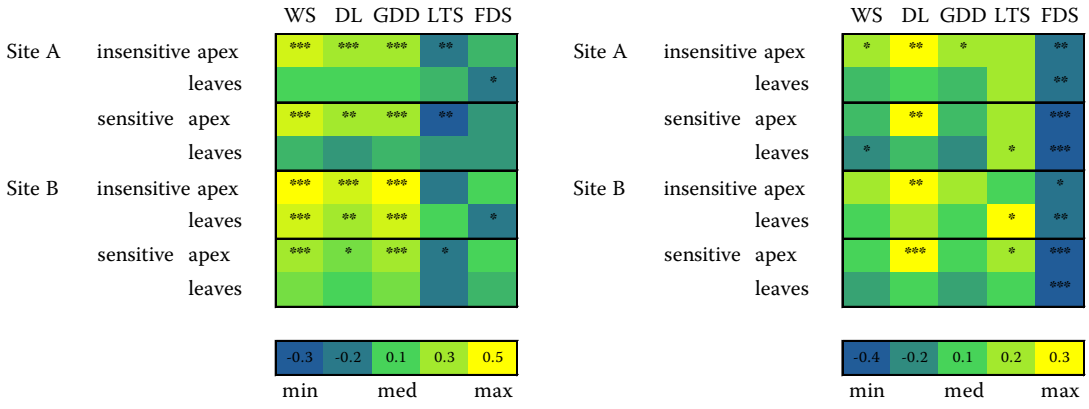

Figure 4. Spearman ρ heat maps (site × type × tissue): *VRN-A1* (A); *VRN-B1* (B)
WS – Waddington scale; DL – daylength; GDD – growing degree days; LTS – low temperature sum; FDS – freezing-degree sum; *, **, ***significant at $P < 0.05, 0.01, 0.001$

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higher *VRN-B1* expression than *Ppd-D1a* genotypes, in clear contrast to the *VRN-A1* pattern. Overall, *VRN-B1* was the homeolog most responsive to microclimate and temperature indices in our field data.

Apex–leaf tissue specificity in *VRN-A1* responses. Across sites, tissues and photoperiod-sensitivity genotypes, Spearman analyses revealed few strong associations ($P < 0.001$; Figure 4), all in the apex. At Site A, apex *VRN-A1* expression increased with apex developmental stage in both *Ppd-D1a* and *ppd-D1b* genotypes ($\rho = 0.4$), and at the colder Site B photoperiod-insensitive *Ppd-D1a* genotypes also showed a positive association with GDD ($\rho = 0.5$). In contrast, no $P < 0.001$ correlations were detected for *VRN-A1* in leaves at Site B, underscoring clear tissue specificity. Apex *VRN-A1* follows gradual, cumulative activation with development and thermal time, whereas leaf *VRN-A1* appears more transient and closely tracks short-term low-temperature fluctuations. Together, these patterns support *VRN-A1* as a primarily apex-localised integrator of vernalisation and thermal time (Li & Dubcovsky 2015), with leaf expression remaining more dynamic and environmentally labile.

***VRN-B1* inversely associates with short-term freezing exposure across sites and tissues.** Across both sites and tissues, *VRN-B1* expression showed a consistent negative association with FDS (Spearman $\rho = -0.3$ to -0.4 in both *Ppd-D1a* and *ppd-D1b* genotypes). This inverse relationship suggests that short-term freezing exposure acts as a short-term "developmental brake", with higher freezing load associated with lower *VRN-B1* and potentially slower apex progression during cold snaps, enhancing survival. Such plasticity is likely most relevant in photoperiod-sensitive genotypes, which progress later and retain greater scope for modulation, whereas earlier, photoperiod-insensitive lines may have less opportunity for this buffering.

CONCLUSION

Under field conditions, *VRN-A1* might follow the developmental progression (apex > leaf) and seasonal forcing (day length, thermal time), whereas *VRN-B1* might be more responsive to short-term microclimate variation, including recent freezing-degree sum and small site differences. Photoperiod background further modulates timing; *Ppd-D1a* tends to advance development. Combining apex *VRN-A1* dynamics, *VRN-B1* sensitivity, and *Ppd-D1* group might guide cultivar choice to site conditions. A key limitation is the comparison across diverse cultivars; targeted sequencing and near-isogenic line tests are needed to validate and further explain these field pattern.

Acknowledgements. The authors thank colleagues at the Field Experimental Station in Žabčice for cultivating the wheat and for technical support during sampling. The extended thanks go also the anonymous reviewers for their thoughtful comments and constructive suggestions, which substantially improved the clarity and scientific rigour of this manuscript.

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Received: September 2, 2025
Accepted: December 31, 2025
Published online: February 9, 2026