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Genes responding to vernalization in hexaploid wheat

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Abstract Genotype-specific gene expression in response to vernalization in common wheat was examined by the differential display method. Two near-isogenic lines of *Vrn-A1* (*Vrn-A1* for the spring type and *vrn-A1* for the winter type) were treated by vernalization of developing embryos in detached-ear cultures. This treatment was effective to promote *vrn-A1* genotypes to head at a time equivalent to that of *Vrn-A1*. Differential cDNA fragments were isolated by the RT-PCR method from embryos subjected to vernalization treatments for 2- and 4-weeks at DAP10 and DAP20 stages, respectively. Among 110 differential cDNA fragments isolated, 48 were examined for their chromosomal locations and designated as *wec* (*w*heat-*e*mbryo *c*old treatment) genes. Seven *wec* genes showed genotype-specific expression in response to vernalization. The statistical analysis utilizing two recombinant inbred lines showed that four *wec* genes were significantly associated with heading factors.

Keywords Vernalization requirement · Heading time · Near-isogenic line · Differential display · QTL analysis

Introduction

The timing of the transition from vegetative growth to flowering in higher plants is one of the important characters for evolutionary adaptation, because plants need to flower under favorable environmental conditions for sexual reproduction. This transition is controlled by interactions between genetical and environmental factors. Vernalization, which is defined as exposure to a low temperature for a certain period, is especially important for some plants that originated from temperate regions in order to ensure flowering in the spring and not during the winter months.

In wheat species, vernalization requirement is a critical determinant between spring-sowing and winter-sowing types, and also one of the major genetic factors for controlling heading time. Although most of the wild relatives of wheat are of the winter type, the advent of spring types has enabled wheat to grow in both higher- and lower-latitude areas. Because of this adaptability to a large range of environments, wheat has become one of the major crop plants. In common wheat, *Triticum aestivum* L. (2n=42), three major homoeologous genes concerning vernalization requirement have been identified, i.e. *Vrn-A1*, *-B1*, and *-D1* on chromosomes 5A, 5B and 5D, respectively (Law et al. 1976; Maystrenko 1980; Galiba et al. 1995; Nelson et al. 1995; Korzun et al. 1997; McIntosh et al. 1998). The degree of requirement to complete heading depends on the *Vrn* genotype. For example, *Vrn-A1* does not require vernalization treatment at all: whereas *Vrn-B1* and *Vrn-D1* require vernalization for 15–30 days, and winter-type wheats recessive for all of these genes require 45–60 days for heading (Maystrenko 1987; Kato 1988). Among these genes, *Vrn-A1* has been mapped in detail on the long arm of chromosome 5A (Sarma et al. 1998; Kato et al. 1999).

Although *Vrn* genes has been genetically analyzed elaborately, their function with respect to heading has not yet been clarified. Moreover, there is little knowledge as to how vernalization treatment promotes the heading of winter-type wheat without the dominant *Vrn* genes. These difficulties may be due to the following: (1) the *Vrn* genes have not been isolated, (2) neither the growth stage nor the organs of the vernalization response have been specified, and (3) since the response to vernalization treatment involves both promotion of heading and metabolic adjustment to a low temperature, the genes involved in heading can not be separated from those responsible for cold acclimation.

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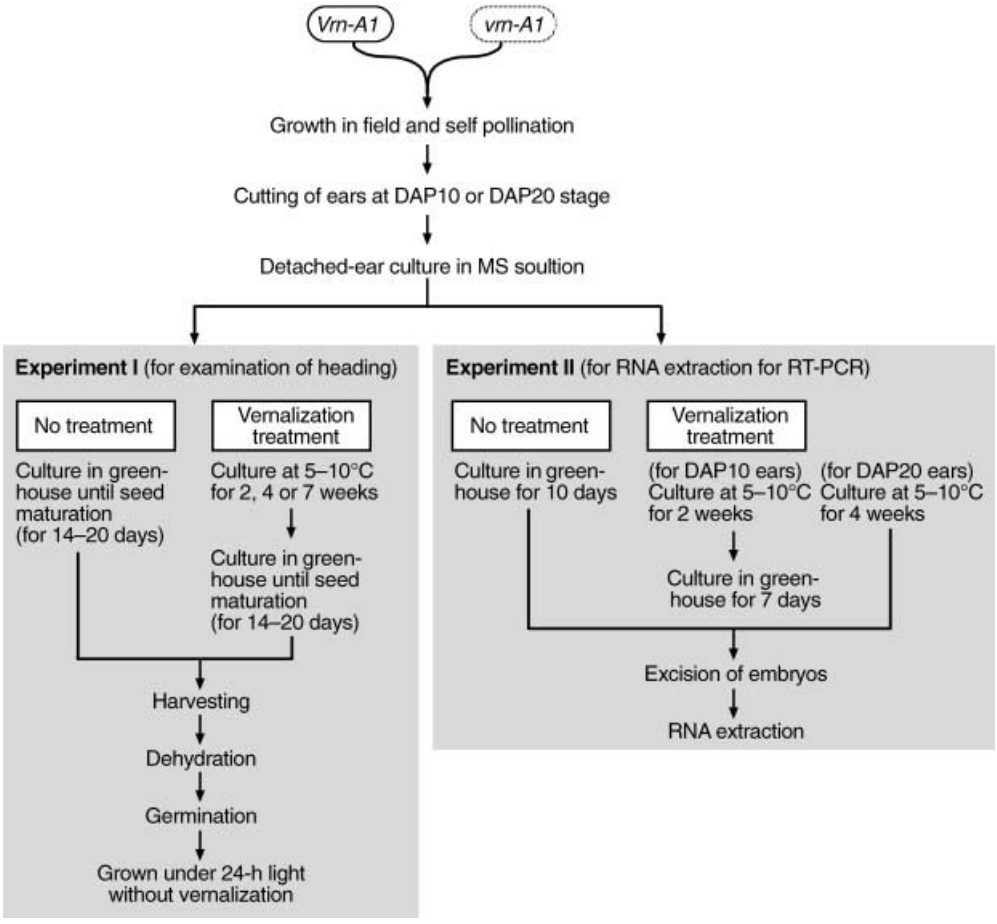

Fig. 1 An outline for vernalization of developing embryos in detached-ear cultures

The utilization of both near-isogenic lines and the application of cold treatment of developing embryos provides a valuable tool for distinguishing between the response to low temperature and that promoting heading. Isogenic lines of *Vrn* genes were established on a common background of cv Triple Dirk (Pugsley 1968, 1971). Kato et al. (1990) reported that the developing embryos have the ability to respond to vernalization treatment. We have established a series of recombinant inbred lines showing clear genetic segregation for the traits concerned (Shindo and Sasakuma, unpublished). These lines and treatments will allow detection of the genes necessary for heading to be expressed at a restricted growth stage and in a specific organ.

In this report, we describe the genotype-specific gene expression in hexaploid wheat that occurs in response to vernalization. The differential display method was applied to vernalized embryos to isolate genes responding to vernalization in a genotype-specific manner. The isolated cDNA fragments were mapped to two recombinant inbred lines of hexaploid wheats and diploid species, respectively, and evaluated for their association with the heading trait by QTL (quantitative trait locus) analysis. This strategy enabled us to examine the relationship between differentially expressed cDNA and heading. The structural characteristics and location were also examined.

Materials and methods

Plant materials

Isogenic lines for *Vrn* genes in a common wheat cultivar Triple Dirk (TD) (Pugsley 1968, 1971) were used for a test of the vernalization response and for isolation of genes responding to vernalization, namely, TD(D) and TD(C) for *Vrn-A1* and *vrn-A1*, respectively. Also, nulli-tetrasomic lines of Chinese Spring derived by Sears (1954) were used to determine the homoeologous groups of the isolated gene products. Two recombinant inbred lines (RILs) of hexaploid (Chinese Spring × spelt wheat) and diploid (*Triticum monococcum* × *Triticum boeoticum*) wheat, RIL1 and RIL5, respectively, were established and utilized for mapping cDNAs and QTL analysis. The genetic analysis of heading factors (narrow-sense earliness, vernalization requirement, and photoperiod sensitivity) indicated that segregation of the genes involved in each factor could be examined individually by growing the wheats under controlled conditions as well as in the field (Shindo and Sasakuma, unpublished).

Vernalization treatment for detached-ear cultures

The vernalization treatment for developing embryos is schematically presented in Fig. 1, which shows the protocols for examination of the effects of vernalization treatment on heading (Experiment I) and RT-PCR analysis (Experiment II). Two isogenic lines, *Vrn-A1* and *vrn-A1*, were grown in the field, and their ears at DAP10 and DAP20 stages were cut at the second internodes and cultured in a solution containing 100 g/ml of sucrose and MS

salts. In experiment I, the detached ears were cultured under a vernalization treatment (5/10 °C with 14-h lighting) in a phytron (Koito Co.) for 2, 4 or 7 weeks. After the vernalization treatment, they were moved to a greenhouse until seed maturation (for 14–20 days). For the control, the detached ears were cultured in the same solution in the greenhouse until maturation (for 14–20 days). Mature seeds were harvested and then dehydrated. This system is here-after referred to as embryo vernalization. The dried seeds were germinated, and ten seedlings were grown in a greenhouse under 24-h light without vernalization treatment. For comparison with the conventional method of vernalization treatment for young seedlings (seedling vernalization), 3-day seedlings were also treated with low temperature (5–10 °C) for 2, 4 or 7 weeks. In this treatment, the control seedlings were germinated 3 days before the end of vernalization in order to adjust the plant growth to same stage. The number of days to the unfolding of the flag leaf from the start of the long-day condition was recorded as days to heading.

In experiment II for RT-PCR analysis, the detached ears were cultured under the same conditions as in Experiment I. Ears of DAP20 with a 4-week treatment were subjected to excision of embryos, whereas those of DAP10 with a 2-week treatment were cultured in the greenhouse for 7 days before the excision. In the non-vernalization treatment, ears of DAP10 and 20 were cultured for 10 days until sampling. The excised embryos were immediately frozen in liquid N_2 and stored at –80 °C until used for RNA extraction.

Synthesis of cDNA and RT-PCR

Total RNA was extracted from about 200 embryos by the phenol/LiCl method (Chirgwin et al. 1979). Poly(A)$^+$ RNA was purified from 1.0 mg of total RNA by using Oligotex-dT30<Super> (TaKaRa). First-strand cDNA was synthesized from 500 ng of poly(A)$^+$ RNA by SuperScript II RNase H$^-$Reverse Transcriptase (GIBCOBRL). DNA fragments were amplified by PCR using a 100-fold diluted solution of the first-strand cDNA as templates. The reaction mixture contained 1 ml of template solution, 10 mM of Tris-HCl (pH 8.3), 50 mM of KCl, 1.5 mM of $MgCl_2$, 200-mM concentration of each dNTP, 400 nM of 10-mer common's primer (BEX), and 0.625 units of *Taq* DNA polymerase (TaKaRa). The amplification reaction was carried in a GeneAmp PCR System 9600 (Perkin-Elmer) for 40 cycles (denaturation at 94 °C for 1 min, annealing at 36 °C for 1 min, and extension at 72 °C for 2 min).

PAGE and silver staining

The amplified cDNA fragments were separated by electrophoresis on a 10% polyacrylamide-0.27% BIS gel in buffer (3% Tris base and 0.15% glycine). The gel after electrophoresis was soaked in 10% acetic acid solution with shaking for 30 min, and then washed with distilled water three times. After the last washing, the gel was soaked in silver solution (0.1% $AgNO_3$ and 0.037% formaldehyde) with gentle shaking for 20 min, and developed by exchanging this solution with the developing solution (22 mM sodium carbonate, 0.037% formaldehyde and 0.002% sodium thiosulfate). Finally, the gel, with the fully developed image, was fixed with stop solution (9.8 mM EDTA-Na_2), and dried on a Cellophane Support (Bio-Rad).

Cloning of DNA fragments eluted from the polyacrylamide gel and sequence analysis

The differentiated cDNA bands, showing genotype- and treatment-specific patterns, were eluted from the gels, and re-amplified with the same primer as used in the primary reaction. Amplified DNA fragments were eluted in TE buffer, purified by phenol treatment and ethanol-precipitation, and ligated to pGEM-T Easy Vector (Promega) according to the manufacturer's instructions. Plasmid DNA was prepared by the alkali-lysis method. DNA sequences were obtained by using a SequiTherm EXCEL II Long-Read DNA Sequencing Kit-LC (EPICENTRE TECHNOLOGIES) and dNA sequencer model 4000L (LI-COR).

Southern and Northern hybridization

Ten micrograms of DNA was digested with restriction enzymes (*Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII and *Xba*I), fractionated by electrophoresis through a 1.0% agarose gel, and blotted with 0.4 M NaOH onto a Hybond N$^+$ filter (Amersham). Membranes were hybridized with the ^{32}P-labeled re-amplified products. After hybridization, the blots were washed with 0.5 × SSC solution at 68 °C, and exposed to X-Omat films (Eastman-Kodak). For Northern analysis, 10 μg of total RNA or 3.0–5.0 μg of poly(A)$^+$ RNA were electrophoresed on 1.2% agarose gels and transferred to Zeta-Probe blotting membranes (Bio-Rad) by the capillary method after denaturation with glyoxal and DMSO. The membranes were hybridized with the ^{32}P-labeled re-amplified products in 20 mM Na_2HPO_4 (pH 7.2) and 5% SDS at 65 °C. The membranes were washed in 20 mM of Na_2HPO_4 (pH 7.2), 5% SDS at 65 °C. Radioactivity of signals was measured on the imaging plates of FUJIX BAS2000 (Fuji Photo Film). The actin gene of rice was used as an internal control for signal standardization.

QTL analysis

Following a screening cDNA polymorphism between the parental lines, the segregation in both RILs was analyzed. The association between cDNA segregation and heading trait among RIL populations was evaluated by single-marker analysis using the QGENE computer program (Nelson 1997) with $\alpha = 0.01$.

Results

Effect of vernalization for developing embryos

After low-temperature treatment for developing embryos in detached-ear cultures, the two isogenic lines (*Vrn-A1* and *vrn-A1*) derived from the matured seeds after treatment were grown under the long-day condition in a greenhouse. There was no significant difference in the days to heading among *Vrn-A1* plants in terms of the stage at which vernalization was started (DAP10 and DAP20) or the period of treatment (0, 2, 4 or 7 weeks, Table 1). In the winter-type *vrn-A1* plants, however, the embryo vernalization promoted heading depending on both the embryo stage and period of treatment (Table 1). At the DAP10 stage, 2-week treatment accelerated heading by 25.6 days, with respect to the control. The longer period of treatment resulted in even earlier heading at the DAP10 stage: acceleration of 84.2 and 99.4 days for the 4- and 7-week treatment, respectively. In contrast to the DAP10 stage, the *vrn-A1* plants with 2-week treatment at DAP20 headed without a significant difference from the control. At the DAP20 stage, the *vrn-A1* plants with 4- and 7-week treatment headed earlier than the control by 49.4 and 86.5 days, respectively.

To compare the effects of the embryo vernalization and the seedling vernalization, the degree of vernalization requirement in *vrn-A1* was presented as the ratio of

Table 1 Effect of vernalization of developing embryos on heading, and comparison with the vernalization of young seedlings

Developmental stages	period of vernalization (weeks)	Days to heading[a]		Degree of vernalization requirement[c]
		Vrn-A1[b]	*vrn-A1*[b]	
Embryo vernalisation				
DAP10	0	39.0 (0.0)	139.8 (0.0)	1.00
	2	38.8 (–0.2)	114.2* (–25.6)	0.75
	4	37.7 (–1.3)	55.6** (–84.2)	0.18
	7	38.0 (–1.0)	40.4** (–99.4)	0.02
DAP20	0	50.4 (0.0)	144.7 (0.0)	1.00
	2	47.7 (–2.7)	144.1 (–0.6)	1.02
	4	51.1 (0.7)	95.3** (–49.4)	0.47
	7	50.7 (0.3)	58.2** (–86.5)	0.08
Seedling vernalization	0	38.1 (0.0)	90.6 (0.0)	1.00
	2	39.8** (1.7)	53.4** (–37.2)	0.26
	4	41.9** (3.8)	48.8** (–41.8)	0.13
	7	43.2** (5.1)	43.8** (–46.8)	0.02

[a] For seedling vernalization, days to heading were counted from the end of vernalization. Figures in parantheses represent the deviation from the 0-week treatment within the lines for each of the developing stages, and the significance of difference of treatment groups from the 0-week group are shown as * and ** at the 5 and 1% level respectively
[b] *Vrn-A1* and *vrn-A1* represented TD(D) and TD(C), respectively
[c] The difference is heading days between the two lines was divided by those of the control condition (0-week treatment)

the difference in heading days between *Vrn-A1* and *vrn-A1* under each condition to that of the control (0-week treatment). This value indicates how-much vernalization treatment enables promotion of heading in the *vrn-A1* line. At both treatments, the 7-week treatment gave a degree of vernalization requirement of less than 0.1% (Table 1). This result indicates that the *vrn-A1* plants required the 7-week treatment to be completely vernalized independent of the developmental stage. In the two developmental stages of the embryos, the degrees of vernalization requirement (or sensitivity to the vernalization treatment) were different. The 2-week treatment for DAP10 promoted heading of *vrn-A1*, whereas the same treatment for DAP20 did not. The degree obtained with the 4-week treatment of DAP20 embryos was 0.47, which was larger than that for the other developmental stage. These results indicate that the DAP10 stage was more-sensitive to vernalization than DAP20.

Differential display

The immature embryos with 2- and 4-week vernalization treatment at the DAP10 and 20 stage, respectively, were employed for isolation of cDNA fragments to identify genotype- and vernalization-specific gene expression. Because these stages were shown to be in middle of the response to the vernalization treatment (Table 1), we expected that mRNA of both induced or suppressed genes responding to vernalization-treatment would be identified. In the differential display, the cDNAs synthesized from poly(A)$^+$ RNA were used as templates for RT-PCR by using 100 primers for each template (represented as DAP10-2W and DAP20-4W in Fig. 1). On average, 20 bands of the amplified fragments were observed on the gel-plates after silver staining per primer (Fig. 2).

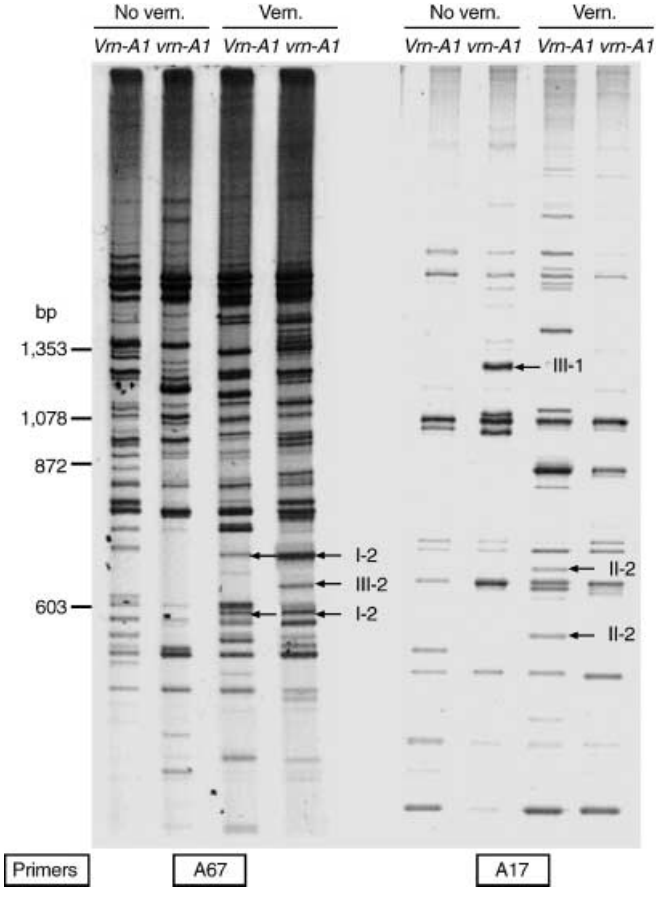

Fig. 2 RT-PCR product profile obtained by silver staining. RT-PCR products were electrophoresed in 10% polyacrylamide gels and stained with $AgNO_3$. Band patterns indicated as I-2 appeared by vernalization treatment common to *vrn* genotypes, while bands marked as II-2, III-1 and III-2 appeared in the genotype-specific response to vernalization. *No vern*, no vernalization treatment; *Vern*, vernalization treatment

Table 2 Differential band patterns of RT-PCR products specific to vernalization and the *Vrn-A1* genotype

Type	Band patterns[a]				No. of bands (%)[b]	
	Non vernalization		Vernalization		DAP10-2W	DAP1020-4W
	Vrn-A1	*vrn-A1*	*Vrn-A1*	*vrn-A1*		
1. Common response to vernalization						
I-1	+	+	–	–	3 (3.7)	5 (6.3)
I-2	–	–	+	+	15 (18.5)	18 (22.5)
2. *Vrn-A1*-specific response to vernalization						
II-1	+	–	–	–	2 (2.5)	5 (6.3)
II-2	–	–	+	–	17 (21.0)	16 (20.0)
II-3	+	+	–	+	1 (1.2)	1 (1.3)
II-4	–	+	+	+	3 (3.7)	1 (1.3)
3. *vrn-A1*-specific response to vernalization						
III-1	–	+	–	–	6 (7.4)	9 (11.3)
III-2	–	–	–	+	21 (25.9)	14 (17.5)
III-3	+	+	+	–	1 (1.2)	3 (3.8)
III-4	+	–	+	+	6 (7.4)	5 (6.3)
4. Reverse response to vernalization						
IV-1	+	–	–	+	4 (4.9)	2 (2.5)
IV-2	–	+	+	–	2 (2.5)	1 (1.3)
Total					81 (100.0)	80 (100.0)

[a] +, presence and –, absence, of transcripts
[b] Amplified transcripts were examined for reproductivity in duplicate experiments. Templates for RT-PCR were derived from the developing embryos at the DAP10 and DAP20 stages with 2- and 4-week vernalization treatment, respectively. Figures in parentheses are the proportion (%) among the total number of the differential bands

The band patterns were examined for reproducible products in duplicate trials. Out of 2,000 bands observed, 161 (8.1%) showed differential amplification between lines and/or treatments, with almost equal frequencies between DAP10-2W and DAP20-4W treatments. They were classified into 12 patterns of possible combinations of the presence (+) or absence (-) among the lines and treatments (Table 2). Pattern I, which showed a common response to vernalization, involved 18 (22%) and 23 (28.8%) products that were amplified from the cDNA templates of DAP10-2W and DAP20-4W, respectively. The other 120 bands showed differential patterns specific to the genotypes. In both *Vrn-A1* and *vrn-A1*, higher frequencies were observed for the bands amplified with vernalization treatment (II-2 and III-2, respectively).

In total, 110 differential bands were selected for further analyses by sequencing and mapping.

Table 3 Homoeologous groups of the differential cDNA fragments and their segregation in RIL populations of hexaploid and diploid wheats

Homoeologous groups[a]	No. of cDNAs	No. of cDNAs segregated in	
		RIL1 (6χ)	RIL5 (2χ)
1	3	3	0
2	6	1	2
3	12	6	4
4	3	2	0
5	8	8	5
6	3	0	2
7	7	2	1
ND	2	1	1
1 and 7	1	1	1
1, 3 and 7	1	0	1
1, 4 and 6	1	1	1
2, 4 and 7	1	1	0
Total	48	26	18

[a] ND, no polymorphism between the genomes

Genetic characterization of differential cDNA

The chromosomal locations of the differential cDNA products were determined by the use of a nulli-tetra series of Chinese Spring. Out of the total of 110 cDNA fragments selected, 46 were assigned to homoeologous groups (Table 3), though two did not show clear polymorphism (presence or absence) between the genomes. These 48 cDNAs were designated as *wec* (*w*heat-*e*mbryo *c*old treatment). They were distributed over all homoeologous chromosome groups, and a quarter of them were located in group 3. The remaining cDNAs (53%) could not be determined on location because of unclear signals or the high background of Southern-hybridization panels.

The differential cDNAs were examined for their segregation in two RIL populations, RIL1 (6χ) and RIL5 (2χ). In RIL1 and RIL5, 26 and 18 cDNAs segregated,

Table 4 The association of *wec* clones with heading traits in RIL1 (6χ) and RIL5 (2χ)[a]

RIL	Clone	Heading trait[b]	Band patterns[c]	(Templates)	Homoeologous groups	Early type[d]	*F* value	$r^2 \times 100$[e]	Probability	Additive effect
RIL1	*wec70*	Vernalization requirement	+ + + –	(DAP20-4W)	1, 4, 6	CS	7.2	10.6	0.0092	–6.74
	wec78	Field	– – – +	(DAP20-4W)	5	SP	14.7	19.2	0.0003	1.70
RIL5	*wec57*	Narrow-sense earliness	+ – – +	(DAP10-2W)	3	mono.	29.1	21.9	0.0000	6.85
	wec70	Vernalization requirement	+ + + –	(DAP20-4W)	1, 4, 6	mono.	9.7	8.6	0.0024	9.77
	wec71	Vernalization requirement	– + + –	(DAP20-4W)	6	mono.	8.9	7.8	0.0036	9.02

[a] RIL1 and RIL5 were the recombinant inbred lines estabilised from hexaploid wheats (Chenese Spring × spelt wheat) and diploid species (*T. monoccocum* × *T. boeoticum*), respectively
[b] Only significant ones with $P < 0.01$ were listed from the single-marker analysis
[c] See description inTable 2
[d] CS, SP and mono.; Chinese Spring, *Triticum spelta*, and *T. monoccocum*, respectively
[e] Coefficient of determination

Table 5 Gene expression of the differential cDNA (*wec*) in the developing embryos treated with vernalization

Expression patterns	*wec* #	RT-PCR pattern	Mean relative density[a]			
			Non vernalization		Vernalization	
			Vrn-A1	*vrn-A1*	*Vrn-A1*	*vrn-A1*
Increased by vernalization	17	– – – +	1.00	1.04	1.21	1.44
Decreased by vernalization	1	– + – –	1.00	0.99	0.65	0.73
	8	– + – –	1.00	1.00	0.88	0.81
	9	+ + + –	1.00	0.98	0.51	0.50
	70	+ + + –	1.00	0.91	0.40	0.53
Decreased by vernalization in *Vrn-A1*	2	– – – +	1.00	0.85	0.82	0.88
	19	– – – +	1.00	0.92	0.77	0.97

[a] The hybridization signals of Northern blots were standardized with the actin gene and represented as values relative to those of non-vernalized *Vrn-A1*

respectively (Table 3). Ten of them were mapped in both RILs. The association between the segregation of *wec* clones in both RILs and heading traits were evaluated by the single-marker analysis by using QGENE. Table 4 summarizes *wec* clones significantly associated (at 1% level) with narrow-sense earliness, vernalization requirement, and heading in the field. With respect to the vernalization requirement, *wec70* was significant in RIL1 and RIL5; and *wec71* only in RIL5. In RIL1, *wec78* was localized on the 5B chromosome associated with heading date in the field; *wec57* explained 21.9 and 46.9% of the variability of narrow-sense earliness and heading in the field, respectively, in RIL5.

Structural analysis and expression analysis

The DNA sequences of these cDNAs were determined and searched for sequence homology with sequences of known function (data not shown). The region from 363 to 1,027-bp of *wec17*, which had a total-length of 1,083-bp, was homologous at 72.4% to the coding region of phosphoglycerate mutase (E.C. 5.4.2.1) isolated from timothy grass (Ferreira et al. 1997). Sequences of other clones were unreported.

The *wec* genes were quantified for their mRNA accumulation by Northern hybridization. The hybridization signals of blots were measured by standardization with the actin gene of rice. The relative values of signal density were measured, and the results are listed in Table 5. The reproducibity of the gene expression was examined by conducting repeated experiments with different lots of RNA. Expression patterns of seven *wec* genes differed from those obtained by RT-PCR. The expression of *wec17*, which had the RT-PCR pattern "- - - +", increased after vernalization commonly in both *Vrn-A1* and *vrn-A1*. The decreased expression after vernalization was observed in four *wec* cDNAs, which showed the pattern III-1 and III-3. *wec2* and *wec19*, categorized as pattern III-2, showed reduced mRNA accumulation by 18 and 23%, respectively, after vernalization specifically in *Vrn-A1*. No difference in the expression pattern of *wec57* and *wec78*, associated with the heading trait in RIL1 and RIL5, was detected between genotypes and treatments.

Discussion

Vernalization is a low-temperature treatment for young seedlings to promote initiation of the inflorescence in higher plants. Kato et al. (1990) were the first to report that low-temperature treatment of developing embryos could promote the heading of winter wheat. In the present study, we reconfirmed that the developing embryo is sensitive to the low-temperature equivalent to seedling vernalization in winter types, and showed that an early stage (DAP10) was more sensitive than at a later stage (DAP20). For both DAP10 and DAP20 stages of embryos, the 7-week treatment enabled promotion of heading completely. Low temperature is perceived mainly by the shoot apex (Bernier et al. 1993). It was reported that mitotically active imbibed seed following imbibition and the meristem in *Arabidopsis* can respond to vernalization, indicating that vigorous cell division is necessary for vernalization (Dennis et al. 1996). The same mechanism would be involved in the immature embryos of wheat. In embryo vernalization, the vernalized state is maintained throughout seed maturation and seedling formation after germination. Because winter-type wheat with this treatment can be compared directly with spring-type wheat under the same growth condition, embryo vernalization is useful for various physiological studies without a time lag. It is also useful to shorten the time for practical breeding programs of winter wheat because the vernalization treatment can be done during seed development. Because the phase transition should be in progress in the developing embryos under cold treatment, the embryo vernalization was employed in this study to identify genes involved in promoting heading.

There are two types of gene functions for heading, promotive and inhibitory. The homoeologous *Vrn-A1*, *-B1*, and *-D1* genes actively promote early heading. Contrarily, unknown genes on group-1 and -6 chromosomes delay heading due to the requirement for increased vernalization treatment (Islam-Faridi et al. 1996; Law et al. 1998). *Vrn-A^{m}2*, located on chromosome 5A^m in *T. monococcum*, was also reported to increase the vernalization requirement (Dubcovsky et al. 1998). To isolate genes with promotive or inhibitory functions, we employed RNAs from embryos at the mid point of the fully vernalized state as templates for differential display. While cDNAs commonly responding to vernalization in both genotypes were 25.5% of the total of 161, the cDNAs specific to *vrn-A1* represented 40.4%, which was twice as much as that specific to *Vrn-A1*. In two genotypes, the patterns induced by vernalization were more-frequent than those repressed by it. These results suggest that the immature embryos with DAP10-2W and DAP20-4W treatments are suitable to isolate genes having either a promotive or inhibitory function in winter wheat bearing *vrn-A1*.

Sequences of the *wec* genes have not been reported before, except in case of *wec17*, which showed high homology to phosphoglycerate mutase (E.C.5.4.2.1). This enzyme is essential for catalysis causing the interconversion of 3-phosphoglycerate and 2-phosphoglycerate during glycolysis and gluconeogenesis. In *Arabidopsis*, the *pgm* mutant, which is starchless and defective in this gene, has been reported to have a late-flowering phenotype that could be suppressed by vernalization treatment (Caspar et al. 1985; Bernier et al. 1993). These findings suggest the relation between the control of flowering time and starch metabolism.

The *wec* genes were located on all homoeologous groups. These *wec* genes would function downstream from the *Vrn* gene in the pathway of heading activated by vernalization. In this study, *wec70* showed reduced gene expression by the vernalization treatment. Thus, *wec70* would function in an inhibitory manner for heading. In the present study, several of the novel genes isolated were associated with heading time. These genes will be useful for understanding the physiological function of vernalization requirement as well as serving as DNA markers for early heading breeding in hexaploid wheat.

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