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Allelic variation and differential expression of *VRN-A1* in durum wheat genotypes varying in the vernalization response

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With 1 figure and 3 tables

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Abstract

The purpose of this research was to study the effect of vernalization on flowering time in thirty accessions of *Triticum turgidum* spp. durum. To this end, screening of allelic variation at *VRN-1* loci was carried out using PCR-based markers and DNA sequencing, and gene expression analysis was performed in two and four developmental stages, with and without cold treatment. In non-vernalized plants, average days to flowering ranged from 34 to 99 days and only one accession was observed to remain at the vegetative stage. Three accessions were found to accelerate flowering after cold treatment. At *VRN-A1* locus, three alleles previously characterized, *Vrn-A1b*, *Vrn-A1c* and *vrn-A1*, were detected. No polymorphisms at *VRN-B1* locus were observed. The differences in vernalization response among the genotypes studied could be explained by allelic variation and *VRN-A1* transcript levels. Variability in flowering time and sensitivity to vernalization in a collection of durum wheat, both potentially useful to the development of new varieties, were observed. This information is key to directing breeding cultivars for adaptation.

Key words: *Triticum turgidum* ssp. *durum* — *VRN-1* alleles — vernalization requirement — *VRN-1* expression

Flowering time influences the adaptability of wheat plants to a large range of environments. Plants can sense environmental signals and delay flowering under favourable seasonal conditions, thus preventing freezing damage to sensitive reproductive tissues (Bergjord et al. 2009). In addition, earliness in flowering is an effective means to avoid high-temperature stress effects during grain filling, particularly in Mediterranean climates. Therefore, flowering time is a major adaptive trait and is a determinant of crop performance and yield (Araus et al. 2008, Kamran et al. 2013).

The main seasonal cues for flowering are temperature and day length. In relation to the former, the vernalization requirement is the physiological process through which plants become competent to flower only after prolonged exposure to cold temperatures. Wheat varieties that require vernalization to flower are described as winter wheats, whereas spring wheats flower in the absence of a long low-temperature period. The genetic network underlying the vernalization response has been thoroughly studied in wheat. Natural variation in vernalization requirements is mainly associated with allelic differences in genes *VRN-1*, *VRN-2* and *VRN-3*.

VRN-1 encodes a MADS-box transcription factor that is closely related to three paralogous *Arabidopsis thaliana* meristem identity genes, namely *APETALA1* (*AP1*), *CAULIFLOWER* (*CAL*) and *FRUITFULL* (*FUL*), all of which are responsible for the transition of the shoot apical meristem from the vegetative to the reproductive stage (Danyluk et al. 2003, Trevaskis et al. 2003, Yan et al. 2003). The wild *VRN-1* form (*vrn-1* allele) confers winter growth habit to genotypes carrying this allele, whereas mutations at one or more of the *Vrn-1* homoeoalleles are the predominant cause of spring growth habit in polyploid wheat (Fu et al. 2005). Variants carrying dominant mutations (*Vrn-1* alleles) in the promoter, retrotransposon insertions or large deletions/insertions in the intron 1 region of *VRN-1* have been studied in wheat and barley (Yan et al. 2004a, Fu et al. 2005, Cockram et al. 2007, Pidal et al. 2009, Chu et al. 2011). Genotypes with a winter growth habit show very low *VRN-1* transcript levels prior to vernalization. In contrast, spring genotypes constitutively express *VRN-1* at high levels (Loukoianov et al. 2005). Differences in vernalization requirements among winter wheats have recently been associated with variation in *VRN-A1* copy number (Díaz et al. 2012, Zhu et al. 2014) and with non-synonymous mutation affecting VRN-1 protein interaction with other flowering pathway proteins (Li et al. 2013).

The *VRN-2* locus includes two tandemly duplicated CCT domain (*CONSTANS*, *CO-like* and *TOC1*) genes, *ZCCT1* and *ZCCT2*, which behave as long-day flowering repressors (Yan et al. 2004b). The 43-amino acid CCT domain is present in proteins involved in photoperiod, light and circadian rhythm signalling and is well conserved among different plant species (Griffiths et al. 2003). Allelic variation in this locus is attributed to either loss-of-function mutations or simultaneous deletion in all *ZCCT* genes (*vrn-2* alleles), thus leading to a recessive spring growth habit in both barley and wheat (Yan et al. 2004b, Distelfeld et al. 2009a).

VRN-3, homologous to *FLOWERING LOCUS T* (*FT*) of Arabidopsis, is the main integrator of vernalization and photoperiod signals (Yan et al. 2006, Hemming et al. 2008). In addition, *VRN-3* transcription is negatively regulated by *VRN-2* through a competition between VRN-2 and CO proteins (CO is an activator of *VRN-3* expression in the photoperiod pathway) to interact with NF-Y transcription factors (Li et al. 2011). The FT protein encoded by *VRN-3* upregulates *VRN-1* transcription by interacting with bZIP transcription factor FD in the *VRN-1* promoter G-box (Li and Dubcovsky 2008). An allelic variant of *VRN-3* associated with early flowering contains a retrotransposon inserted in the promoter region (*Vrn-3*) and has been found at low frequency in wheat germplasm (Yan et al. 2006). Furthermore, an increased

copy number of *FT* has been associated with a spring growth habit in barley (Nitcher et al. 2013).

VRN-1, *VRN-2* and *VRN-3* participate in a regulatory loop to control the timing of flowering. In contrast to its Arabidopsis homolog *AP1,* which is mainly expressed in the apical meristem, *VRN-1* is also expressed at high levels in leaves. In the shoot apex, *VRN-1* expression promotes the transition to the reproductive development to the extent that leaf production ceases and inflorescence development begins (Danyluk et al. 2003). In leaves, *VRN-1* expression unlocks the long-day flowering response, leading to the acceleration of flowering during spring (Hemming et al. 2008). Vernalization induces *VRN-1* and down-regulates *VRN-2* in leaves, and in the absence of *VRN-2*, *VRN-3* transcription is upregulated and the resulting FT protein is transported to the apical meristem where it upregulates *VRN-1* transcription to the levels required for the transition to the reproductive phase (Chen and Dubcovsky 2012).

Allelic variation at *VRN-1* of A genome has been reported to be the most important determinant of variations in growth habit compared to the other homologous copies in polyploid wheat (Sun et al. 2009). Although allelic variation at *VRN-A1* in diploid and hexaploid wheat and barley has been thoroughly studied, little is known on durum accessions (Fu et al. 2005, Golovnina et al. 2010, Oliveira et al. 2012). To our knowledge, the relationship between *VRN-A1* expression and the effect of vernalization on flowering in durum germplasm still remains unexplored.

In Argentina, durum crop currently consists of only eight varieties, all of which are spring materials with narrow variability in terms of cycle length and winter hardiness (Larsen and Jensen 2014). There is an increasing interest in exploring flowering time variability, which could, in turn, help expand the cultivation zone currently restricted to the southern portion of the province of Buenos Aires. Learning about the genetic underpinnings of variation in flowering initiation and vernalization requirements is key to directing attempts to breed cultivars for adaptation. Whether durum genotypes possess total, partial or null vernalization requirements as previously described for hexaploid wheat (Gororo et al. 2001) or barley (Mohammadi et al. 2013) remains unknown. Moreover, allele characterization at *VRN-1* loci can provide useful information concerning the ability of a genotype to withstand low temperatures, due to the demonstrated effect of *VRN-1* on cold-responsive gene expression (Zhu et al. 2014).

Therefore, to get a more detailed insight into the molecular bases of growth habit as well as of vernalization gene variability in durum wheat, we studied cultivars and advanced breeding lines which have not been previously characterized for the vernalization requirement. To this end, the effect of vernalization treatment on flowering time was analysed and a screening of allelic variation at *VRN-1* locus was carried out. The expression patterns of *VRN-A1* in genotypes differing in vernalization response were also investigated. Our findings explain heading time variability in response to low temperatures in terms of allelic variation and expression patterns of *VRN-A1* locus.

Materials and Methods

Plant materials and growth conditions: Thirty genotypes of durum wheat, *Triticum turgidum* spp. *durum*, including cultivars and advanced breeding lines from Argentina, Mexico, Italy, France, and Hungary were used in this study (Table 1). This set of accessions was formed taking

Table 1: Accessions of durum wheat, geographic origin and pedigree

Origin	Accessions	Pedigree	Supplied by[1]
Argentina	'Buck Ambar'	NA	Buck Seeds
	'Buck Esmeralda'	NA	Buck Seeds
	'Buck Cristal'	NA	Buck Seeds
	'Bonaerense INTA Cariló'	TGBB/CANDEF/3/BERK/GDOVZ516//MTTE"S"/4/LAKOTA/**CANDO**	INTA
	'Bonaerense INTA Cumenay'	CAPP/TGBB/GDO516	INTA
	'Bonaerense INTA Facón'	**STN"S"**/3/CHUR"S"/HUI"S"//POC"S"/4/MO"S"	INTA
	B#25	NA	Buck Seeds
	B#27	NA	Buck Seeds
	CBW 0101	BGTO//CATA"S"/**STN"S**"/3/LK/**CANDO**	INTA
	CBW 0105	BGTO//CATA"S"/**STN"S**"/3/LK/**CANDO**	INTA
	CBW 0112	BGTO//CATA"S"/**STN"S"**/3/F.LUNGA/GDO 645	INTA
	CBW 0141	BONVAL//F.LUNGA/GDO 645	INTA
	CBW 0153	BONVAL/BAMB	INTA
	CBW 0156	BONVAL/BAMB	INTA
	CBW 0200	BONVAL//F.LUNGA//GDO645/3/PROB611/ALTAR84	INTA
	VF 0136	CHEN/ALTAR 84/4/SRN//HUI/YAV79/3/SKARV/	INTA
	VF 0154	SORD 1/PLATA 16	INTA
	VF 0167	CDK/2620.89/PROB611/ALTAR 84	INTA
CIMMyT-Mexico	65-IAT2	AJAIA_12/F3LOCAL(SEL.ETHIO.135.85)//PLATA_13	ACA
	69-IAT2	PLATA_1/SNM//PLATA_9	ACA
	71-IAT2	SOOTY_9/RASCON_37	ACA
	80-IAT2	YAVAROS-TALL	ACA
France	'Exeldur'	VALDUR//REGAL	Buck Seeds
	'Sachem'	NA	Buck Seeds
	'Vivadur'	NA	Buck Seeds
Italy	'Cantico'	PLATANI//GIANNI	INTA
	'Ciccio'	APPULO/VALNOVA//VALFORTE/PATRIZIA	ACA
	Co 1937	COLOSSEO//PROSEME line	INTA
	'Concadoro'	SIMETO//CAPEITI/VALFORTE	INTA
Hungary	MVTD 10-98	NA	INTA

NA, Data not available.
[1]Breeding Institute/Companies supplier of seeds. Bold type: ancestor with winter growth habit.

into account genotypes currently involved in Argentinean durum breeding programmes designed by private companies (ACA, Buck Seeds) and the Instituto Nacional de Tecnología Agropecuaria (INTA). These accessions lack of formal studies describing vernalization responsive and *VRN-1* alleles.

Plants were grown in a glasshouse (20–25°C) under long-day conditions (16-h light) using a randomized design with five replicates. For vernalization experiments, seedlings were germinated at 25°C, maintained at 4°C for 6 weeks in the dark and then transferred to the glasshouse (16-h light). For each genotype, days to heading of both vernalized and non-vernalized plants were scored as the number of days between the two-leaf stage and the time at which the first spike overtopped 50% of the flag leaf (Z5.5, Zadoks scale). Glasshouse experiments were terminated 130 days after emergence. The effects of vernalization treatment on days to heading were analysed by one-tailed Student's *t* test, in comparison with non-vernalized plants.

PCR amplification, cloning and sequencing: Total DNA was isolated from 200-mg leaf samples from a pool of plants using a standard CTAB method (Rogers and Bendich 1985). DNA quality and quantity were determined on agarose gels and by spectrophotometry under UV light.

Specific primers previously designed (Table 2) were used to amplify and sequence promoter and intron 1 regions of gene *VRN-1*. PCRs were performed in a volume of 25 μl containing 1× *Taq* Buffer, 3 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 μM each primer, 50–100 ng DNA template and 1 U *Taq* DNA polymerase (Promega, USA). Amplifications were performed in a MyCycler thermal cycler (Bio-Rad, USA) programmed as follows: 94°C for 3 min, followed by 38 cycles of 94°C for 1 min, annealing at the indicated temperature in Table 2 for 45 s and 72°C for 1 min, with a final extension step of 72°C for 10 min. PCR products were separated on 1% agarose gels and visualized with SYBR Safe (Invitrogen, USA).

Allele composition of *PPD-A1* photoperiod sensitivity gene was also determined. Amplifications were performed using primer combination and PCR conditions proposed by Wilhelm et al. (2009) according to which sensitive and insensitive alleles can be recognized by differences in the amplified fragment size as a result of deletions in the promoter region (Table 2).

Sets of 15 and 13 genotypes were selected to sequence promoter and intron 1 of *VRN-A1* gene, respectively (Table 3). PCR products were cloned into pGEM-T Easy vector (Promega) following the manufacturer's instructions. Competent *E. coli* cells (strain DH5α) were transformed with the recombinant vector and plated onto LB-agar–ampicillin–X-Gal–IPTG plates. White colonies were picked and amplified in liquid LB with ampicillin, and plasmids including inserts were purified using the Wizard Plus SV Minipreps DNA Purification System (Promega). Each amplicon was sequenced on both strands to generate a consensus sequence and to minimize amplification and sequencing errors. Sequencing reactions were conducted at SIGYSA (INTA, Castelar, Argentina) using an ABI 3130XL Genetic Analyzer (Applied Biosystems, USA). Sequence identities were confirmed using algorithm BLASTN against non-redundant nucleotide collection, deposited at NCBI (NCBI, http://www.ncbi.nlm.nih.gov/). Multiple sequence alignments for comparison analysis were prepared with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) using the default settings.

Reverse transcription–PCR (RT-PCR) analysis of *VRN-1*: Semiquantitative RT-PCR was performed to study the expression patterns of a subset of five genotypes with different vernalization sensitivity. Total RNA was extracted from leaves of plants at two- and five-leaf stages growing at either 22°C (non-vernalized) or 4°C (vernalized) under long-day conditions (16-h light). Two genotypes were additionally analysed at 10- and 15-leaf stages.

After the treatments, leaves were collected and immediately frozen in liquid nitrogen and stored at −80°C until use. Total RNA was extracted

Table 2: Primers used to detect allelic variation (PCR) at the *VRN-1* and *Ppd-A1* loci and differential expression (RT-PCR) of *VRN-1* in durum wheat

	Locus	Gene Region	Primers	Sequence (5′ → 3′)	Target allele	Expected size (bp)	AT (°C)
PCR					*vrn-A1*	~500	56
	VRN-A1	Promoter[1]	VRN1A-F//VRN1-R	GAAAGGAAAAATTCTGCTCG	*Vrn-A1a*	~750	
				TGCACCTTCCC(C/G)CGCCCCAT	*Vrn-A1b*	<500	
					Vrn-A1d		
					Vrn-A1e		
	VRN-B1	Promoter[1]	VRN1B-F//VRN1-R	CAGTACCCCTGCTACCAGTG	*vrn-B1*	~1300	56
				TGCACCTTCCC(C/G)CGCCCCAT			
	VRN-A1	Intron 1[2]	Ex1/C/F//Intr1/A/R3	GTTCTCCACCGAGTCATGGT	*Vrn-A1c*	522	56
				AAGTAAGACAACACGAATGTGAGA			
	VRN-A1	Intron 1[2]	Intr1/C/F//Intr1/AB/R	GCACTCCTAACCCACTAACC	*vrn-A1*	1068	56
				TCATCCATCATCAAGGCAAA			
	VRN-B1	Intron 1[2]	Intr1/B/F//Intr1/B/R3	CAAGTGGAACGGTTAGGACA	*Vrn-B1*	709	58
				CTCATGCCAAAAATTGAAGATGA			
	VRN-B1	Intron 1[2]	Intr1/B/F//Intr1/B/R4	CAAGTGGAACGGTTAGGACA	*vrn-B1*	1149	56
				CAAATGAAAAGGAATGAGAGCA			
	Ppd-A1	Promoter[3]	Ag5del_F1//	GTATGCGATTCGCCTGAAGT	*Ppd-A1b*	453	52
			Ag5del_F2//	CGTCACCCATGCACTCTGTT	*Ppd-A1a (deletion 1027)*	381	
			Ag5del_R2	CTGGCTCCAAGAGGAAACAC	*Ppd-A1a (deletion 1117)*	290	
RT-PCR	*VRN-A1*	Exon 6-7[4]	VRN-A1F//VRN-A1R	TCGTGGAGAAGCAGAAGGC		140	55
				CTCTGCCCTCTCGCCTGTT			
	VRN-B1	Exon 4-7[4]	VRN-B1F//VRN-B1R	CCAACTTATGCACGAATCCATTT		260	65
				ATCCTCTGCCCTCTCTCCTGAT			
	ACTIN	Exon 3[5]	ACTIN-F//ACTIN-R	ATGTGGATATCAGGAAGGA		85	49
				CTCATACGGTCAGCAATAC			

1, Yan et al. (2004a); 2, Fu et al. (2005); 3, Wilhelm et al. (2009); 4, Ishibashi et al. (2007); 5, Yan et al. (2003).

Table 3: Days to heading (mean ± SE) for 30 durum wheat accessions grown at 20–25°C during 16-h light under non-vernalization and vernalization conditions. Genotypes showing a significant response to vernalization in heading date are indicated with asterisks (Student test): ***P ≤ 0.0001. MVTD 10-98 did not flower without vernalization. Alleles at the *VRN-A1* (promoter// intron 1) and *PPD-A1* loci are listed and genotypes selected for sequence analysis are highlighted in bold

Genotype	Non-vernalization	Vernalization	Student test	*VRN-A1* locus Promoter//intron 1	*PPD-A1* locus Promoter
Concadoro	32.40 ± 2.28	37.49 ± 2.62		*vrn-A1//Vrn-A1c*	*Ppd-A1b*
B# 25	34.74 ± 1.23	44.49 ± 3.66		*vrn-A1//Vrn-A1c*	*Ppd-A1a*
Ciccio	35.20 ± 1.66	37.19 ± 1.64		*vrn-A1//Vrn-A1c*	*Ppd-A1b*
VF0136	36.60 ± 2.07	42.57 ± 1.96		*vrn-A1//Vrn-A1c*	*Ppd-A1a*
Cántico	37.83 ± 1.23	37.73 ± 1.14		*vrn-A1//Vrn-A1c*	*Ppd-A1b*
B. Esmeralda	39.80 ± 1.79	39.20 ± 2.68		*vrn-A1//Vrn-A1c*	*Ppd-A1b*
71-IAT 2	40 ± 3.16	44.88 ± 2.10		*vrn-A1//Vrn-A1c*	*Ppd-A1a*
B.I. Facón	41 ± 2	40.28 ± 2.56		*vrn-A1//Vrn-A1c*	*Ppd-A1a*
CBW0141	41.20 ± 1.10	39.60 ± 5.18		*vrn-A1//Vrn-A1c*	*Ppd-A1b*
Exeldur	42.27 ± 3.12	48.42 ± 2.42		*vrn-A1//Vrn-A1c*	*Ppd-A1b*
B.Cristal	42.27 ± 2.92	51.53 ± 3.58		*vrn-A1//Vrn-A1c*	*Ppd-A1b*
Co1937	42.30 ± 0.45	44.60 ± 2.01		*vrn-A1//Vrn-A1c*	*Ppd-A1b*
B# 27	42.75 ± 1.26	51.75 ± 5.91		*vrn-A1//Vrn-A1c*	*Ppd-A1b*
65-IAT2	42.87 ± 2.10	44.73 ± 2.71		*vrn-A1//Vrn-A1c*	*Ppd-A1a*
80-IAT2	43.20 ± 1.92	42 ± 2.55		*vrn-A1//Vrn-A1c*	*Ppd-A1a*
Vivadur	43.93 ± 1.69	51.20 ± 2.66		*vrn-A1//Vrn-A1c*	*Ppd-A1b*
B.I.Cariló	44.25 ± 0.96	43.50 ± 4.51		*vrn-A1//Vrn-A1c*	*Ppd-A1b*
CBW0200	45 ± 1.74	59.21 ± 7.66		*vrn-A1//Vrn-A1c*	*Ppd-A1b*
Dupri	45.67 ± 2.08	52 ± 1.73		*vrn-A1//Vrn-A1c*	*Ppd-A1b*
69-IAT2	46.03 ± 1.22	47.80 ± 0.84		*vrn-A1//Vrn-A1c*	*Ppd-A1a*
Sachem	46.50 ± 2.20	55.96 ± 4.81		*vrn-A1//Vrn-A1c*	*Ppd-A1b*
CBW0112	47.33 ± 1.83	48.5 ± 7.65		*vrn-A1//Vrn-A1c*	*Ppd-A1b*
VF0167	49.17 ± 0.76	59.5 ± 1.32		*vrn-A1//Vrn-A1c*	*Ppd-A1b*
VF0154	49.25 ± 9.95	45.50 ± 11.03		*vrn-A1//Vrn-A1c*	*Ppd-A1a*
CBW0101	49.80 ± 1.79	49.60 ± 0.89		*vrn-A1//Vrn-A1c*	*Ppd-A1b*
B. Ambar	50.80 ± 3.90	53 ± 8.25		*vrn-A1//Vrn-A1c*	*Ppd-A1b*
CBW0153	52.92 ± 2.87	43.92 ± 3.27	***	*vrn-A1//Vrn-A1c*	*Ppd-A1b*
B.I. Cumenay	56.80 ± 2.02	42.77 ± 0.98	***	*Vrn-A1b//vrn-A1*	*Ppd-A1b*
CBW0105	98.71 ± 2.61	35.88 ± 1.55	***	*vrn-A1//vrn-A1*	*Ppd-A1b*
MVTD10-98		57.25 ± 2.06		*vrn-A1//vrn-A1*	*Ppd-A1b*

using SV Total RNA Isolation System (Promega, USA) following the manufacturer's instructions. cDNA was synthesized using the First Strand cDNA Synthesis Kit (Fermentas). Reverse transcription was performed with 75 ng total RNA in 20 µl reaction mixtures containing 1 mM each dNTP, 20 U RiboLock RNase Inhibitor, 40 U M-MuLV reverse transcriptase, 5× reaction buffer and 0.5 µg Oligo(dT)18 primer. The second-strand synthesis was carried out using specific primers (Table 2) as previously described for *VRN-1* promoter and intron 1 amplifications with the following PCR program: 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 45 s, with a final extension step of 72°C for 10 min. The *ACTIN* gene was used as an endogenous control. The expression profiles were analysed by electrophoresis through 2% agarose gels stained with ethidium bromide under UV light and imaged with a Kodak Easy share Z7590 zoom digital camera. Light intensity of the RT-PCR products was measured using IMAGE J software (http://rsb.info.nih.gov/nih-image/). Gene expression levels were semi-quantitatively assessed by obtaining the ratio of the intensity of the PCR product of target gene with the intensity of *ACTIN*. The experiments were conducted on leaves from two plants per genotype, with three technical replicates.

Results

Variability in flowering time and response to vernalization

Thirty durum wheat varieties and lines with different geographical origins (Table 1) were studied in relation to flowering time. Days to head emergence were monitored in non-vernalized plants under long-day conditions. Genotypes differed in flowering time, with average days to flowering ranging from 32 to 99 days (Table 3). The largest group (17 genotypes) exhibited 32–45 days to heading. 'Concadoro' was the earliest genotype with <33 days to flowering, whereas heading of 'B.I.Cumenay', CBW 0153 and CBW 0105 occurred after 52 days (Table 3). No flowering was observed in MVTD 10-98 accession at the end of the experiment, that is 130 days after sowing.

Compared to non-vernalized conditions, in vernalized plants MVTD 10-98 was observed to flower in an average of 52 days after cold exposure, whereas flowering time was shorter in genotypes CBW 0105, 'B.I.Cumenay' and CBW 0153 ($P < 0.0001$; Table 3). Accessions CBW 0105, 'B.I.Cumenay' and CBW 0153 were found to accelerate flowering by 63, 15 and 9 days, respectively, with respect to non-vernalized plants. In CBW 0105, the effect of vernalization was very strong on account of the fact that under this condition, it showed the shortest heading time (mean 35.88 days).

Allelic variation in the *VRN-A1* promoter region

Analysis of allelic variation was initiated in the 5`-untranslated region (UTR) and in *VRN-A1* promoter containing key transcriptional regulatory sites (Yan et al. 2004a). The PCR screening assays of *VRN-A1* promoter carried out in a set of 30 accessions (Table 1) using genome-specific primers (Table 2) showed two alleles based on the amplified fragment size (Fig. 1). All accessions produced a ~500-bp fragment except 'B.I.Cumenay', from which a smaller amplification product was obtained. Sequence analysis in a subset of 15 accessions revealed that the ~500-bp fragment was 99% homologous to *vrn-A1* allele (AY616455) from the hexaploid wheat Triple Dirk C. In contrast, the fragment amplified from 'B.I.Cumenay' was 100% homologous to *Vrn-A1b allele* (AY616461), characterized by a 20-bp deletion close to the CArG box and a 2-bp deletion/SNPs (single nucleotide polymorphisms) within the putative Vrn-box (Pidal et al. 2009). On the other hand, when nucleo-

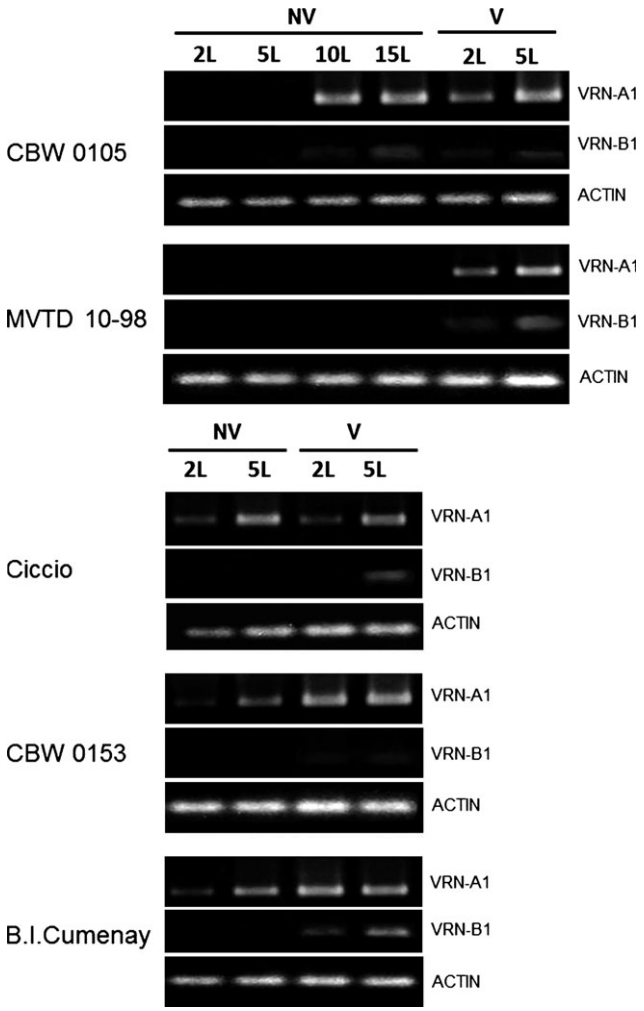

Fig. 1: Expression profiles of *VRN-1* homoeoalleles assayed by RT-PCR. RNA was extracted from leaves of plants at 2-leaf (2L), 5-leaf (5L), 10-leaf (10L) and 15-leaf (15L) stages, after growth at 22°C (NV) or 4°C (V) under long-day conditions (16-h light). CBW 0105 and MVTD 10-98 carry *vrn-A1* allele (wild-type for the promoter and intron 1 regions); Ciccio and CBW 0153 carry *Vrn-A1c* allele (with a 7222-bp deletion in the intron 1 region); B.I.Cumenay carries *Vrn-A1b* allele (the promoter contains a 20-bp deletion close to CArG-box and a 2-bp deletion + SNPs within the putative VRN-Box). All five durum accessions carry *vrn-B1* allele. *ACTIN* was used as endogenous control.

tide differences were analysed within promoter sequences in 15 accessions, only one T/G substitution at position -436 relative to ATG codon was detected from the multiple sequence alignment. In this site, CBW 0153 and CBW 0105 were observed to have nucleotide T, whereas the rest of the accessions were found to have nucleotide G.

Allelic variation in the *VRN-A1* intron 1 region

We next analysed *VRN-A1* intron 1 region using primers previously designed to detect intron 1 length (Table 2). In 27 accessions, amplification resulted in a ~500-bp fragment, whereas three accessions produced a ~1000-bp fragment.

These fragment sizes correspond to intron 1 deletion (*Vrn-A1c*) or full-length (*vrn-A1*) alleles, respectively (Table 3) (Fu et al. 2005). DNA sequence analysis in a subset of 13 accessions revealed that the ~500-bp fragments were almost 100% homologous to spring *Vrn-A1c* allele (AY747598) cv. 'Langdon'. Among genotypes with *Vrn-A1c* allele, six transitional SNPs were detected within the 522-bp intron 1 region relative to the sequence from 'Langdon': 80-IAT2 878T>C and 886T>C, 'B.I.Cariló' 881T>C, B#25 933C>T, 'B.Esmeralda' 1152C>T and CBW 0101 1166A>G. On the other hand, the ~1000-bp fragment sequences presented 99% homologous to winter *vrn-A1* allele (AY747601) Triple Dirk D, 'B.I.Cumenay' showed polymorphic sites relative to the sequence of Triple Dirk D: 1-bp indels at position 5103 and one SNP G>C at position 5470, and CBW 0105 presented three A>G SNPs at positions 5148, 5612 and 5647.

Allelic variation at *VRN-B1* locus

To study the natural diversity of *VRN-B1* locus, PCR screening was carried out in all 30 accessions using genome-specific primers for both promoter and intron 1 regions (Table 2). Amplification of genomic DNA from the promoter region using primers VRN1BF and VRN1R produced a ~1300-bp fragment that corresponds to *vrn-B1* allele (AY616453) cv. 'Langdon' (Yan et al. 2004a). No size differences were found among genotypes. PCRs were subsequently performed using primers previously designed to detect full-length or deleted intron 1 (Fu et al. 2005). Amplifications resulted in a ~1150-bp fragment corresponding to full-length *vrn-B1* allele from Triple Dirk C (AY747604) or 'Langdon' (AY747602), where no differences in amplified fragment size were observed among genotypes**.** The Intr1/B/F and Intr1/B/R3 primers designed to detect a deletion in *Vrn-B1* intron 1 yielded no amplification in none of the 30 cultivars/lines.

Allelic variation at *Ppd-A1* locus

Twenty-two genotypes were observed to carry wild-type alleles conferring photoperiod sensitivity (*Ppd-A1b*), whereas eight genotypes were found to exhibit the 1117-pb deletion (*Ppd-A1a*) associated with photoperiod insensitivity (Table 3).

Expression patterns of *VRN-1* during the development of vernalized and non-vernalized plants

To perform the expression analysis of *VRN-1*, a set of five accessions differing in heading date and vernalization response was selected. The transcriptional levels of the two *VRN-1* homoeologs in the stages analysed under vernalized and non-vernalized conditions are shown in Fig. 1.

In non-vernalized plants, *VRN-A1* transcripts were detected in 'Ciccio', 'B.I.Cumenay' and CBW 0153 in the 2-leaf stage, and stronger signal bands were detected in the 5-leaf stage (Fig. 1). In the case of CBW 0105, no *VRN-A1* transcripts were observed in non-vernalized plants in the 2- and 5-leaf stages. Nonetheless, transcripts were clearly detected approximately in the 10-leaf stage and remained the same in the 15-leaf stage. No transcripts were detected in non-vernalized plants of MVTD 10-98 until 15-leaf stage.

When transcription was analysed in plants growing under vernalization conditions, all genotypes showed detectable levels of *VRN-A1* transcript at the initial sampling point of 2-leaf stage (Fig. 1). Intensity of *VRN-A1* band in the 5-leaf stage of 'Ciccio', CBW 0105 and MVTD 10-98 was stronger than that in the previous stage, whereas 'B.I.Cumenay' and CBW 0153 showed similar signal intensity between 2-leaf stage and 5-leaf stage.

No *VRN-B1* transcripts were detected before the cold treatment except in CBW0105 from the 10-leaf stage (Fig. 1). Obvious differences were observed between the transcripts from the *VRN-A1* and *VRN-B1* loci in all genotypes; *VRN-B1* always showed low signal intensity with respect to *VRN-A1*.

Discussion

In this study, attempts were made to assess variation in the response to vernalization in a collection of durum wheat accessions. To this end, allelic variation in *VRN-1* gene was analysed using PCR amplification and DNA sequencing. Five genotypes differing in vernalization sensitivity were specifically compared via expression analysis.

No evidence of polymorphisms in size was observed in the promoter and intron 1 regions of *VRN-1* in the B genome. Previous studies have reported the absence of polymorphisms in the promoter region between dominant and recessive *VRN-B1* alleles (Yan et al. 2004a, Golovnina et al. 2010). Recent research has described a novel *Vrn-B1* allele in durum wheat which harbours a retrotransposon in its promoter at ~100 bp upstream start codon, conferring spring growth habit (Chu et al. 2011). In contrast, considerable variability in terms of deletions of several sizes and duplications associated with spring growth habit has been detected in the intron 1 region of *VRN-B1* (Fu et al. 2005, Milec et al. 2012, Shcherban et al. 2012). On account of the fact that the *VRN-B1* fragments amplified in this study were observed to exhibit the typical size of wild-type winter alleles, polymorphisms responsible for the variation in the vernalization response were not found.

The effect of vernalization on flowering was further analysed taking into account allelic variation at *VRN-A1* and transcription patterns of *VRN-A1* and *VRN-B1*. MVTD 10-98 was observed to exhibit flowering behaviour, vernalization response and allelic constitution for *VRN-1* (Table 3) as typically observed in a winter variety, that is a variety that is unable to progress to the reproductive phase under non-vernalized conditions because the essential genes for flowering remain repressed (Shitsukawa et al. 2007). Induction of *VRN-1* and subsequent progression to the reproductive phase were observed to occur only after vernalization. As previously observed (Loukoianov et al. 2005), comparisons of level of transcripts between *VRN-1* homeologous loci revealed that transcripts from *VRN-A1* accumulated at higher levels than *VRN-B1* (Fig. 1).

As observed with MVTD 10-98, CBW 0105 showed a strong vernalization response (Table 3) with vernalized plants flowering 63 days earlier than non-vernalized plants. Although these two genotypes harbour the same wild-type *vrn-1* allele in both genomes (Table 3), CBW 0105 flowered after 99 days even in the absence of a prolonged exposure to cold temperatures, thus indicating that its vernalization requirement is not absolute. The difference in flowering behaviour between MVTD 10-98 and CBW 0105 was clearly associated with *VRN-1* expression profiles. Unlike MVTD 10-98, CBW 0105 was found to evidence induction of *VRN-A1* in the 10-leaf stage under non-vernalized conditions (Fig. 1), thus indicating a differential regulation of *VRN-A1* between both genotypes. In the limited sequenced region of intron 1, three SNPs located towards the 3′ end of the intron 1 region, at >5 kb from intron 1 start, differentiated CBW 0105 from the full-winter MVTD 10-98. Most of the mutations in the intron 1 region associated with elevated expression of *VRN-1* in non-vernalized plants consist of size-variable indels affecting the core regulatory region in wheat and barley (Fu et al. 2005, Hemming et al. 2009). The single T/G substitution detected in the promoter region of CBW 0105 and MVTD 10-98 is equally present in winter *vrn-A1* (AY616455) and spring *Vrn-A1d* (AY616462) and *Vrn-A1e* (AY616463) alleles, thus suggesting that this substitution is not associated with growth habit. Summing up, the polymorphisms detected in the promoter and intron 1 regions were outside of the previously described regulatory sites (Fu et al. 2005, Kane et al. 2007, Li and Dubcovsky 2008, Pidal et al. 2009), thus suggesting that these SNPs are not likely to be responsible for the differences in *VRN-A1* expression.

Transcripts from *VRN-B1* could also be detected in non-vernalized plants but with lower level of expression than *VRN-A1* (Fig. 1), indicating that most of the RNA transcripts originated from the *VRN-1* locus of genome A (Loukoianov et al. 2005). Given that the transcriptional activity found in this study corresponds to the form *VRN-A1* > *VRN-B1,* the presence of a dominant *VRN-B1* homoeoallele activating the recessive allele at *VRN-A1* through coordinated transcription (Loukoianov et al. 2005, Shcherban et al. 2013) can be discarded. In addition, hexaploid wheat lines having dominant alleles in the B genome and showing coordinated *VRN-1* regulation take <55 days to flowering (Allard et al. 2012), but this is not the case of CBW 0105 (~99 days).

A plausible explanation for the difference in *VRN-1* expression between MVTD 10-98 and CBW 0105 (Fig. 1) could be found in the flowering regulatory model that includes *VRN-1*, *VRN-2* and *VRN-3* (Distelfeld et al. 2009b). Winter habit determination requires functional copies of *VRN-2* (Tranquilli and Dubcovsky 2000) to such an extent that *VRN-2* maintains the repression of the *VRN-3* flowering promoter (Distelfeld et al. 2009b). In lines lacking *VRN-2*, there is no repressor for *VRN-3* and induction of *VRN-1* occurs via a *VRN-3*-mediated photoperiod signal. Differences in days to heading were observed between the *Vrn-2* and *vrn-2* allelic classes within the *vrn-1* class in *T. monoccocum* (Tranquilli and Dubcovsky 2000). This mechanism requires an inductive light regime of long days, as followed in our experimental study, and a photoperiod-sensitive allele in the *PPD-1* locus (Hemming et al. 2008) whose presence was confirmed via PCR analysis in CBW 0105 (Table 3). Thus, CBW 0105 flowering behaviour completely agrees with a class of winter barley genotype known as 'facultative' in which the full vernalization requirement is eliminated because *VRN-2/ZCCT* genes are deleted (von Zitzewitz et al. 2005). CBW 0105 origin strongly supports this hypothesis. This is a durum line derived from winter × spring crosses (C. Jensen and A. Larsen, personal communication) where recombinant genotypes '*vrn-1/vrn-2*' can be obtained.

Other forms of genetic variation among winter alleles of *VRN-1* as SNPs in exon 4 (Chen et al. 2009) and non-synonymous mutations in exon 7 (Li et al. 2013) lead to quantitative differences in the vernalization requirement in common wheat. These mutations affect the vernalization requirement at the protein level, and they therefore do not represent a plausible explanation for the differential regulation of *VRN-A1* observed in this study. In turn, Díaz et al. (2012) reported that cultivars carrying duplications of *VRN-A1* display late flowering and concluded that plants with a high copy number have an increased vernalization requirement. Variation in copy number of *VRN-A1* among winter varieties from different regions of the world has been recently confirmed (Zhu et al. 2014). Additional research is still necessary to determine whether MVTD 10-98 and CBW 0105 differ in *VRN-1* copy number.

The remaining genotypes included in this study were observed to carry mutations at the *VRN-A1* locus that are expected to modify its basal transcription level expression profile in comparison with the wild-type winter allele. 'B.I.Cumenay' is a relatively late-flowering genotype (Table 3) with a moderate vernalization response and with evidence of *VRN-A1* transcription under non-vernalized conditions (Fig. 1). At the *VRN-A1* locus, 'B.I.Cumenay' carries the *Vrn-A1b* allele in the promoter region and the full-length allele in the intron 1 region (Table 3). *Vrn-A1b* allele contains deletions and SNPs at a regulatory site (putative VRN-box) in the promoter region that causes *VRN-A1* transcription under non-inductive temperatures (Pidal et al. 2009). This explains the presence of transcripts in the 2-leaf stage of non-vernalized plants in 'B.I.Cumenay'. The transcriptional activation of *VRN-1* during vernalization (Fig. 1) is likely to be responsible for the earlier flowering observed under cold conditions. As 'B.I.Cumenay' carries a full-length and therefore inducible intron 1, a positive effect of vernalization on *VRN-A1* transcription through the activation of changes in chromatin could occur (Oliver et al. 2009). The pedigree of 'B.I.Cumenay' includes the Italian variety 'Cappelli', the Emmer wheat derivative 'Yuma' from USA and 'Taganrog', originally cultivated in Russia (Engineer Carlos Jensen and Engineer Adelina Larsen, personal communication). Which of these ancestral accessions is the donor of the *Vrn-A1b* allele present in 'B.I.Cumenay' still remains unknown.

The rest of the genotypes analysed carry the *Vrn-A1c* allele which presents a large deletion in the intron 1 region, thus affecting regulatory regions (Table 3; Fu et al. 2005, Cockram et al. 2007). This mutation causes *VRN-A1* to be transcribed under non-inductive low-temperature conditions. A typical member of this class of genotypes is 'Ciccio'. The latter showed the same expression profile under vernalization and non-vernalization conditions (Fig. 1); that is, *VRN-A1* transcripts were detected in the 2-leaf stage and expression seemed to increase as developmental stages progressed. These observations agree with 'Ciccio′s' null response to vernalization in heading date. These results characterize 'Ciccio' and they also seem to characterize the rest of the accessions carrying the deletion intron 1 (Table 3), such as spring genotypes whose exposure to cold does not accelerate flowering. An exception to this behaviour was CBW 0153. Although it contains the deletion in the *VRN-A1* intron 1 region (Table 3), it exhibited a moderate response to vernalization, thus accelerating flowering in nine days. Expression profiles were consistent with this sensitivity on account of the fact that cold-treated plants of CBW 0153 showed a stronger expression signal in *VRN-A1* compared to non-vernalized plants in 2- and 5-leaf developmental stages (Fig. 1). Previous studies that analysed overlapping deletions of various sizes in the intron 1 region showed that alleles with high basal expression as a result of large deletions can still be induced by low temperatures (Hemming et al. 2009, Oliver et al. 2013). In addition, because CBW 0153 carries the same allelic constitution in *VRN-A1* as the rest of the spring accessions (Table 3), its response to vernalization cannot be explained by means of the gene regions examined. However, *VRN-A1* expression profile in CBW 0153 was observed to fully follow this flowering behaviour. Further studies involving other *VRN-1* regions or loci affecting *VRN-A1* regulation are therefore necessary to determine the causes of *VRN-1* induction via cold temperature in CBW 0153.

Interestingly, it could be observed in the present study that two independent mutations in gene *VRN-A1* determine the same flowering phenotype. 'B.I.Cumenay' and CBW 0153 were found to carry altered forms of promoter and intron 1, respectively. Although these mutations affect different *VRN-A1* regulatory sites, both genotypes not only flower at a similar time under glasshouse conditions but also accelerate flowering similarly under vernalized conditions.

Common and durum wheat accessions differ markedly in spring and winter allele frequencies. A recent study carried out on ~700 *T. aestivum* cultivars from different geographical origins reported that only 7% of the samples analysed carried spring alleles in *VRN-A1* (Kiss et al. 2014). Our analysis of 30 durum wheat accessions with specific molecular markers demonstrated a clear preponderance of spring dominant alleles at the *VRN-A1* locus (93% of accessions). Durum breeding programmes often perform winter × spring crosses tending to enlarge the genetic basis of a predominantly spring germplasm. In this respect, CBW 0105 and CBW 0101 are sister lines originated from winter × spring crosses (Engineer Carlos Jensen and Engineer Adelina Larsen, personal communication), showing differences in the allelic status of *VRN-A1* and in the vernalization response (Table 3).

In the current study, only one genotype, 'B.I.Cumenay', showed the spring *VRN-A1* allele originated by mutations in the promoter region. The low frequency of this *VRN-A1b* agrees with findings from previous studies according to which deletion in the intron 1 region is the most frequent determinant of spring habit in tetraploid wheats (Yan et al. 2004a, Fu et al. 2005, Golovnina et al. 2010, Chu et al. 2011). Molecular screening in tetraploid wheat showed that intron 1 deletion is highly frequent in *T. turgidum* subspecies, while it is nearly absent in *T. dicoccoides* and *T. dicoccum* emmer wheats (Oliveira et al. 2012). This has been attributed to the different selection procedures followed to obtain cultivars adapted to a diversity of climates.

Considering the first week of August to be the typical sowing date, in the durum wheat cultivation zone in Argentina, commercial durum varieties head within a limited range of 71–78 days (Larsen and Jensen 2014). Here, our analysis of the days to heading under conditions in which light was not limiting revealed phenotypic variability in a durum collection that could potentially be useful to the development of new varieties. Wheats with longer cycles allow earlier sowing and extended vegetative periods, thus increasing yield. Varieties with moderate vernalization requirements adapt to a wider range of sowing time and thus fit better to crop rotation schemes.

The effect of vernalization on flowering has long been categorized in relation to bread wheat and barley. The integration of sequencing and transcriptional data from durum wheat collected in the present study secures a molecular framework within which the previously established phenotypic categories, namely cold obligate, cold stimulated, cold neutral (Gardner and Barnett 1990), can be understood. This relevant information stimulates advances in durum breeding including frost tolerance and promotes efforts to expand its cultivation area.

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References

Allard, V., V. Otto, K. Bela, M. Rousset, J. Le Gouis, and P. Martre, 2012: The quantitative response of wheat vernalization to environmental variables indicates that vernalization is not a response to cold temperature. J. Exp. Bot. **63**, 847—857.

Araus, J. L., G. Slafer, C. Royo, and M. D. Serret, 2008: Breeding for yield potential and stress adaptation in cereals. Crit. Rev. Plant Sci. **27**, 377—412.

Bergjord, A. K., A. K. Bakken, and A. O. Skjelva, 2009: The relationship between frost tolerance and generative induction in winter wheat (*Triticum aestivum* L.) under field conditions. Can. J. Plant Sci. **89**, 1031—1039.

Chen, A., and J. Dubcovsky, 2012: Wheat TILLING mutants show that the vernalization gene *VRN1* down-regulates the flowering repressor *VRN2* in leaves but is not essential for flowering. PLoS Genet. **8**, 1—13.

Chen, Y., B. F. Carver, S. Wang, F. Zhang, and L. Yan, 2009: Genetic loci associated with stem elongation and winter dormancy release in wheat. Theor. Appl. Genet. **118**, 881—889.

Chu, C. G., C. T. Tan, G. T. Yu, S. Zhong, S. S. Xu, and L. Yan, 2011: A novel retrotransposon inserted in the dominant Vrn-B1 allele confers spring growth habit in tetraploid wheat (*Triticum turgidum* L.). G3 (Bethesda) **1**, 637—645.

Cockram, J., E. Chiapparino, S. A. Taylor, K. Stamati, P. Donini, D. A. Laurie, and D. M. O'Sullivan, 2007: Haplotype analysis of vernalization loci in European barley germplasm reveals novel *VRN-H1* alleles and a predominant winter *VRN-H1/VRN-H2* multi-locus haplotype. Theor. Appl. Genet. **115**, 993—1001.

Danyluk, J., N. A. Kane, G. Breton, A. E. Limin, D. B. Fowler, and F. Sarhan, 2003: TaVRT-1, a putative transcription factor associated with vegetative to reproductive transition in cereals. Plant Physiol. **132**, 1849—1860.

Díaz, A., M. Zikhali, A. S. Turner, P. Isaac, and D. A. Laurie, 2012: Copy number variation affecting the photoperiod-B1 and vernalization-A1 genes is associated with altered flowering time in wheat (*Triticum aestivum*). PLoS ONE **7**, e33234.

Distelfeld, A., G. Tranquilli, C. Li, L. Yan, and J. Dubcovsky, 2009a: Genetic and molecular characterization of the *VRN2* loci in tetraploid wheat. Plant Physiol. **149**, 245—257.

Distelfeld, A., C. Li, and J. Dubcovsky, 2009b: Regulation of flowering in temperate cereals. Curr. Opin. Plant Biol. **12**, 178—184.

Fu, D., P. Szűcs, L. Yan, M. Helguera, J. S. Skinner, J. von Zitzewitz, P. M. Hayes, and J. Dubcovsky, 2005: Large deletions within the first intron in *VRN-1* are associated with spring growth habit in barley and wheat. Mol. Genet. Genomics **273**, 54—65.

Gardner, F. P., and R. D. Barnett, 1990: Vernalization of wheat cultivars and a triticale. Crop Sci. **30**, 166—169.

Golovnina, K. A., E. Y. Kondratenko, A. G. Blinov, and N. P. Goncharov, 2010: Molecular characterization of vernalization loci *VRN1* in wild and cultivated wheats. BMC Plant Biol. **10**, 168.

Gororo, N. N., R. G. Flood, R. F. Eastwood, and H. A. Eagles, 2001: Photoperiod and vernalization responses in *Triticum turgidum* × *T. tauschii* synthetic hexaploid wheats. Ann. Bot. Lond. **88**, 947—952.

Griffiths, S., R. P. Dunford, G. Coupland, and D. A. Laurie, 2003: The evolution of CONSTANS-like gene families in barley, rice, and Arabidopsis. Plant Physiol. **131**, 1855—1867.

Hemming, M. N., W. J. Peacock, E. S. Dennis, and B. Trevaskis, 2008: Low temperature and day length cues are integrated to regulate FLOWERING LOCUS T in barley. Plant Physiol. **147**, 355—366.

Hemming, M. N., S. Fieg, W. J. Peacock, E. S. Dennis, and B. Trevaskis, 2009: Regions associated with repression of the barley (*Hordeum vulgare*) VERNALIZATION1 gene are not required for cold induction. Mol. Genet. Genomics **282**, 107—117.

Ishibashi, M., F. Kobayashi, J. Nakamura, K. Murai, and S. Takumi, 2007: Variation of freezing tolerance, Cor/Lea gene expression and vernalization requirement in japanase common wheat. Plant Breed. **126**, 464—469.

Kamran, A., H. S. Randhawa, C. Pozniak, and D. Spaner, 2013: Phenotypic effects of the flowering gene complex in Canadian spring wheat germplasm. Crop Sci. **53**, 84—94.

Kane, N. A., Z. Agharbaoui, A. O. Diallo, H. Adam, Y. Tominaga, F. Ouellet, and F. Sarha, 2007: TaVRT2 represses transcription of the wheat vernalization gene TaVRN1. Plant J. **51**, 670—680.

Kiss, T., K. Balla, O. Veisz, L. Láng, Z. Bedő, S. Griffiths, P. Isaac, and I. Karsai, 2014: Allele frequencies in the *VRN-A1*, *VRN-B1* and *VRN-D1* vernalization response and *PPD-B1* and *PPD-D1* photoperiod sensitivity genes, and their effects on heading in a diverse set of wheat cultivars (*Triticum aestivum* L.). Mol. Breed. **34**, 297—310.

Larsen, A. O., and C. A. Jensen, 2014: Evaluación de cultivares de trigo candeal en Barrow-Campaña 2013–2014. EEAI INTA Barrow. Available at: http://inta.gob.ar/documentos/evaluacion-de-cultivares-de-trigo-candeal-en-barrow-campana-2013-2014 (last accessed March 14, 2014).

Li, C., and J. Dubcovsky, 2008: Wheat FT protein regulates *VRN1* transcription through interactions with FDL2. Plant J. **55**, 543—554.

Li, C., A. Distelfeld, A. Comis, and J. Dubcovsky, 2011: Wheat flowering repressor VRN2 and promoter CO2 compete for interactions with NUCLEAR FACTOR-Y complexes. Plant J. **67**, 763—773.

Li, G., M. Yu, T. Fang, S. Cao, B. F. Carver, and L. Yan, 2013: Vernalization requirement duration in winter wheat is controlled by TaVRNA1 at the protein level. Plant J. **76**, 742—753.

Loukoianov, A., L. Yan, A. Blechl, A. Sanchez, and J. Dubcovsky, 2005: Regulation of *VRN-1* vernalization genes in normal and transgenic polyploidy wheat. Plant Physiol. **138**, 2364—2373.

Milec, Z., L. Tomková, T. Sumíková, and K. Pánkova, 2012: A new multiplex PCR test for the determination of Vrn-B1 alleles in bread wheat (*Triticum aestivum* L.). Mol. Breeding **30**, 317—323.

Mohammadi, M., D. Torkamaneh, and H.-R. Nikkhah, 2013: Correlation of vernalization loci VRN-H1 and VRN-H2 and growth habit in barley germplasm. Int. J. Plant Genomics **2013**, 1—9, doi:10.1155/2013/924043.

Nitcher, R., A. Distelfeld, C. Tan, L. Yan, and J. Dubcovsky, 2013: Increased copy number at the HvFT1 locus is associated with accelerated flowering time in barley. Mol. Genet. Genomics **288**, 261—275.

Oliveira, H. R., M. G. Campana, H. Jones, H. V. Hunt, F. Leigh, D. I. Redhouse, D. L. Lister, and M. K. Jones, 2012: Tetraploid wheat landraces in the Mediterranean Basin: taxonomy, evolution and genetic diversity. PLoS ONE **7**, e37063.

Oliver, S. N., E. J. Finnegan, E. S. Dennis, W. J. Peacock, and B. Trevaskis, 2009: Vernalization-induced flowering in cereals is associated with changes in histone methylation at the VERNALIZATION1 gene. Proc. Natl Acad. Sci. USA **106**, 8386—8391.

Oliver, S. N., W. Deng, M. C. Casao, and B. Trevaskis, 2013: Low temperatures induce rapid changes in chromatin state and transcript levels of the cereal VERNALIZATION 1 gene. J. Exp. Bot. **64**, 2413—2422.

Pidal, B., L. Yan, D. Fu, F. Zhang, G. Tranquilli, and J. Dubcovky, 2009: The CArG-Box located upstream from the transcriptional start of wheat vernalization gene *VRN1* is not necessary for the vernalization response. J. Hered. **100**, 355—364.

Rogers, S. O., and A. J. Bendich, 1985: Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. Plant Mol. Biol. **5**, 69—76.

Shcherban, A. B., T. T. Efremova, and E. A. Salina, 2012: Identification of a new Vrn-B1 allele using two near-isogenic wheat lines with difference in heading time. Mol. Breeding **29**, 675—685.

Shcherban, A. B., E. K. Khlestkina, T. T. Efremova, and E. A. Salina, 2013: The effect of two differentially expressed wheat VRN-B1 alleles on the heading time is associated with structural variation in the first intron. Genetica **141**, 133—141.

Shitsukawa, N., C. Ikari, S. Shimada, S. Kitagawa, K. Sakamoto, H. Saito, H. Ryuto, N. Fukunishi, T. Abe, and S. Takumi, 2007: The einkorn wheat (*Triticum monococcum*) mutant, maintained vegetative phase, is caused by a deletion in the *VRN1* gene. Genes Genet. Syst. **82**, 167—170.

Sun, Q. M., R. H. Zhou, L. F. Gao, G. Y. Zhao, and J. Z. Jia, 2009: The characterization and geographical distribution of the genes responsible for vernalization requirement in Chinese bread wheat. J. Integr. Plant Biol. **51**, 423—432.

Tranquilli, G., and J. Dubcovsky, 2000: Epistatic interactions between vernalization genes Vrn-Am 1 and Vrn-Am2 in diploid wheat. J. Hered. **91**, 304—306.

Trevaskis, B., D. J. Bagnall, M. H. Ellis, W. J. Peacock, and E. S. Dennis, 2003: MADS box genes control vernalization-induced flowering in cereals. Proc. Natl Acad. Sci. USA **100**, 13099—13104.

Wilhelm, E. P., A. S. Turner, and D. A. Laurie, 2009: Photoperiod insensitive Ppd-A1a mutations in tetraploid wheat (*Triticum durum* Desf.). J. Theor. Appl. Genet. **118**, 285—294.

Yan, L., A. Loukoianov, G. Tranquilli, M. Helguera, T. Fahima, and J. Dubcovsky, 2003: Positional cloning of wheat vernalization gene *VRN1*. Proc. Natl Acad. Sci. USA **100**, 6263—6268.

Yan, L., M. Helguera, K. Kato, S. Fukuyama, J. Sherman, and J. Dubcovsky, 2004a: Allelic variation at the *VRN-1* promoter region in polyploid wheat. Theor. Appl. Genet. **109**, 1677—1686.

Yan, L., A. Loukoianov, A. Blechl, G. Tranquilli, W. Ramakrishna, P. San Miguel, J. L. Bennetzen, V. Echenique, and J. Dubcovsky, 2004b: The wheat *VRN2* gene is a flowering repressor down-regulated by vernalization. Science **303**, 1640—1644.

Yan, L., D. Fu, C. Li, A. Blechl, G. Tranquilli, M. Bonafede, A. Sanchez, M. Valarik, and J. Dubcovsky, 2006: The wheat and barley vernalization gene *VRN3* is an orthologue of FT. Proc. Natl Acad. Sci. USA **103**, 19581—19586.

Zhu, J., S. Pearce, A. Burke, D. R. See, D. Z. Skinner, J. Dubcovsky, and K. Garland-Campbell, 2014: Copy number and haplotype variation at the VRN-A1 and central FR-A2 loci are associated with frost tolerance in hexaploid wheat. Theor. Appl. Genet. **127**, 1183—1197.

von Zitzewitz, J., P. Szűcs, J. Dubcovsky, L. Yan, N. Pecchioni, E. Francia, A. Casas, T. H. H. Chen, P. M. Hayes, and J. S. Skinner, 2005: Molecular and structural characterization of barley vernalization genes. Plant Mol. Biol. **59**, 449—467.