Level of *VERNALIZATION 1* expression is correlated with earliness in extra early-flowering mutant wheat lines

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Four extra early-flowering mutants, named *extra early-flowering1* (*exe1*), *exe2*, *exe3*, and *exe4*, were identified in *Triticum monococcum* strain KU104-1 following heavy-ion beam mutagenesis. The four *exe* mutants fell into two groups, namely Type I (moderately extra early-flowering type; *exe1* and *exe3*) and Type II (extremely extra early-flowering type; *exe2* and *exe4*). Analysis of plant development in a growth chamber showed that the speed of leaf emergence was accelerated in *exe* mutants at the reproductive stage compared to wild-type (WT) plants. The speed of leaf emergence was faster in Type II than Type I plants. Analysis of *VERNALIZATION 1* (*VRN1*), a flowering promoter gene, showed that it was more highly expressed in seedlings at early developmental stages in Type II mutants than Type I mutants. These findings indicate that the difference in earliness between Type I and Type II mutants is associated with the level of *VRN1* expression. The original KU104-1 is an einkorn wheat strain that carries a null allele of the *VRN2* gene, a repressor of flowering. Thus, our results indicate that the level of *VRN1* expression controls earliness in *exe* mutants independently of *VRN2*.

Key Words: early-flowering, ion beam mutagenesis, *VERNALIZATION 1*, wheat, *Triticum monococcum*.

Introduction

Many plant species display an adaptation termed vernalization in which an extended period of low temperature in winter results in competence to flower during the following spring. Interestingly, recent studies have revealed that vernalization systems evolved independently in different plant groups (Ream *et al.* 2013). In temperate cereals, such as wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), three genes have been found to control the need for vernalization, namely, *VERNALIZATION 1* (*VRN1*), *VRN2* and *VRN3* (Distelfeld *et al.* 2009a).

VRN1 encodes an APETALA1/FRUITFULL-like (AP1/FUL-like) MADS-box transcription factor that is up-regulated by vernalization (Danyluk *et al.* 2003, Murai *et al.* 2003, Trevaskis *et al.* 2003, Yan *et al.* 2003). The expression of *VRN1* is epigenetically suppressed in seedlings at early stages of the vegetative growth phase; this suppression is caused by modification of histones at the *VRN1* locus (Diallo *et al.* 2012, Oliver *et al.* 2009). The repressive histone state is modified by signals induced by vernalization, resulting in the up-regulation of *VRN1*. A recent study found that rapid increases in histone acetylation levels at the *VRN1* locus in response to a 24 hr cold treatment trigger the ver-

nalization effect (Oliver *et al.* 2013). The level of *VRN1* expression gradually increases during the seedling growth stage without the need for vernalization (Kitagawa *et al.* 2012, Murai *et al.* 2003), suggesting that the epigenetic status of *VRN1* is also modified by internal signals such as aging. Furthermore, *VRN1* shows a diurnal expression pattern that is affected by the length of daylight, with a long photoperiod producing up-regulation of its expression level (Shimada *et al.* 2009). These observations indicate that *VRN1* expression is controlled by autonomous and photoperiodic pathways as well as by the vernalization pathway.

The *VRN2* locus consists of two linked genes, *ZCCT1* and *ZCCT2*, which encode a protein with a zinc finger motif and a CCT domain (Yan *et al.* 2004). There are no clear homologs of *VRN2* in the *Arabidopsis* genome. Natural variations have been identified in the *VRN2* locus. Simultaneous deletion or non-functional mutations of the two *ZCCT* genes result in a plant that has a spring habit (Distelfeld *et al.* 2009b, Dubcovsky *et al.* 2005, Yan *et al.* 2004), indicating that *VRN2* is a flowering repressor gene. A high level of *VRN2* expression is observed in seedlings at the 1-leaf stage, while expression is down-regulated by vernalization and aging. By contrast, *VRN1* shows the opposite pattern with low expression in seedlings and up-regulated expression after vernalization (Shimada *et al.* 2009). It has also been reported that *VRN2* shows a diurnal expression pattern and that a long photoperiod up-regulates its expression level (Dubcovsky *et al.* 2006, Trevaskis *et al.* 2006). However, as

Communicated by T. Komatsuda

Received January 27, 2014. Accepted May 1, 2014.

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plants encounter a long photoperiod during spring to early summer when they are at the reproductive growth stage, it is unlikely that *VRN2* is up-regulated by a long photoperiod in the field.

VRN3 encodes a Raf kinase inhibitor-like protein with a high similarity to the *Arabidopsis* FLOWERING LOCUS T (FT) protein, which is a florigen (Yan *et al.* 2006). Therefore, *VRN3* is also called *FT1* in barley (Hemming *et al.* 2008, Sasani *et al.* 2009) and *WFT* (wheat FT) in wheat (Shimada *et al.* 2009). Transgenic wheat plants overexpressing *VRN3* show an extra early flowering phenotype without the need for vernalization (Shimada *et al.* 2009, Yan *et al.* 2006), indicating that *VRN3* is a strong flowering promoter. Under long day conditions, *VRN3* shows a diurnal expression pattern; however, expression is very low under short day conditions (Kitagawa *et al.* 2012, Shimada *et al.* 2009). In this study, we refer to *VRN3* as *WFT*, as in our previous studies (Kitagawa *et al.* 2012, Shimada *et al.* 2009).

VRN2 and *WFT* are preferentially expressed in leaves (Yan *et al.* 2004, 2006), while *VRN1* is expressed in both leaves and shoot apical meristems (SAMs) (Kinjo *et al.* 2012, Sasani *et al.* 2009, Yan *et al.* 2003). These expression patterns suggest that *VRN1* and *VRN2* might cooperate in the up-regulation of florigenic WFT proteins in leaves. Kinjo *et al.* (2012) postulated that WFT proteins are transported from the leaves to the SAMs, where they interact with *VRN1* to induce flowering.

To further elucidate the mechanisms that control flowering in wheat, especially the relationship between *VRN1* and *WFT*, we are developing a large-scale panel of mutants in diploid einkorn wheat (*Triticum monococcum*). These mutants were induced by heavy-ion beam mutagenesis and are being systematically screened for effects on flowering time (Murai *et al.* 2013). Heavy-ion beam irradiation is effective at producing gene deletions (null mutations) (Kazama *et al.* 2011, 2013). Here we describe four newly identified extra-early flowering mutant lines in diploid einkorn wheat, which have been named *extra early-flowering 1* (*exe1*), *exe2*, *exe3*, and *exe4*. Based on their phenotypes in the field, the four *exe* mutants were classified into two groups: Type I (moderately extra early-flowering type; *exe1* and *exe3*) and Type II (extremely extra early-flowering type; *exe2* and *exe4*). Our analyses showed that the expression level of *VRN1* was correlated with earliness in *exe* mutants. The parental strain, KU104-1, carries a null allele of *VRN2*, which is a repressor of flowering. Our expression analyses indicate that the level of *VRN1* expression controls earliness in *exe* mutants independently of *VRN2*. Here, we present a model for flowering in temperate cereals in which environmental signals, such as cold and long photoperiods, and internal signals, such as aging, are cues for flowering; *VRN1* is envisaged as functioning in the acquisition of flowering competency in leaves.

Materials and Methods

Plant material

Seeds of the diploid einkorn wheat (*Triticum monococcum*, $2n = 2x = 14$, genome constitution $A^m A^m$) strain KU104-1 were given 50 Gy of carbon-ion irradiation at a linear energy transfer (LET) of $50 \text{ keV } \mu\text{m}^{-1}$ and then sown in the field. It has been demonstrated previously that ion beam is an efficient inducer of mutations in plants and to predominantly cause null mutations (Kazama *et al.* 2011, 2013). The spikes of M_1 plants were bagged and selfed seeds were harvested from each spike and used to produce the M_2 lines. From approximately 1,200 M_2 lines, we identified 4 mutations that showed an extra early-flowering phenotype; we termed these mutations as *extra early-flowering* (*exe*) 1, 2, 3 and 4. Wild-type (WT) strain KU104-1 and the M_3 plants of all 4 *exe* mutations were used in the experiments. The original strain KU104-1 lacks *VRN2* locus. Spring einkorn wheat strains are classified into three types in the *VRN2* allele: WT, R/W mutant-type and deletion-type (Yan *et al.* 2004). KU104-1 is the *VRN2* deletion-type einkorn wheat.

Field experiments

WT and *exe* mutant plants were sown in the middle of October in an experimental field at Fukui Prefectural University. The heading dates of each line were scored. WT and *exe* mutant plants were screened at the maturation stage to examine the following morphological characters: spike number per plant, spike length, and spikelet number per spike.

Growth chamber experiments

To examine leaf emergence speed (plastochron), the total number of leaves and heading times, WT and *exe* mutant plants were cultivated in a growth chamber under long day (LD; 16 h light/8 h dark) conditions or short day (SD; 10 h light/14 h dark) conditions at 20°C (light intensity $\sim 100 \mu\text{E m}^{-2} \text{ s}^{-1}$). The phenotypes of 10 WT plants and 10 plants of each *exe* mutation were assessed. The growth stage with transition from vegetative to reproductive phases was estimated by the initiation timing of stem elongation of the main shoot. Heading time was measured as the number of days from 1-leaf unfolding to the flag leaf unfolding. For the diurnal expression analysis of *VRN1*, non-vernalized WT plants were grown under LD conditions at 20°C ($100 \mu\text{E m}^{-2} \text{ s}^{-1}$).

Gene expression analysis

Expression of *VRN1* and *WFT* in WT and *exe* mutant plants at each leaf stage was analyzed in non-vernalized plants grown under LD or SD conditions at 20°C . Seedlings from each line were sampled from the 1-leaf stage to the 6-leaf stage for analysis. Leaves were sampled 2 hr after the start of the light period. For the analysis of the diurnal expression of *VRN1*, seedlings at the 7-leaf stage were sampled every 2 hr over a 26 hr period. In this study, the 1-leaf stage was assumed to last from the unfolding of the 1-leaf to

the unfolding of the 2-leaf.

Total RNAs were extracted from leaves using ISOGEN (Nippon Gene, Japan); cDNAs were synthesized from the total RNAs using an oligo dT primer in accordance with the protocol for the Ready-To-Go T-primed First-Strand Kit (GE Healthcare Life Sciences). Real-time PCR analyses were performed using a LightCycler 2.0 (Roche Diagnostics GmbH) with the following gene-specific primer sets: *VRN1*, TaMADS#11-545L (5'-GGAGAGGTCAGTGCAGGAGGA-3') and TaMADS#11-698R (5'-GCCGCTGGATGAATGCTG-3') at an annealing temperature of 65°C; *WFT*, TaFT-mono3F (5'-GGTACAACTGGTGCCTCGTT-3') and TaFT-mono3R (5'-GTTGTAGAGCTCGGCGAAGT-3') at an annealing temperature of 64°C. The relative quantities of the transcripts were determined using a SYBR Green-labelled amplification product from *Ubiquitin* prepared with the primers Ubi-1L (5'-GCATGCAGATATTTGTGA-3') and Ubi-1R (5'-GGAGCTTACTGGCCAC-3') at an annealing temperature of 58°C. The primer sets for *VRN1* and *Ubiquitin* were identical to those used in our previous study (Kitagawa *et al.* 2012). Two biological replicates with three technical replications were performed. Each biological sample contained leaves from two individual plants.

Results

Classification of the *exe* mutants

The *extra early-flowering* (*exe*) mutants were initially identified during the development of a large-scale mutant panel produced using heavy-ion beam mutagenesis (Murai *et al.* 2013, Nishiura *et al.* 2013). For *exe1* mutation, two mutant plants were identified out of six M₂ plants derived from one ear of M₁ plant. One each mutant plant out of ten, nine or seven M₂ plants were detected for *exe2*, *exe3* or *exe4* mutation, respectively. The M₃ plants derived from one M₂ mutant plants were uniform and not segregated in earliness (data not shown). These suggest that *exe* mutations are recessive with relatively small number of locus.

The *exe* mutants showed extra early heading-time compared to WT plants in the field in seasons 2011/2012 and 2012/2013 (Table 1). In early spring 2013, the plants in the experimental field were damaged by rabbits, which had a particularly adverse effect on early growing tillers. For this reason, heading of the *exe* mutants was delayed in season

Table 2. Mean ($\pm$ SE) spike length and the number of spikelets per spike in wild-type (WT) and *exe* mutant plants in the field

Line	No. of plants examined	No. spikes/plant	Spike length (cm)	No. spikelets/spike	Type
WT	3	9~17	6.9 $\pm$ 0.21 ^d	32.5 $\pm$ 0.63 ^d	–
<i>exe1</i>	7	3~11	5.9 $\pm$ 0.09 ^c	27.8 $\pm$ 0.37 ^c	I
<i>exe2</i>	6	3~11	4.9 $\pm$ 0.44 ^{ab}	19.8 $\pm$ 1.22 ^b	II
<i>exe3</i>	3	3~7	5.7 $\pm$ 0.24 ^{bc}	26.0 $\pm$ 0.91 ^c	I
<i>exe4</i>	9	5~12	4.7 $\pm$ 0.19 ^a	17.1 $\pm$ 0.56 ^a	II

ANOVA (analysis of variance) was performed. The same superscript means no difference significantly ($p = 0.05$) from each other when Fisher's LSD (least significant difference) analysis is applied.

2012/2013 compared to 2011/2012. Nevertheless, the data on heading dates in the field indicated that the *exe* mutants fell into two distinct groups: a moderately extra early-flowering type (Type I), which included *exe1* and *exe3*; and an extremely extra early-flowering type (Type II), which included *exe2* and *exe4* (Table 1).

The *exe* mutant plants tended to have fewer spikes than WT plants (Table 2). Furthermore, compared to WT plants, the spikes were smaller and had fewer spikelets. The Type I and Type II *exe* mutants clearly differed with respect to these traits: Type I (*exe1* and *exe3*) had spikes of 5.7–5.9 cm length with 26–28 spikelets, whereas Type II (*exe2* and *exe4*) had spikes of 4.7–4.9 cm length with 17–20 spikelets. The smaller spikes in Type II *exe* mutants are probably associated with their extremely extra early-flowering phenotype.

Leaf emergence speeds in *exe* mutants

Leaf emergence timing in WT and *exe* mutant plants was determined by screening leaf unfolding in seedlings cultivated in a growth chamber under LD or SD conditions (Fig. 1). Under LD conditions, WT plants averaged about 180 days from sowing to flag-leaf unfolding. In contrast, Type I *exe* mutants took about 65 days and Type II about 50 days (Fig. 1A). WT plants transited from the vegetative to reproductive growth phase at the 9-leaf stage. However, *exe* mutants showed earlier phase transition: at the 5-leaf stage for *exe1* and *exe3* (Type I), and the 4-leaf stage for *exe2* and *exe4* (Type II). The production of successive leaves ceased following the initiation of the flag leaf, which could be distinguished from the other leaves by its short blade and the emergence of a spike from its leaf sheath. In WT plants, the flag-leaf occurred at the 18-leaf stage, while in *exe1*, *exe2*, *exe3*, and *exe4* the flag leaf appeared at the 9-leaf, 8-leaf, 9-leaf, and 7-leaf stages, respectively.

Under SD conditions, WT plants did not unfold the flag-leaf until at least 200 days from sowing. In contrast, Type I *exe* mutants showed flag leaf unfolding at about 110 days and Type II at about 60 days from sowing (Fig. 1B). WT plants transited from the vegetative to reproductive growth phase at the 13-leaf stage. By contrast, *exe1* and *exe3* mutants (Type I) transited at the 8-leaf stage, while *exe2* and *exe4* mutants (Type II) transited at the 4-leaf stage. Interestingly, the timing of phase transition was early (at the 4-leaf

Table 1. Heading dates of wild-type (WT) and *exe* mutant plants in the field and the relative earliness (difference) of the mutants

Line	Season 2011/2012		Season 2012/2013		Type
	Heading date	Difference	Heading date	Difference	
WT	6 Jun	0	8 Jun	0	–
<i>exe1</i>	7 May	–30	15 May	–24	I
<i>exe2</i>	22 Apr	–45	4 May	–35	II
<i>exe3</i>	7 May	–30	14 May	–25	I
<i>exe4</i>	25 Apr	–42	4 May	–35	II

Sowing dates were Oct 18 for Season 2011/2012 and Oct 16 for Season 2012/2013.

stage) in Type II *exe* mutants under both SD and LD conditions. Flag-leaf emergence occurred at the 13-leaf, 8-leaf, 13-leaf, and 8-leaf stages in *exe1*, *exe2*, *exe3*, and *exe4*, respectively. The delay in flag-leaf emergence in Type I *exe* mutants under SD conditions compared to LD conditions indicates that these mutants retained photoperiodic sensitivity. However, Type II *exe* mutants had lost almost all photoperiodic sensitivity.

Fig. 1. Leaf emergence speed in non-vernalized *exe* mutants and wild-type (WT) plants grown in a growth chamber. Arrows indicate timing of phase transition: black, WT; red, *exe1*; pink, *exe2*; blue, *exe3*; and green, *exe4*. (A) Days from germination to leaf unfolding at each leaf stage grown under long day (LD) conditions. The data at the 2-leaf stage are missing. The final leaf of each line indicates flag-leaf. (B) Days from germination to leaf unfolding at each leaf stage under short day (SD) conditions. The data at the 2-leaf stage are missing. The final leaf of each *exe* line indicates flag-leaf. WT did not head before the 20-leaf stage.

The timing of emergence of the first five leaves was very similar in WT and *exe* mutant plants (Fig. 1); subsequently, however, leaf emergence was more rapid in the *exe* mutants compared to WT plants. This finding indicates that rapid leaf emergence in the *exe* mutants was caused by a more rapid rate of leaf initiation (plastochron) in the mutants. Furthermore, the plastochron appeared to be shorter at the reproductive growth phase (after phase transition) in the mutants. Under SD conditions, Type II *exe* mutants (*exe2* and *exe4*) displayed a shortened plastochron compared to Type I (*exe1* and *exe3*) mutants (Fig. 1B).

Altered photoperiodic sensitivity in exe mutants

To compare photoperiodic sensitivities of WT and *exe* mutant plants, the timing from 1-leaf unfolding to flag-leaf unfolding (D1f) was determined under LD and SD conditions (Table 3). Relative photoperiodic sensitivity was estimated using the ratio of the averaged D1fs in the LD and SD regimes. WT plants had a ratio greater than 2.60, whereas those of Type I and Type II *exe* mutants were 1.76 and 1.17–1.24, respectively. The differences in these ratios clearly indicate that the *exe* mutants have a decreased photoperiod sensitivity compared to WT plants; this difference is in line with the differences in leaf numbers under LD or SD conditions in the three plant groups (Fig. 1). Furthermore, the results also indicate that Type II *exe* mutants have almost completely lost photoperiodic sensitivity.

Patterns of VRN1 expression in exe mutants

We used real-time PCR analysis to compare the levels of *VRN1* expression in WT and *exe* mutant plants (Fig. 2). Based on the PCR and sequence analyses, it was confirmed that mutations did not occur in *VRN1* of *exe* mutants (data not shown). cDNAs were obtained from leaves of non-vernalized plants at the 1-leaf to 6-leaf stages. Under LD conditions, WT plants showed relatively low *VRN1* expression level during these stages, but the level of *VRN1* expression increased with time from the 1-leaf stage in the *exe* mutants. Expression of *VRN1* was higher at the 3-leaf to 5-leaf stages in Type II (*exe2* and *exe4*) than Type I (*exe1* and *exe3*) mutant plants (Fig. 2A). Notably, similar levels of

Table 3. Mean ($\pm$ SE) number of days from 1-leaf unfolding to flag-leaf unfolding (D1f) under long day (LD) or short day (SD) conditions, and relative photoperiod sensitivities

Line	D1f		Degree of photoperiod sensitivity (SD/LD)	Type
	LD	SD		
WT	192.5 $\pm$ 5.06 ^c	>500.0	>2.60	–
<i>exe1</i>	57.8 $\pm$ 0.80 ^b	101.7 $\pm$ 0.99 ^c	1.76	I
<i>exe2</i>	43.9 $\pm$ 0.84 ^a	51.4 $\pm$ 0.50 ^a	1.17	II
<i>exe3</i>	54.9 $\pm$ 0.31 ^b	96.9 $\pm$ 2.37 ^b	1.76	I
<i>exe4</i>	41.4 $\pm$ 0.56 ^a	51.3 $\pm$ 0.68 ^a	1.24	II

ANOVA (analysis of variance) was performed. The same superscript means no difference significantly ($p = 0.05$) from each other when Fisher's LSD (least significant difference) analysis is applied.

VRN1 expression were observed during phase transition in each *exe* mutant: at the 5-leaf stage for Type I and at the 4-leaf stage for Type II. This suggests that the level of *VRN1* expression is associated with phase transition from vegeta-

tive to reproductive growth and may be a threshold for flowering competency in the plants.

The pattern of *VRN1* expression under SD conditions was similar in Type II mutants to that observed under LD conditions; thus, the mutants showed a significantly higher level of expression at earlier leaf stages than WT plants (Fig. 2B). By contrast, *VRN1* expression under SD conditions was not up-regulated in Type I mutants in a similar manner to that seen under LD conditions; as a consequence, the mutant plants showed a delay in heading times. Under SD conditions, Type II *exe* mutants (*exe2* and *exe4*) showed phase transition at the 4-leaf stage, whereas Type I *exe* mutants (*exe1* and *exe3*) were still vegetative at the 6-leaf stage. This indicates that a threshold for flowering competency exists under both SD and LD conditions.

It has been reported that *VRN1* shows a diurnal expression pattern in the spring bread wheat cultivar Triple Dirk (Shimada *et al.* 2009). We therefore examined the WT plants used here (einkorn spring wheat strain KU104-1) for diurnal expression of *VRN1* (Fig. 3). At the 7-leaf stage of vegetative growth, the WT plants showed diurnal variation in *VRN1* expression under LD conditions. *VRN1* expression peaked during the light period and was lower during the dark period. This pattern is consistent with that reported for the Triple Dirk cultivar (Shimada *et al.* 2009). The occurrence of a diurnal expression pattern (Fig. 3) and the photoperiod-dependent expression of *VRN1* (Fig. 2) indicate that this gene is regulated by a light-dark cycle in wheat leaves.

Fig. 2. Patterns of *VRN1* expression in non-vernalized WT and *exe* mutant plants at the 1-leaf to 6-leaf stages. The data at the 2-leaf stage are missing. The expression level of *VRN1* was determined by real-time PCR. The *Ubiquitin (Ubi-1)* gene was used as an internal control for calculating the relative levels of expression of the *VRN1* gene. Means and standard errors are indicated. The dotted lines indicate the expression level of *VRN1* at the phase transition, i.e., the threshold for flowering competency. (A) Expression patterns in seedlings at each leaf stage under LD conditions. Leaf stage numbers (1L, 3L, etc.) are shown for *exe4* only. (B) Expression patterns in seedlings at each leaf stage under SD conditions. Leaf stage numbers (1L, 3L, etc.) are shown for *exe4* only. ANOVA (analysis of variance) was performed to detect the difference of gene expression level at the 3L and 4L stages. The same superscript means not significantly different ($p = 0.05$) from each other when Fisher's LSD (least significant difference) analysis is applied.

Fig. 3. Diurnal expression pattern of *VRN1* in spring wheat KU104-1 under long day conditions. The level of *VRN1* expression was analyzed by real-time PCR using non-vernalized seedlings at the 7-leaf stage. The *Ubiquitin (Ubi-1)* gene was used as an internal control for calculating the relative levels of *VRN1* genes. Each point indicates means and standard errors. The white and black bars along the horizontal axis represent light and dark periods, respectively.

Patterns of *WFT* expression in *exe* mutants

Using PCR analysis and sequence analysis, we confirmed that mutations were not involved in *WFT* of *exe* mutants (data not shown). During vegetative growth (1-leaf to 6-leaf stages), WT plants showed a low level of *WFT* expression under LD and SD conditions (Fig. 4). Under LD

Fig. 4. Expression patterns of *WFT* in non-vernalized WT and *exe* mutants plants at the 1-leaf stage to 6-leaf stages. The data at the 2-leaf stage are missing. The level of *WFT* expression was determined by real-time PCR. The *Ubiquitin (Ubi-1)* gene was used as an internal control for calculating the relative levels of expression of the *WFT* gene. Means and standard errors are indicated. (A) Expression patterns in seedlings at each leaf stage under LD conditions. Leaf stage numbers (1L, 3L, etc.) are shown for *exe4* only. (B) Expression patterns in seedlings at each leaf stage under SD conditions. Leaf stage numbers (1L, 3L, etc.) are shown for *exe4* only. ANOVA (analysis of variance) was performed to detect the difference of gene expression level at the 3L and 4L stages. The same superscript means not significantly different ($p = 0.05$) from each other when Fisher's LSD (least significant difference) analysis is applied.

conditions, the level of *WFT* expression level was higher in *exe* mutants compared to the WT plants; Type II *exe* mutants (*exe2* and *exe4*) showed a much higher level of expression at the 3-leaf to 5-leaf stages than Type I *exe* mutants (*exe1* and *exe3*) (Fig. 4A). By contrast, *WFT* expression in Type I mutants was reduced under SD conditions to a similar extent as in WT plants (Fig. 4B). Significant up-regulation of *WFT* was observed in Type II mutants, presumably as a consequence of their loss of photoperiodic response which would otherwise suppress *WFT* expression.

Discussion

In this study, we compared the characteristics of four *extra early-flowering* mutants that had been induced by heavy-ion beam irradiation. The mutants fell into two categories, namely Type I *exe* mutants (*exe1* and *exe3*) that showed a moderately extra early-flowering phenotype in the field and Type II *exe* mutants (*exe2* and *exe4*) that had an extremely extra early-flowering phenotype (Table 1). Growth chamber analyses indicated that both Type I and II mutants had reduced photoperiodic sensitivity, i.e., the mutants showed a reduced delay in flowering under SD conditions (Table 3). Type II mutants showed a greater loss of photoperiodic sensitivity than Type I mutants and could flower earlier under short day (SD) conditions. The early-flowering phenotype of Type II *exe* mutants under SD conditions was due to the up-regulation of *WFT* expression (Fig. 4B). Normally under SD conditions, *WFT* expression is down-regulated; this indicates that the *WFT* protein can act as a florigen under LD conditions (Fig. 4B, Kitagawa *et al.* 2012, Shimada *et al.* 2009, Yan *et al.* 2006). The unusual up-regulation of *WFT* expression in Type II *exe* mutants under SD conditions might be associated with the higher level of *VRN1* expression (Fig. 2B).

The up-regulation of *VRN1* expression in *exe* mutants was associated with earliness in flowering under LD conditions (Fig. 2A). The extremely extra early-flowering in Type II mutants was associated with a more rapid up-regulation of *VRN1* expression than in Type I mutants. It is notable that a similar level of *VRN1* expression was observed at the timing of phase transition in each *exe* mutant: at the 5-leaf stage for Type I mutants and at the 4-leaf stage for Type II mutants (Fig. 2A). This finding suggests that the level of *VRN1* expression is correlated with the phase transition from vegetative to reproductive growth and may act as a threshold for flowering competency in the plants.

The transition from vegetative to reproductive growth can be divided into two steps: first, the systemic establishment of flowering competency; and second, the determination of floral meristems in the shoot apex (Preston and Kellogg 2008). In a previous study, we analyzed the roles of three novel wheat *API/FUL*-like genes, *WFUL1* (identical to *VRN1*), *WFUL2* and *WFUL3* during this transition period (Kinjo *et al.* 2012). We found that *WFUL1/VRN1* was expressed in leaves as well as spike primordia of non-vernalized plants at

the vegetative stage just before phase transition, while *WFUL2* and *WFUL3* were not expressed in leaves. This indicated that *WFUL1/VRN1* performs a distinct role in leaves before phase transition. The findings in the present study concur with those from the earlier report in suggesting that the *VRN1* expression level in leaves confers flowering competency in wheat plants.

Based on data from expression, transgenic and mutant analyses, we previously proposed a gene network model for the interaction of *VRN1*, *VRN2* and *WFT/VRN3* in leaves (Shimada *et al.* 2009). In this model, *VRN1* is upstream of *WFT* and activates *WFT* expression under LD conditions. Vernalization down-regulates *VRN2* and up-regulates *VRN1* independently of each other. *VRN2* is also suggested to be down-regulated by *WFT*. The proposed up-regulation of *WFT* by *VRN1* is based on the expression analysis of einkorn wheat (*T. monococcum*) lines with winter or spring habit and of the *maintained vegetative phase (mvp)* mutant. The *mvp* mutant lacks a genomic region including the *VRN1* locus, and is unable to flower (Distelfeld and Dubcovsky 2010, Shitsukawa *et al.* 2007). The proposed activity of *WFT* in the down-regulation of *VRN2* is based on the analysis of transgenic wheat plants with over-expression of *WFT* (Shimada *et al.* 2009). Overall, the model can explain why *VRN1* is able to repress *VRN2* expression (Loukoianov *et al.* 2005, Trevaskis *et al.* 2006). Thus, *VRN1* is proposed to play a role as an integrator of the vernalization and photo-periodic signals. This model is consistent with our suggestion here that the level of *VRN1* expression functions as a threshold for flowering competency in wheat plants.

Trevaskis (2010) put forward an alternative gene network model for *VRN1*, *VRN2* and *FT1/VRN3*; this model was based on the results of investigations using barley. This alternative model postulates that *VRN1* and *FT1* mutually up-regulate each other: *VRN1* first activates *FT1* expression, and then *FT1* further activates *VRN1*. The model was referred to as the “flowering model for temperate cereals” in a recent review paper on flowering in plants (Andrés and Coupland 2012). Our suggestion here that the level of *VRN1* expression functions as a threshold for flowering competency is compatible with this model. However, the mechanism for the mutual activation of *VRN1* and *FT1* is still unknown.

More recently, another model of flowering in wheat and barley was suggested by Chen and Dubcovsky (2012). This model proposes that *VRN1* is activated by *WFT/VRN3* and then suppresses *VRN2* expression. Under this model, *VRN1* is not essential for flowering. The latter conclusion was drawn from an analysis of a novel TILLING *VRN1* mutant line that could transit to the reproductive growth phase and produce normal flowers (Chen and Dubcovsky 2012). However, it is not certain that this mutant line is a true *VRN1* knock-out, because its genetic alteration is a point mutation and *VRN1* mRNA is transcribed. In the present study, we showed that the level of *VRN1* expression was associated with earliness in *exe* mutants (Fig. 2). The *exe* mutants do not have a functional *VRN2* locus because the original

Fig. 5. Model for the extra early-flowering phenotype in *exe* mutants. This model is based on that proposed by Shimada *et al.* (2009). Under this model, *VRN1* acts as an integrator of the vernalization and photo-periodic pathways, which are also coordinated with the autonomous pathway. *VRN1* up-regulates the florigen gene *WFT/VRN3*. In *exe* mutants, the mechanism for suppressing the expression of *VRN1* under SD conditions must be disrupted. High levels of accumulation of *VRN1* transcripts induce *WFT* expression, resulting in the extra early-flowering phenotype. Note that *exe* mutants do not have an active *VRN2* allele. Arrows and T-bars indicate promotion and suppression effects, respectively. Arrows with wider lines show stronger effects.

einkorn strain KU104-1 carried a *VRN2* deletion. Thus, our present results indicate that the level of *VRN1* expression can control earliness in *exe* mutants without the need for *VRN2* expression. It is not possible to reconcile our conclusion on the function of *VRN1* expression in flowering earliness with the expectations of Chen and Dubcovsky's (2012) model. Furthermore, the WT strain KU104-1 shows a relatively weak vernalization effect that accelerates flowering (data not shown). It has also been reported in barley that vernalization accelerates flowering (spike primordial formation) in lines lacking *VRN2* (Hemming *et al.* 2008, Sasani *et al.* 2009). Again, the model of Chen and Dubcovsky (2012) does not explain these observations.

VRN1 is transcriptionally activated by prolonged cold during winter to promote flowering. In barley, analysis of transgenic plant having *VRN1* promoter fused to GFP (Green Fluorescent Protein) reporter gene revealed that GFP fluorescence increased at the shoot apex and leaves after prolonged cold treatment (Alonso-Peral *et al.* 2011). The barley cultivar used in this study lacks *VRN2*, indicating that *VRN2* is not involved in the up-regulation of *VRN1* and flowering promotion. This observation also rules out the third model of Chen and Dubcovsky (2012).

In bread wheat, it has been known that winter cultivar quantitatively requires prolonged cold temperature to obtain the vernalization saturation. Some winter cultivars require 2–4 weeks of low temperature to reach the maximum vernalization effect on heading, and the others require more than 4 weeks. The positional cloning of the gene for vernalization requirement duration was performed by Li *et al.* (2013), and they demonstrated that this trait is controlled by recessive *VRN1* gene on A genome at the protein level. They also revealed that binding ability of the VRN1 protein to TaHOX1 (the first homeobox protein in wheat) was associated with requirement of low temperature duration to reach vernalization saturation. The present findings support our model of gene network for flowering: *VRN1* plays a central role in vernalization pathway (Shimada *et al.* 2009).

In conclusion, the present expression analyses of *VRN1* and *WFT* in *exe* mutants indicate that the difference in earliness between Type I and Type II mutants is associated with differences in the patterns of *VRN1* expression. Thus, *VRN1* is essential for flowering in wheat, and the level of expression of *VRN1* determines flowering competency and, therefore, time of flowering (Fig. 5). In Type II *exe* mutants, the mechanism for down-regulating expression of *VRN1* under SD conditions appeared to be disrupted. The accumulation of *VRN1* transcripts induced *WFT* expression, resulting in the extra early-flowering phenotype.

To avoid rainy season for harvesting, early-flowering or early-heading is one of the most important characteristics for bread wheat in East Asia including Japan. The early-heading is closely associated with photoperiodic insensitivity there. The combination of photoperiod-insensitive alleles of *Ppd-A1*, *Ppd-B1* and *Ppd-D1*, affects heading time and adjustability to each area in Japanese bread wheat cultivars (Seki *et al.* 2011, 2013). The present study revealed the relationship between photoperiodic insensitivity and *VRN1* expression level in wheat. The identification of novel photoperiod-insensitive genes and their natural variations affecting the up-regulation of *VRN1* may be useful tools for fine-tuning breeding of heading time. The genes should be identified by the expression QTL analysis of *VRN1* with appropriate populations.

Acknowledgements

We are grateful to the National Bioresource Project - Wheat (NBRP-KOMUGI) for providing the WT wheat strain. This work was supported in part by the Grant-in-Aid for Scientific Research on Innovative Areas from The Ministry of Education, Culture, Sports, Science and Technology (MEXT) KAKENHI Grant Number 24113517 to K. Murai.

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